# Cytotoxicity and T-B cell Crosstalk in Belatacept-treated Kidney Transplant Patients

Gretchen N. de Graav

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# Cytotoxicity and T-B Cell Crosstalk in Belatacept-Treated Kidney Transplant Patients

Cytotoxiciteit en T-B cel samenspel

in niertransplantatiepatiënten behandeld met belatacept

Proefschrift

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op gezag van de rector magnificus

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**Gretchen Norine de Graav**

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“The function of education is to teach one to think intensively and to think critically.
Intelligence plus character - that is the goal of true education”

Martin Luther King Jr.

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# Chapter 1

## General introduction and outline of the thesis

Partly based on:

**T follicular helper cells in transplantation: the target to attenuate antibody-mediated allogeneic responses?**

C.C. Baan, G.N. de Graav and K. Boer

Department of Internal medicine, Erasmus MC, University Medical Center, Rotterdam, the Netherlands

Current Transplantation Reports (2014) 1:166–172

***and***

**Therapeutic drug monitoring of belatacept in kidney transplantation**

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Therapeutic Drug Monitoring (2015) 37:560–567

## Improvements in kidney transplantation

Kidney transplant patients are no longer balancing on the edge of life and death, residing in a plastic bubble for weeks after their transplantation in order to protect them from infections under their heavy immunosuppression. This intensive immunosuppression was deemed necessary to prevent them from losing their graft to hyper acute antibody-mediated or acute T-cell mediated rejection.(1, 2) Nowadays, quality of life and outcomes of kidney transplant patients are significantly improved by the implementation of pre-emptive transplantation and careful human leukocyte antigen (HLA)-matching(3-5), but mostly by the development of a greater therapeutic arsenal, including calcineurin inhibitors (CNIs).(6-8) The downside of potent immunosuppressive reagents like CNIs is the increased risk of infections and malignancies.(9, 10) In addition, CNIs are known for their nephrotoxicity, which can cause kidney graft failure.(11, 12) Another problem of CNI-based regimens is the insufficient prevention of the formation of donor-specific anti-HLA antibodies (DSA) and therefore antibody-mediated rejection, which also decreases kidney graft survival.(13-15) Clearly, there is still room for improvements in the treatment of kidney transplant patients. The development of immunosuppressive drugs that have less adverse effects, but are more potent in preventing the development of DSA could contribute to improved long-term outcomes in kidney transplantation.

## The immune reaction of T cells

Understanding the basic principles of the immune reaction of T cells is important to comprehend alloreactive processes in kidney transplantation, because the current most widely-used immunosuppressive regimen targets predominantly this cell population, but not its interaction with other immune cells. This regimen consists of anti-CD25 (anti-IL-2 receptor) induction therapy with basiliximab, and maintenance therapy with the CNI tacrolimus, the cell cycle inhibitor mycophenolate mofetil (active compound = mycophenolic acid) and glucocorticoids. This combination intervenes on different points in the hereafter described immune cascade in T cells (Figure 1)(16) and effectively blocks the functions of aggressive cytotoxic T cells,(17) one of the key mediators in a classical T-cell mediated rejection response.

The immune reaction in T cells comprises 3 signals (Figure 1): 1) T-cell activation via the T-cell receptor, 2) costimulation, and 3) cytokine receptor up-regulation and cytokine production. In detail, the T-cell receptor is activated by antigen bound to an HLA molecule on the antigen-presenting cell (APC), the first signal. In case of transplantation, this APC can be from the recipient that presents antigen in an indirect manner or from the allograft donor in the case of direct antigen presentation.(18) For proper T-cell activation, a second signal, the so-called costimulatory signal, is required. This second signal is provided when multiple costimulatory molecules expressed on APCs interact with their specific receptors on the surface of T cells. Important costimulatory molecules are CD80 and CD86 (also named B7-1 and B7-2, respectively) expressed by APCs, which activate T cells by binding their CD28. This T-cell activation leads to the third signal in the immune cascade: production of soluble activation proteins called interleukins (IL). Mainly the production of IL-2 and the up regulation of the IL-2 receptor, resulting in abundant IL-2 production, are important for the positive feedback loop that augments the T-cell response. After T-cell activation, the co-inhibitory molecule CTLA-4 is up regulated on the surface of T-cells to control this augmented activation. The CTLA-4 molecule has a much stronger interaction

with CD80/86 than CD28 and has an inhibitory effect on activated T cells. Subsequently, T-cell activation will be dampened through this CD80/86–CTLA-4 pathway.

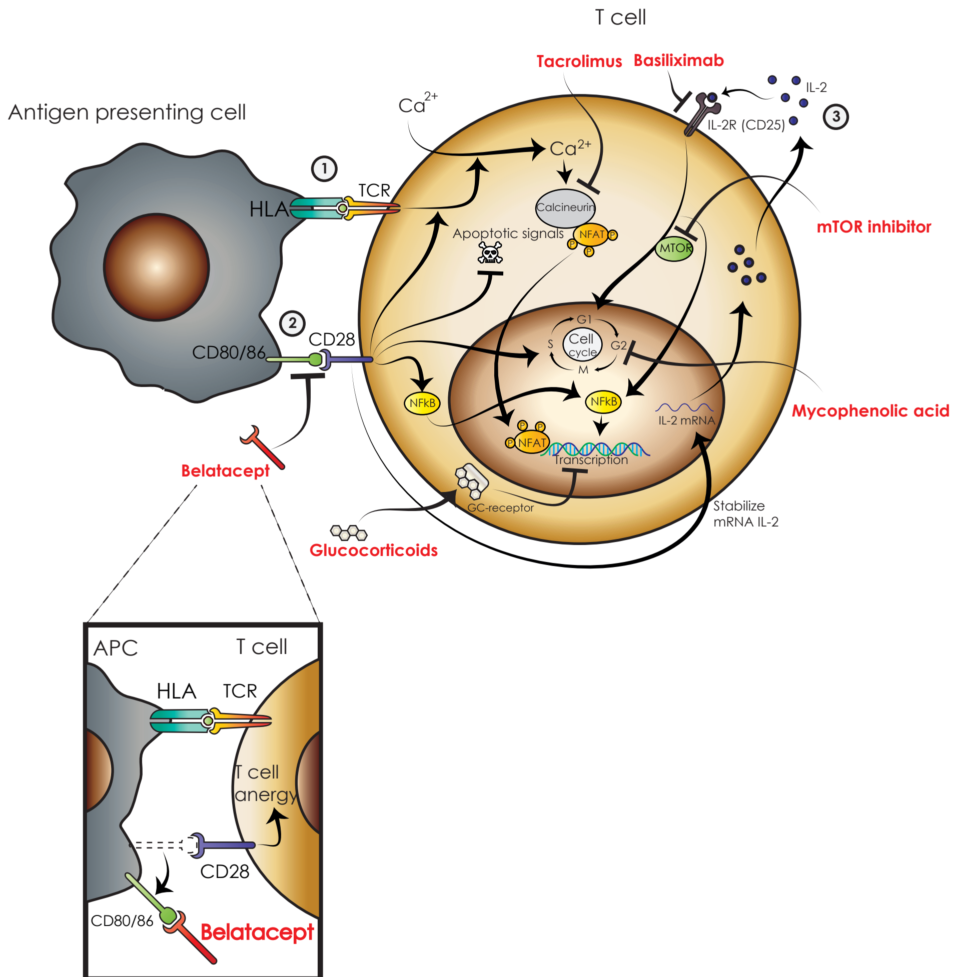



**Figure 1. Immune reaction by T cells and different interference points of immunosuppressive drugs.**

The immune reaction comprises 3 signals: 1) T-cell activation via the T-cell receptor, 2) costimulation, and 3) cytokine receptor upregulation and cytokine production. The current most widely used immunosuppressive regimen in kidney transplantation consists of anti-CD25 induction therapy with basiliximab, the calcineurin inhibitor tacrolimus, the cell cycle inhibitor mycophenolate mofetil (active compound = mycophenolic acid) and glucocorticoid maintenance therapy, and intervenes on different points in above described immune cascade. Belatacept, an alternative immunosuppressant for tacrolimus, binds CD80 and CD86 on antigen-presenting cells, and consequently prevents signal 2 necessary for T-cell activation, leading to T-cell anergy.

APC, antigen-presenting cells; $Ca^{2+}$, calcium ion; CD, cluster of differentiation; G, Gap phase; HLA, human leukocyte antigen; IL, interleukin; M, mitosis phase; mRNA, messenger ribonucleic acid; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; NFkB, nuclear factor kappa B; P, phosphor; S, synthesis phase; TCR, T-cell receptor

## Follicular T helper cells, a recently discovered T helper cell subset

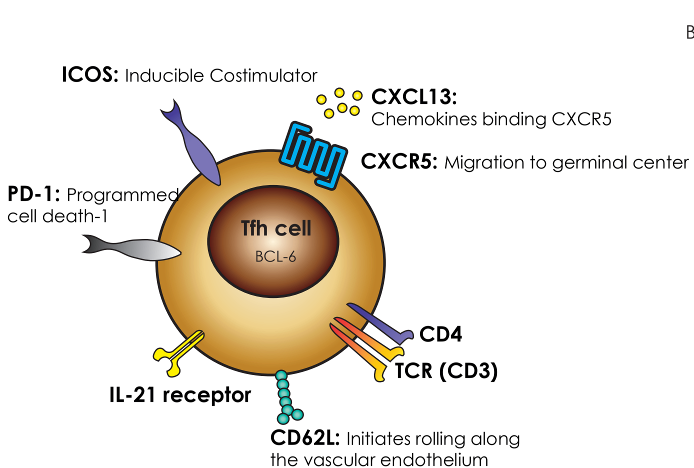
As previously mentioned, the current most widely-used immunosuppressive regimen in kidney transplantation is less effective against DSA formation resulting from T-B cell interactions (19-21) than in suppressing cytotoxic T cells. Over the last decade, the importance of B cells is recognized in acute alloreactivity after transplantation.(15, 22-28) B cells are present in biopsies of apparent T cell-mediated rejections, probably functioning as APCs.(23-28) Furthermore, B cells produce important interleukins for T-cell activation, proliferation and differentiation, like IL-6 and TNFa.(29, 30)

Important mediators in B-cell activation and differentiation are follicular T helper (Tfh) cells. These specialized cells support differentiation and immunoglobulin production when co-cultured with B cells (Figure 2), and express high levels of CXCR5, which, in conjunction with the loss of CCR7, enables them to localize to B-cell follicles and germinal centers of secondary lymphoid organs during T-cell-dependent immune responses.(31, 32) The capacity of Tfh cells to provide B cell-help depends upon the acquisition of molecules that are known to play functional roles in T–B cell interactions, like the costimulatory molecules CD40 ligand, inducible costimulator (ICOS), programmed death 1 (PD-1), and the cytokine interleukin (IL)-21 (Figure 2). The transcriptional suppressor B cell lymphoma-6 (Bcl-6) is the master regulator for Tfh-cell differentiation.(32-34) Recent identification of these Tfh cells in the circulation created the possibility to study the immunological functions and molecular composition of antigen-activated Tfh cells in detail.(32, 33) In contrast to Tfh cells present in the secondary lymphoid tissues, their peripheral counterparts, characterized as $CXCR5^{+}CD4^{+}$ T cells, do not express Bcl-6 and express lower levels of ICOS and PD-1.(31, 33) Both Tfh and peripheral Tfh cells secrete IL-21 upon stimulation, which has an essential role in activation and expansion of  B cells, plasma cell generation and immunoglobulin production, including DSA. An immunosuppressive agent specifically targeting Tfh-B cell interaction would be a useful contribution to the improvement of outcomes in kidney transplantation. Studies on peripheral Tfh cells could be very helpful in this aspect.

## Belatacept, the latest approved immunosuppressant in kidney transplantation

An immunosuppressive agent that might effectively inhibit Tfh-B cell interaction is the costimulatory signal inhibitor belatacept (Nulojix®, formerly known by its study name LEA29Y and produced by Bristol-Myers Squibb).(35) This inhibitor of the CD28-CD80/86 pathway is the first drug in its class approved for the prevention of kidney allograft rejection. Belatacept binds CD80 and CD86 on APCs, and consequently prevents signal 2 necessary for T cell activation, leading to T cell anergy (Figure 1).  In June 2011, this compound was approved by the Food and Drug Administration of the Unites States of America and the European Medicines Agency. The drug is also marketed in several countries in South America. Belatacept is prescribed in combination with mycophenolate mofetil and glucocorticoids and allows for CNI-free immunosuppression. Because belatacept is not nephrotoxic and lacks many of the other troublesome side effects of the CNIs tacrolimus and cyclosporine, the drug has great potential for transplantation medicine. Belatacept is a fusion-protein that consists of the modified Fc-fragment of the human immunoglobulin G1 linked to the extracellular domain of human cytotoxic T-lymphocyte antigen (CTLA)-4 (Figure 3).(35) The latter binds CD80 and CD86 with high affinity, *i.e.*, the strength of

A.

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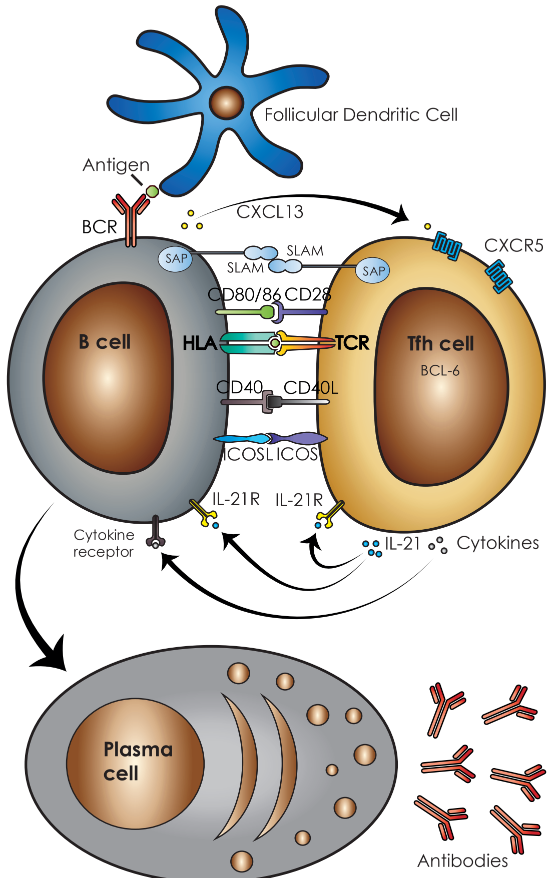


**Figure 2. Follicular T helper (Tfh) cells are important mediators of humoral reactivity.**

Tfh cells express high levels of CXCR5, the chemokine receptor for CXCL13, which enables them to localize to B-cell follicles and germinal centers of secondary lymphoid organs during T-cell dependent immune responses. The transcriptional suppressor B-cell lymphoma-6 (Bcl-6) is the master regulator for Tfh-cell differentiation (A). The capacity of Tfh cells to provide B cell-help depends upon the acquisition of molecules that are known to play functional roles in T–B cell interactions, like the co-stimulatory molecules CD40 ligand, inducible costimulator (ICOS), programmed death 1 (PD-1), and the cytokine interleukin (IL)-21 (B).

APC, antigen-presenting cells; Bcl-6, B-cell lymphoma 6; BCR, B-cell receptor; CD, cluster of differentiation; CXCL, C-X-C motif ligand; CXCR, C-X-C chemokine receptor; HLA, human leukocyte antigen; IL, interleukin; L, ligand; SAP, SLAM-associated protein; SLAM, signaling lymphocytic activation molecule; TCR, T-cell receptor; Tfh cell, follicular T helper cell

interaction between belatacept and CD80/86 is high. By changing the 2 amino acids L104E and A29Y, the belatacept fusion-protein is a higher affinity variant of CTLA4-immunoglobulin (Ig) or abatacept, that is used to treat rheumatoid arthritis.(35) Belatacept was developed because CTLA4-Ig was not effective enough in inhibiting alloreactivity in a pancreatic islet and kidney transplantation model in nonhuman primates.(36, 37) Belatacept inhibits T-cell activation by blocking costimulatory signals from antigen-presenting cells (APCs), which include dendritic cells, but also monocytes and B cells. This costimulatory inhibitor therefore has the potential to efficiently interfere with Tfh-B cell interaction. In animal studies, combination therapy with belatacept effectively prevented germinal center and Tfh-cell formation, IL-21 production, clonal B-cell expansion and DSA production.(20, 21) In line with this, belatacept-treated patients had significantly lower DSA levels than patients treated with the CNI cyclosporine A seven years after kidney transplantation.(19, 38) So far, belatacept has not been compared to the most widely-used CNI, tacrolimus, in this aspect.

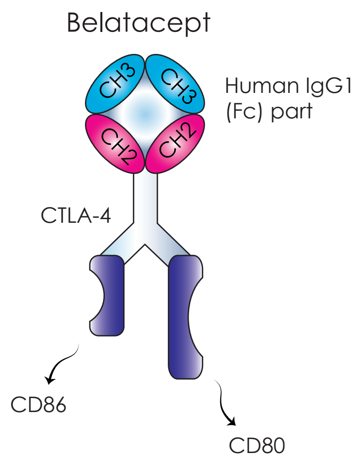


**Figure 3. Structure of the costimulatory inhibitor, belatacept.**

Belatacept is a fusion-protein that consists of the modified Fc-fragment of the human immunoglobulin G1 linked to the extracellular domain of human cytotoxic T lymphocyte antigen (CTLA)-4. The latter binds to CD80 and CD86 with high affinity.

CH, constant domain heavy chain; CTLA-4, cytotoxic T lymphocyte antigen 4; Fc, fragment crystallizable region; IgG, immunoglobulin G

## Aim and outline of this thesis

Despite the promising results in the large randomized controlled trials comparing belatacept *vs.* cyclosporine A, treatment with this costimulatory inhibitor has downsides, e.g. a higher acute rejection rate.(19, 38) At present, no reliable immunological biomarkers are available that can predict who is at risk for allograft rejection under belatacept treatment.

In this thesis, we aimed to learn more about the immune mechanisms involved in alloreactivity in patients treated with belatacept or tacrolimus after kidney transplantation. Particularly, we focused on the effects of belatacept on effector T cells and Tfh-B cell interaction, and on the question which cell types are less susceptible to the effects of belatacept. In detail, the following aims were investigated:

- To determine if Tfh cells still mediate humoral alloreactivity shortly after kidney transplantation in a tacrolimus-based regimen – **Chapter 2**
- To study whether belatacept more efficiently inhibits alloreactive Tfh-B cell interaction than tacrolimus in kidney transplantation – **Chapter 3**
- To assess if belatacept effectively suppresses its indirect targets, CD28-positive T cells – **Chapter 4**
- To identify a biomarker for belatacept-resistant rejection and to compare clinical outcomes of belatacept- and tacrolimus-treated kidney transplant patients in a randomized controlled trial – **Chapter 5**
- To immunologically analyze a severe steroid-resistant rejection under belatacept-treatment, leading to graft loss – **Chapter 6**

In **Chapter 7** and **Chapter 8** the findings of abovementioned studies are summarized and put into perspective of the clinical practice in kidney transplantation.

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# Chapter 2

## Follicular T-helper cells and humoral reactivity in kidney-transplant patients

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Clinical and Experimental Immunology (2014) 180: 329–340

## Summary

Memory B cells play a pivotal role in alloreactivity in kidney transplantation. Follicular T-helper (Tfh) cells play an important role in the differentiation of B cells into immunoglobulin-producing plasmablasts (through IL-21). It is unclear to what extent this T cell subset regulates humoral alloreactivity in kidney-transplant patients. Therefore we investigated the absolute numbers and function of peripheral Tfh cells ($CD4^{POS}CXCR5^{POS}$ T cells) in patients before and after transplantation. In addition, we studied their relationship with the presence of donor specific anti-HLA antibodies (DSA), and the presence of Tfh cells in rejection biopsies. After transplantation, peripheral $T_{FH}$-cell numbers remained stable, while their IL-21-producing capacity decreased under immunosuppression. When isolated after transplantation, peripheral Tfh cells still had the capacity to induce B-cell differentiation and immunoglobulin production, which could be inhibited by an IL-21-receptor-antagonist. After transplantation the quantity of Tfh cells was the highest in patients with pre-existent DSA. In kidney biopsies taken during rejection, Tfh cells co-localized with B cells and immunoglobulins in follicular-like structures. Our data on Tfh cells in kidney transplantation demonstrate that Tfh cells may mediate humoral alloreactivity, which is also seen in the immunosuppressed milieu.

## Introduction

Memory B cells are important in alloreactivity in kidney transplantation. During the last decade it has become clear that acute T cell mediated rejection biopsies contain numerous B cells as well as conventional T cells.(1) A significant proportion of acute rejections can therefore be classified as a mixed rejection, rather than a pure, T-cell mediated rejection.(2, 3) Hence, B cells are also involved in acute T-cell mediated rejection next to chronic antibody-mediated rejection, which involves donor-specific anti-human leucocyte antigen (HLA) antibodies (DSA) produced by B cells that differentiated into plasmablasts or plasma cells.(4) The latter are formed after the differentiation of donor-specific memory B cells. Follicular T helper (Tfh) cells are of importance for the survival of memory B cells and the differentiation of B cells into immunoglobulin-producing plasmablasts or plasma cells.(5) These Tfh cells are a heterogeneous population of $CD4^{POS}$ T helper cells, which are active in secondary lymphoid organs(6) and may be present in tertiary lymphoid structures formed in kidney allografts.(7) Tfh cells also produce interleukin (IL)-21, which is an important cytokine for B cell stimulation and differentiation.(5, 8, 9) The transcription factor for the formation of Tfh cells, B cell lymphoma 6 (Bcl-6), suppresses the transcription factors required for formation of other T helper subsets.(5, 9, 10) Chemokine Receptor 5 (CXCR5) allows migration of Tfh cells towards the germinal center. (11-13) Peripheral $CD4^{POS}CXCR5^{POS}$ T cells are the counterparts of Tfh cells in blood and express low levels of ICOS and PD1, and lack Bcl-6. (5, 14)

Recent studies revealed the pivotal role of Tfh cells in B-cell mediated immune responses in auto-immunity(15-17) and chronic viral infections(18-23) in humans. In autoimmunity, frequencies of Tfh cells are positively correlated with the peripheral blood levels of disease-specific auto-antibodies(16, 17) and with disease-activity.(15) Accordingly, the percentage of circulating Tfh cells decreased in patients with autoimmune thyroiditis after treatment.(17) IL-21 is an important disease mediator in these autoimmune disorders.(15-17) Also in chronic viral infections, such as hepatitis B and HIV, Tfh cells are expanded in peripheral blood(21-23) or in lymph nodes,(18, 19) and they can function as markers for the response to anti-viral treatment.

No studies have been conducted which define the role of Tfh cells in B cell activation and humoral alloreactivity in immunosuppressed kidney transplant patients.(24) In CD4-reconstituted rodents, Tfh cells induce long-lasting immunoglobulin (Ig)G alloantibody responses after heart transplantation,(25) which are potentially dangerous for the graft. These cells also mediated humoral responses towards the kidney allograft in an immunosuppressed non-human primate model.(26) In addition, Tfh cells from intestinal transplant patients maintain germinal centers in isolated lymphoid follicles in the transplanted intestines and subsequently ensure IgA synthesis to control commensal micro flora, despite the immunosuppressed milieu.(27) As B cells play a pivotal role in both cellular and humoral alloreactivity, the interaction between Tfh cells and B cells is an important target for immunosuppression. We hypothesize that in immunosuppressed kidney transplant patients Tfh cells can still stimulate B cells, which leads to their differentiation into immunoglobulin-producing plasmablasts and plasma cells. The frequency and function of peripheral Tfh cells were therefore examined before and after kidney transplantation, as well as their relationship with the presence of donor specific antibodies (DSA). In addition, we stained acute rejection biopsies to examine the presence of intragraft Tfh cells, B cells and immunoglobulins.

## Material and methods

*Study population in which peripheral Tfh cells were studied*

Thirty consecutive renal transplant recipients were included and followed for one year post-transplantation. Sixteen age and gender-matched controls (healthy volunteers) were also included. Patients receiving a kidney transplant from a living donor all participated in a randomized controlled clinical trial with the primary aim to study the efficacy of a genotype-based approach to tacrolimus dosing (Dutch trial registry number NTR 2226; http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=2226). All patients received induction therapy with basiliximab (Simulect®, Novartis; 20 mg i.v. on day 0 and day 4), tacrolimus (Prograf®, Astellas Pharma; aiming for predose concentrations of 10 - 15 ng/mL in weeks 1 - 2, 8 - 12 ng/mL in weeks 3 - 4, and 5 - 10 ng/mL, thereafter), mycophenolate mofetil (Cellcept®, Roche; starting dose of 1 g b.i.d., aiming for predose concentrations of 1.5 – 3.0 mg/L), and glucocorticoids. Prednisolone was tapered to 5 mg at month 3 and withdrawn at month 4 - 5. For inclusion in the study, as well as the current substudy, written informed consent was required from the patient. The study was approved by the Medical Ethical Committee of the Erasmus MC (MEC number 2010-080, EudraCT 2010-018917-30). Rejections were defined as biopsy-proven acute rejection (BPAR) according to the Banff-classification 2009.(28)

*Numbers of peripheral Tfh cells and cytokine production capacity*

Peripheral Tfh cells were defined as $CD3^{POS}CD4^{POS}CXCR5^{POS}$ lymphocytes and measured in fresh whole blood samples obtained 1 day before and 3 months after transplantation. In these cells the intracellular IL-21 production-capacity was determined *ex vivo* after 4 hours of stimulation with PMA 0.5 µg/ml and Ionomycine 10 µg/ml (Sigma-Aldrich, St. Louis, MO) at 37°C. The following monoclonal antibodies (mAbs) were used: CD3 AmCyan; CD4 Pacific Blue; CXCR5 AF647; IL-21 phycoeryhtrin (PE); and the IL-21 isotope mouse IgG1-PE (all from BD Biosciences, San José, CA). To measure absolute numbers of CD3 and CD4, BD multi-test 6-color® was used in BD TruCount Tubes® (San Jose, CA). Absolute numbers of the subsets were calculated using the percentages of these subsets within the total CD3 and CD4 populations.

*Co-culture experiments of peripheral Tfh cells and memory B cells*

To determine the function of peripheral Tfh cells, co-culture experiments with memory B cells were conducted. First, peripheral Tfh cells, i.e. $CD3^{POS}CD4^{POS}CXCR5^{POS}$ T cells, and memory B cells, i.e. $CD19^{POS}CD27^{POS}$ cells, were isolated by sorting with BD-FACSAria II SORP™ (purities≥95%) from defrosted peripheral blood mononuclear cells (PBMCs). These PBMCs were obtained from patients one day before transplantation and three months after transplantation (thus isolated from an immunosuppressed milieu). As a control, PBMCs of healthy volunteers were used. mAbs were used as described above, including viability staining solution 7-aminoactinomycin (7-AAD) peridinin chlorophyll (PerCP) (BD Biosciences). Secondly, memory B cells were co-cultured for 7 days with $CD4^{POS}CXCR5^{POS}$ Tfh cells in the presence of the superantigen Staphylococcus Aureus Antigen B (SEB, Sigma-Aldrich). Subsequently, differentiation of B cells into plasmablasts after 7 days was determined with flow cytometry. Plasmablasts were defined as $CD3^{NULL}CD4^{NULL}CD19^{POS}CD20^{NULL}CD27^{POS}CD38^{HIGH}$ cells(14, 29) using the following mAbs: CD3 AmCyan (BD Biosciences); CD4 Pacific Blue (Becton Dickinson, BD, Frankin Lakes, NJ); CD19 fluorescein isothiocyanate (FITC) (BD); CD20 PerCP (BD); CD27 PE-Cy7 (eBioscience, San Diego,

CA); and CD38 PE (eBioscience). Finally, after 7 days, IgM and IgG production were measured with a sandwich ELISA in the supernatants of the co-cultures.

To determine the role of IL-21 in the interaction of Tfh cells with memory B cells, 5 µg/mL of IL-21-receptor blocking antibody (IL-21-R-Fc R&D Systems, Minneapolis, MN) was added to the co-cultures of four patients (pretransplantation samples) and three healthy volunteers according to the concentration used for blocking immunoglobulin production by 50-75% in cells of healthy controls,(14) thus sufficiently blocking the IL-21-receptor without completely inhibiting plasmablast formation and immunoglobulin production. An isotype-matched control (IgG1-Fc R&D Systems) was used. Thereafter, differentiation into plasmablasts and immunoglobulin production were measured after 7 days.



*Donor-specific anti-HLA antibodies (DSA)*

DSA, including C1q-binding capacity, were determined in thawed heparin plasma samples before and 3, 6 and 12 months after kidney transplantation. For all patients the complement-dependent cytotoxicity (CDC) cross-match before transplantation was negative for both current and historic sera. DSA presence before transplantation was considered as pre-existent DSA, and DSA developing after transplantation as *de novo* DSA. Plasma samples were centrifuged for 10 minutes at 14 170 rpm. Thirty µL of the plasma was incubated with 100 µl/mL Adsorb Out microbeads (One Lambda®) to minimize false positive staining. Subsequently, 20 µl plasma was incubated for 30 minutes with 2 µl Single Antigen beads mix from LABScreen (One Lambda®) Single Antigen class I and class II kits. After protocol washing procedures, plasma samples were incubated with 1 µl goat anti-human IgG-PE per well (One Lambda®). Microbeads were analyzed with a Luminex Labscan™ 100 analyzer using both Luminex 100IS and HLA Fusion 3.0 software. All samples fulfilled the quality criteria for reactivity of the control beads.

*Immunohistochemistry*

Kidney biopsies, diagnosed as type I acute rejection (three of type 1A and two of type 1B), were paraffin-embedded, formalin-fixed and cut into 4 µm sections. Immunohistochemistry was performed by routine diagnostics on the Benchmark Ultra Stainer (Ventana, Basel), using the following mAbs: CD3 (1:150 dilution, DAKO, Denmark) was used to detect pan-T cells; CD4 (undiluted, Ventana, Arizona) for T helper cells; CD8 (1:50, DAKO) for cytotoxic T cells; CD20 (1:400 dilution, DAKO) for B cells; C4d (1:60 dilution, Biomedica Gruppa, Vienna, Austria) for complement factor C4d; Bcl-6 (1:15 dilution, Novocastra/Leica, Solms, Germany) for the transcription factor of Tfh cells; IgM (1:80 dilution, Biogenex, Fremont, CA) and IgG (1:200 dilution, DAKO) for the immunoglobulin production.

Incubation with antibodies was done for 30 minutes and anti-rabbit or anti-mouse amplifiers were used. As positive control for Bcl-6, tonsillar sections obtained from a pediatric tonsillectomy were used.

To identify intragraft Tfh cells, sections were double-labeled with CD3 (polyclonal rabbit; DAKO; 1:200) and Bcl-6 (mouse monoclonal, Novocastra; 1:50). After incubation overnight at 4°C with the primary antibody Bcl-6, sections were subsequently incubated with a biotinylated horse-anti-mouse Ab (1:500) and Dyelight®594 (red) conjugated with streptavidin (Vector Laboratories, Burlingame, CA; 1:500) to visualize the Bcl-6 expression. Next, sections were

incubated for 1 hour with the CD3-antibody and subsequently with the Dyelight®488 (green) goat-anti-rabbit Ab (Vector Laboratories; 1:200).

**Table 1:** Baseline characteristics and outcomes

| | Study Group (n=30) |
|---|---|
| Recipient age in years (median, range) | 52 (19-75) |
| Recipient Gender (%M) | 67% |
| HLA-A Mismatches (mean±SD) | 0.9 (±0.8) |
| HLA-B Mismatches (mean±SD) | 1.1 (±0.8) |
| HLA-DR Mismatches (mean±SD) | 1.0 (±0.6) |
| Cause of end-stage renal disease | |
| • Hypertensive nephropathy | 23.3% (7) |
| • Glomerulonephritis | 16.7% (5) |
| • Polycystic kidney disease | 13.3% (4) |
| • Focal segmental glomerulosclerosis | 10% (3) |
| • Diabetic nephropathy | 6.7% (2) |
| • Congenital dysplasia | 6.7% (2) |
| • Miscellaneous | 23.3% (7) |
| Previous kidney transplantation | 20% (6) |
| • Third kidney transplantation | 6.7% (2) |
| • Second kidney transplantation | 13.3% (4) |
| Renal replacement therapy before transplantation | 53.3% (16) |
| • Hemodialysis | 26.7% (8) |
| • Peritoneal dialysis | 26.7% (8) |
| Rejection within 12 months of transplantation | 36.7% (11) |
| • Type I T-cell mediated rejection | 54.5% (6) |
| • Type II T-cell mediated rejection | 27.3%(3) |
| • Antibody-mediated rejection | 0% (0) |
| • Mixed rejection | 18.2% (2) |
| Time to rejection in days (median, range) | 99 (7-261) |
| One-year death-censored graft survival | 100% |
| One-year patient survival | 96.7% |

Patient number is depicted between brackets unless otherwise specified.

C4d, complement factor 4d; M, male; SD, standard deviation

*Statistical analyses*

Differences between measurements before and after transplantation were analyzed using the Wilcoxon-signed-rank test. Using the Mann-Whitney U-test, the differences were analyzed between transplant patients and healthy controls; and between patients with pre-existent DSA and patients without pre-existent DSA. For comparing dichotomous outcomes, we

used Fisher's exact test.

IBM SPSS 20 (New York, NY) was used for statistical analysis. P-values with a 2-sided a of 0.05 were considered statistically significant. When not otherwise specified, medians [+range] are presented.



## Results

*Study population*

Table 1 depicts the baseline characteristics of the patients from whom we studied the peripheral Tfh cells. Two patients discontinued tacrolimus within three months of transplantation. In one case this was necessitated by tacrolimus-induced thrombotic micro-angiopathy and in the other because of severe acute nephrotoxicity. One patient underwent a complicated transplantation with small bowel perforation and subsequently did not receive mycophenolate mofetil (MMF). The remaining 27 patients were all on a tacrolimus/MMF/prednisolone regimen for the first 3 months after transplantation. Eleven patients (36.7%) suffered from rejection within twelve months of transplantation. Median time to rejection was 99 days, ranging from 7 to 261 days. Six patients had an early BPAR, i.e. within the first three months of transplantation. One-year dead-censored graft survival was 100% and one-year patient survival was 96.7%. One patient died from gastric adenocarcinoma 9 months after transplantation.

*Numbers of peripheral Tfh cells remain stable after kidney transplantation, while their IL-21 production capacity decreases*

We studied the number of peripheral Tfh cells before and 3 months after kidney transplantation. Absolute numbers of $CD4^{POS}CXCR5^{POS}$ T cells were lower in patients before and after transplantation compared to the healthy controls ($p<0.01$), but remained stable after transplantation (Figure 1A and 1B).

To confirm that IL-21 is produced by $CD4^{POS}CXCR5^{POS}$ T cells, their IL-21 production capacity upon PMA/Ionomycin stimulation was compared with that of $CD4^{POS}CXCR5^{NULL}$ T cells. A representive example of the IL-21-production by $CD4^{POS}CXCR5^{POS}$ and $CD4^{POS}CXCR5^{NULL}$ T cells is illustrated (Figure 1C). A higher percentage of $CD4^{POS}CXCR5^{POS}$ T cells produced IL-21 than $CD4^{POS}CXCR5^{NULL}$ T cells (6.9% [1.5-12.6%] vs. 3.2% [0.4-8.5%], $p<0.0001$).

The numbers of IL-21-producing Tfh cells after stimulation were comparable between healthy controls and patients before transplantation (Figure 1D). After transplantation, a decrease in IL-21 production capacity of $CD4^{POS}CXCR5^{POS}$ T cells was observed, $p<0.05$. (Figure 1D). Patients (n=6) with a BPAR within 3 months after transplantation, which consequently received high doses of pulse steroids, had similar numbers of $CD4^{POS}CXCR5^{POS}$ T cells. However, a lower IL-21 production capacity was observed compared to patients who did not reject within the first three months after transplantation (n=24), $p<0.05$ (Supplementary figure 1).

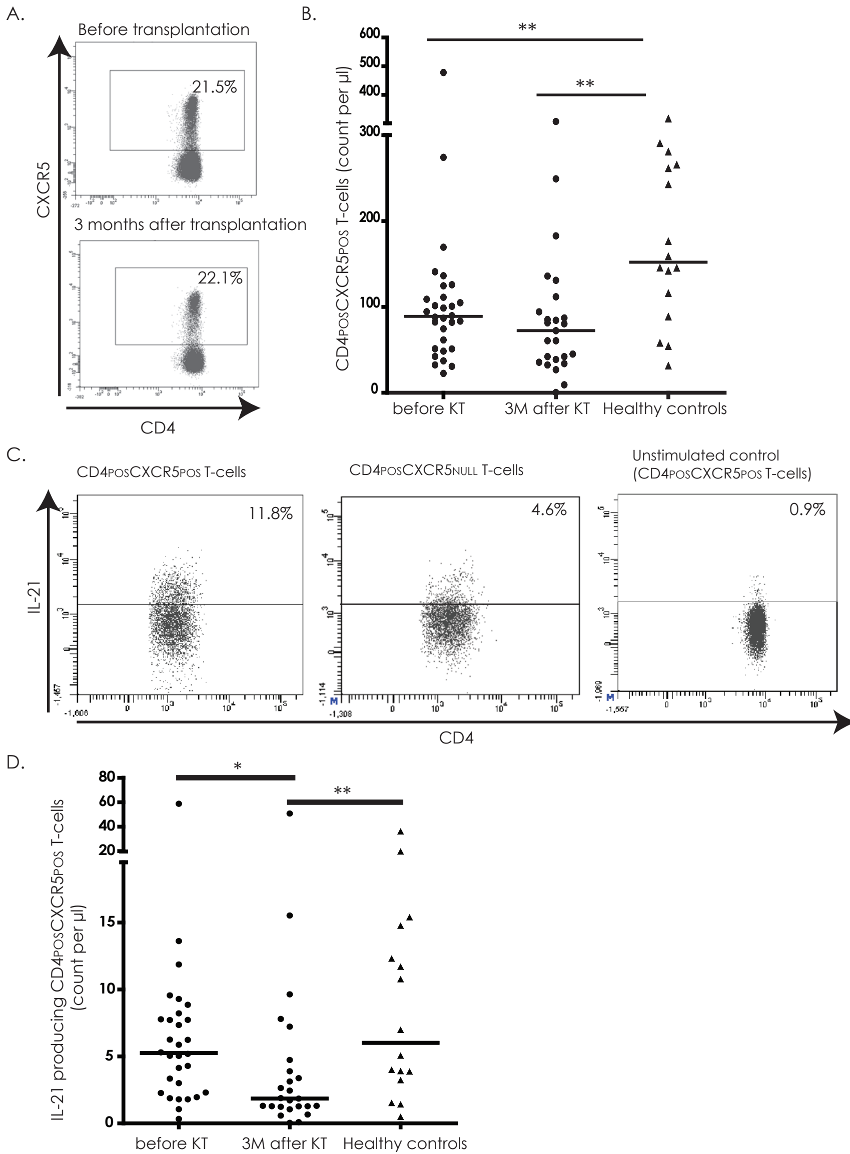



**Figure 1. Numbers of peripheral (CD4POSCXCR5POS) follicular T helper (Tfh) cells remained equal after transplantation, while interleukin (IL)-21-production capacity decreased after transplantation.**
(A) CD4POSCXCR5POS were gated from CD3-positive cells within the lymphocytes, which were defined by forward- and sided-scatter. A typical example of CXCR5-expression within CD4-positive T cells is shown for a patient before and 3 months after kidney transplantation.
(B) Absolute numbers of CD4POSCXCR5POS T cells were compared between before and 3 months after transplantation (n=30), and between patients and healthy controls (n=16).
(C) The proportion of IL-21-producing cells is shown upon 4 hours phorbol myrisate acetate (PMA)/ionomycine stimulation in typical examples for CD4POSCXCR5POS T cells and CD4POSCXCR5NULL T cells (total measured in n=30). An unstimulated (i.e. negative) control for CD4POSCXCR5POS T cells is depicted.
(D) Absolute numbers of IL-21-producing CD4POSCXCR5POS T cells upon 4 hours PMA/ionomycine stimulation are depicted before and after transplantation (n=30), and in healthy controls (n=16).

**Table 2:** Baseline characteristics of patients with pre-existent DSA and without pre-existent DSA when a cut-off of an MFI≥4000 is used

| | No Pre-existent DSA (n=16) | Pre-existent DSA (n=7) | p-value (two-sided) |
|---|---|---|---|
| Recipient age in years (median+range) | 58 (19-75) | 51 (22-58) | 0.02 |
| Donor age in years (median+range) | 55 (26-86) | 48 (25-54) | 0.08 |
| Recipient Gender (%M) | 31% (5) | 57% (4) | 0.36 |
| HLA-A Mismatches (mean±SD) | 1.1 (±0.7) | 0.9 (±0.9) | 0.56 |
| HLA-B Mismatches (mean±SD) | 1.3 (±0.8) | 1.1 (±0.9) | 0.86 |
| HLA-DR Mismatches (mean±SD) | 1.1 (±0.6) | 1.1 (±0.4) | 0.97 |
| Cause of end-stage renal disease | | | 0.02 |
| • Hypertensive nephropathy | 31.2% (5) | 14.3% (1) | |
| • Glomerulonephritis | 0% (0) | 28.6% (2) | |
| • Polycystic kidney disease | 18.8% (3) | 0% (0) | |
| • Focal segmental glomerulosclerosis | 0.0% (0) | 28.6% (2) | |
| • Diabetic nephropathy | 12.5% (2) | 0% (0) | |
| • Congenital dysplasia | 12.5% (2) | 0% (0) | |
| • Miscellaneous | 25% (4) | 28.6% (2) | |
| Previous kidney transplantation[1] | 0% (0) | 43% (3) | 0.02 |
| • Third kidney transplantation | - | 14.3% (1) | |
| • Second kidney transplantation | - | 28.6% (2) | |
| Replacement therapy before transplantation | 43.8% (7) | 71.4% (5) | 0.37 |
| • Hemodialysis | 31.2% (5) | 14.3% (1) | |
| • Peritoneal dialysis | 12.5% (2) | 57.1% (4) | |
| Rejection within 12 months of transplantation | 31.3% (5) | 42.9% (3) | 0.47 |
| Time to rejection in days (median+range) | 78 (7-261) | 129 (8-216) | 0.71 |

Patient number is depicted between brackets unless otherwise specified.[1]Two patients received two previous grafts: one received a graft from a post-mortal donor and from a living donor; the other one received both previous grafts from a post-mortal donor. Four patients received one previous graft, all from a living donor.

DSA, donor-specific anti-human leukocyte antigen antibody; HLA, human leukocyte antigen; M, male; SD, standard deviation

*The in-vitro function of peripheral Tfh cells is conserved in patients after transplantation*

To determine the functionality of peripheral ($CD4^{POS}CXCR5^{POS}$) Tfh cells, we performed co-culture experiments of pure, isolated Tfh cells and memory B cells from patients from whom materials were available before and after transplantation (n=11). For gating strategies see Figure 2A-2C. After transplantation, less Tfh-cell dependent B-cell differentiation into plasmablasts was

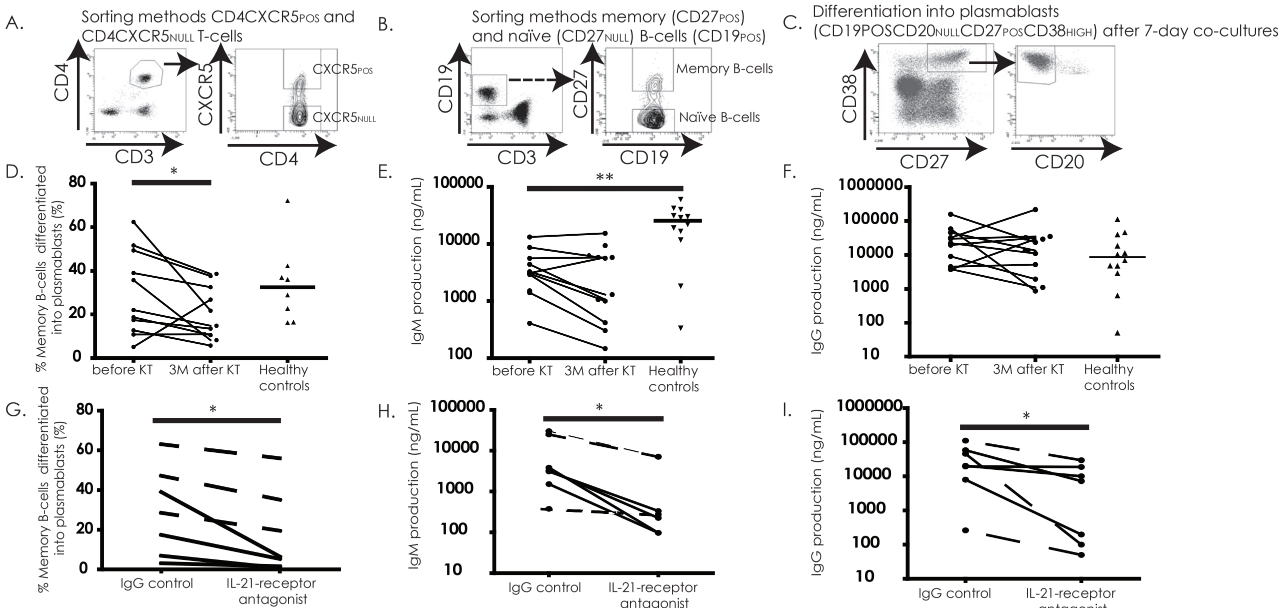



**Figure 2. After kidney transplantation CD4POSCXCR5POS T cells were still capable of stimulating memory B cells to differentiate into immunoglobulin-producing plasmablasts. This process was IL-21 dependent.**

(A) A typical example of the FACS-sorting strategy to obtain CD4POSCXCR5POS T cells is depicted. Cells were gated from viable (i.e. 7-AAD negative) lymphocytes, which were defined by the forward- and side-scatter.

(B) A typical example of the FACS-sorting strategy to obtain memory B cells is depicted. Cells were gated from viable (i.e. 7-AAD negative) cells, which were defined by the forward- and side- scatter.

(C) A typical example of the FACS measurement of differentiation of memory B cells into plasmabast is depicted (after 7 day co-culture with CD4POSCXCR5POS T cells).

(D-F) CD4POSCXCR5POS T cells were obtained before and after transplantation (n=11). Their capacity to stimulate memory B cell differentiation into plasmablasts (D), and to stimulate IgM (E) and IgG production (F) is compared between before and after transplantation. Also the stimulation-capacity of healthy controls' CD4POSCXCR5POS T cells is depicted (n=8).

(G-I) The percentage of memory B cells differentiated into plasmablasts (G), the IgM-production (H) and the IgG-production (I) are given at day 7 in the presence or absence of an anti-IL-21R-antagonist (5µg/ml IL21R-Fc). Dashed lines represent the healthy controls (n=3) and solid lines the patients (n=4).

*p<0.05, **p<0.01, ***p<0.001. N.B.: Y-axes for immunoglobulin production are log linearly scaled. 3M=3 months; KT=kidney transplantation

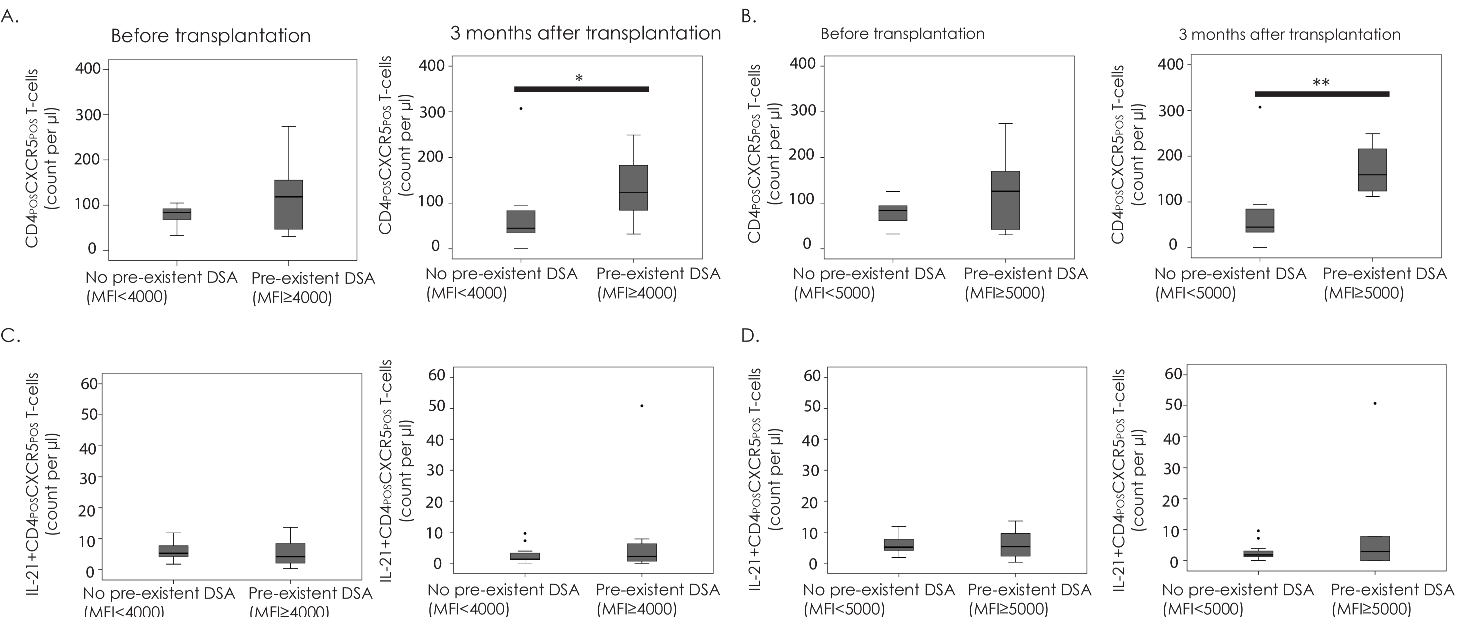



**Figure 3. After kidney transplantation, the numbers of CD4POSCXCR5POS T cells were higher in patients with pre-existent donor-specific anti-human leukocyte antigen (HLA) antibodies (DSA) than in patients without pre-existent DSA.**

(A) The study population (n=23) is divided in patients with pre-existent (=pre-transplantation) DSA (n=16) and patients without pre-existent DSA (n=7). In this analysis an MFI of 4000 or higher was considered as positive DSA. Numbers of CD4POSCXCR5POS T cells were compared between patients with DSA and patients without DSA, before and after transplantation.

(B) The study population (n=23) is divided in patients with pre-existent (=pre-transplantation) DSA (n=17) and patients without pre-existent DSA (n=6). In this analysis an MFI of 5000 or higher was considered as positive DSA. Numbers of CD4POSCXCR5POS T cells were compared between patients with DSA and patients without DSA, before and after transplantation.

(C) The study population (n=23) is divided in patients with pre-existent (=pre-transplantation) DSA (n=16) and patients without pre-existent DSA (n=7). In this analysis an MFI of 4000 or higher was considered as positive DSA. Numbers of IL21⁺CD4POSCXCR5POS T cells were compared between patients with DSA and patients without DSA, before and after transplantation.

(D) The study population (n=23) is divided in patients with pre-existent (=pre-transplantation) DSA (n=17) and patients without pre-existent DSA (n=6). In this analysis an MFI of 5000 or higher was considered as positive DSA. Numbers of IL21⁺CD4POSCXCR5POS T cells were compared between patients with DSA and patients without DSA, before and after transplantation.

N.B.: Black lines represent the median; the upper and lower border of the boxes represent the 25th and 75th percentile; the error lines represent the minimal and maximal value within 1.5 quartile distances of the box; values above 1.5 quartile distances of the box are considered outliers and represented by a dot.

observed than before transplantation (Figure 2D, $p<0.05$). Nevertheless, this did not lead to altered IgM and IgG production levels: among samples obtained before and 3 months after transplantation, comparable levels of IgM and IgG were measured in the co-cultures with $CD4^{POS}CXCR5^{POS}$ T cells (Figure 2E-2F). Tfh-cell dependent IgM production was lower in co-cultures of patient-derived cells than in co-cultures of healthy control-derived cells (Figure 2E, $p<0.01$). This difference was not observed for the Tfh-cell dependent IgG production (Figure 2F).

*Plasmablast formation and immunoglobulin production regulated by peripheral Tfh cells are dependent on IL-21*

The importance of IL-21 in the functional interaction between Tfh cells and B cells was established by adding an anti-IL-21-R antibody to co-cultures containing $CD4^{POS}CXCR5^{POS}$ T cells and memory B cells from healthy controls or patients before transplantation. Differentiation of memory B cells into plasmablasts was inhibited by 48.4% (ranging from 11.3 to 89.9%, $p<0.05$, Figure 2G). Subsequently, both IgM and IgG production were inhibited by 89.2% [27.4-97.5%[, $p<0.05$ (Figure 2H) and 80.4% [3.6-99.8%], $p<0.05$ (Figure 2I).

*After transplantation, numbers of peripheral Tfh cells are higher in patients with pre-existent DSA than in patients without pre-existent DSA*

To study whether Tfh cells may induce the B cell differentiation *in vivo*, we determined the relation between peripheral Tfh ($CD4^{POS}CXCR5^{POS}$) cell numbers and the presence of DSA. For this purpose the absolute numbers of $CD4^{POS}CXCR5^{POS}$ T cells, before and after transplantation, were compared between patients with pre-existent DSA and patients without pre-existent DSA (Figure 3). To cover the different definitions for DSA-positivity, different mean fluorescence intensity (MFI) cut-offs were used to determine DSA-positivity or negativity. No differences were found at MFI cut-offs ≥1000, ≥2000 or ≥3000 (data not shown). When cut-offs for the MFI of ≥4000 or ≥5000 were used, patients with pre-existent DSA had more peripheral Tfh cells after transplantation than patients without pre-existent DSA (Figure 3A and 3B, $p<0.05$ and $p<0.001$). See Table 2 for baseline characteristics in patients with and without pre-existent DSA at an MFI cut-off ≥4000. All pre-existent DSA (MFI≥4000) were initially C1q-negative and were persistent after transplantation. In two patients the pre-existent DSA converted into C1q-positive, i.e. complement-binding DSA, after transplantation. In the other patients the DSA remained C1q-negative after transplantation. Five patients developed *de-novo* DSA after transplantation. In three of these patients DSA *de novo* were C1q-positive, two of whom suffered from an acute rejection within the first year after transplantation. Despite the limited numbers of patients with *de-novo* DSA, we compared them with patients without *de-novo* DSA and found no differences among the numbers of peripheral Tfh cells (data not shown).

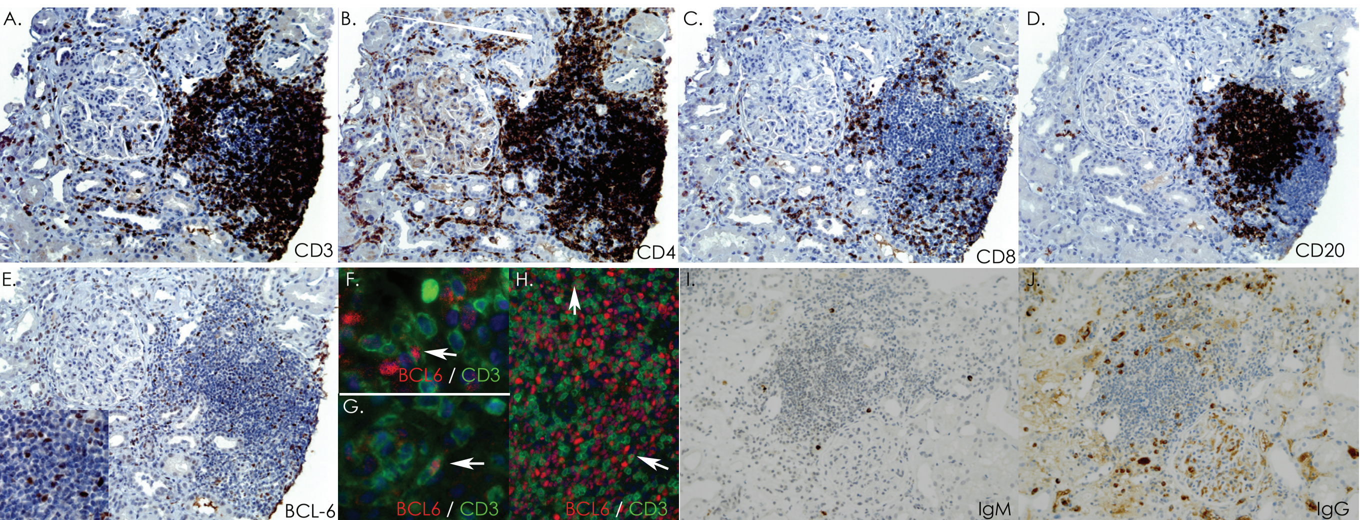



**Figure 4. In a typical acute T cell-mediated rejection biopsy Bcl-6POS T cells were co-localized with CD20POS B cells and immunoglobulins in follicular-like structures.**
(A-D) Immunohistochemistry for CD3 (A), CD4 (B), CD8 (C) and CD20 (D) was performed to determine the location of respectively T cells, T-helper cells, cytotoxic T cells and B cells in the same acute T-cell mediated rejection biopsy.
(E) Immunohistochemistry for Bcl-6 was performed to determine the location of this transcription factor for follicular T-helper cells among others in the same acute cellular renal rejection biopsy.
(F, G) CD3 and Bcl-6 double staining showed the presence of the transcription factor for follicular T-helper cells in T cells.
(H) Positive control for double-staining procedure on a tonsil
(I-J) Immunohistochemistry of IgM (I) and IgG (J) to determine the presence of IgM and IgG in the same acute cellular renal rejection biopsy.
Magnification of A to E: 20x; insert in E: 40x; F and G: 63x; H to J: 20x.

Chapter 2

**Table 3:** Immunofluorescence on 5 biopsies of kidney-transplants undergoing acute T cell mediated rejection

| | PA diagnosis | CD3 | CD4 | CD20 | BCL6 | IgM | IgG |
|---|---|---|---|---|---|---|---|
| 1 | TCMR I | ++ | ++ | ++ | + | ++ | ++ |
| 2 | TCMR I | ++ | ++ | ++ | ++ | not enough material | not enough material |
| 3 | TCMR I | ++ | ++ | ++ | ++ | - | - |
| 4 | TCMR I | ++ | ++ | ++ | + | + | +++ |
| 5 | TCMR I | ++ | ++ | +++ | + | ++ | ++ |

+++, very strongly positive; ++, strongly positive; +, positive; +/-, weakly positive; -/+, very weakly positive; -, negative; TCMR I, T cell (cellular) mediated rejection type I

*Tfh cells are present in follicular-like structures in acute T cell-mediated rejection kidney biopsies*

Acute T-cell mediated rejection biopsies (n=5) were stained to determine the presence of Tfh cells (representative example in Figure 4). Semi-quantitative analyses of the immunohistochemistry are depicted in Table 3. Follicular-like structures containing $CD4^{POS}$ T cells and $CD20^{POS}$ B cells were observed. Simultaneous staining of CD3 and Bcl-6 revealed Bcl-6-expressing T cells on the T-B cell border of the follicular-like structures, suggesting that these cells physically interact at the graft site to regulate B-cell function (Figure 4F-4G). Confirming this, the immunohistochemistry data showed that these biopsies were also positive for IgM and IgG (Figure 4I-4J). All biopsies were C4d-negative (data not shown).

## Discussion

We have studied the involvement of Tfh cells in B-cell mediated immune responses after kidney transplantation *in vitro* and *in vivo*. Several new insights were gained: (i) despite the decreased capacity of peripheral Tfh cells to stimulate B-cell differentiation after transplantation, the levels of IgM and IgG production capacity by plasmablasts in co-cultures were not affected by the immunosuppressive drugs *in vivo*; (ii) Tfh cells numbers post-transplantation were associated with high DSA titers before transplantation; (iii) graft infiltrating Tfh cells co-localized with B cells in follicular like structures; and (iv) locally-produced immunoglobulins were present in transplanted kidneys during rejection. Overall, the combination of *in-vitro* and *in-vivo* measurements in samples of patients before and after transplantation emphasize the importance of Tfh cells in humoral alloreactivity.

Even though the immunoglobulin production remained intact after transplantation, Tfh-cell dependent B-cell differentiation into plasmablasts was reduced, which suggests that either the Tfh cells or the B cells are partially influenced by immunosuppressive drugs. As IgM and IgG levels were comparable after transplantation, this indicates that at the single cell level, differentiated B cells produced higher amounts of IgM and IgG. (30, 31) The sustained Tfh-cell mediated immunoglobulin production after transplantation by differentiated B cells *in vitro* indicates that peripheral Tfh cells are capable to support B cells. Meier et al. also concluded

that Tfh cells are present and functional in the immunosuppressed milieu of intestinal transplant patients.(27) IgM-production capacity of the cells isolated from pre-transplant patients was lower than in healthy controls (Figure 2E), which may be explained by an impaired immune response caused by uremia in end-stage renal disease patients,(32) resulting in a lower antibody response towards new antigen.(33) IgG production by plasma cells represents a memory response, which was not impaired in our study cohort, suggesting that memory B cell or plasmablast functions are not susceptible to the devastating effects of end-stage kidney disease. Because in our study the function of peripheral Tfh cells was demonstrated by the differentiation of memory B cells into IgM and IgG producing plasmablasts in the presence of a super-antigen, further studies will have to establish whether these peripheral Tfh cells are fully capable to provide B cell help during allogeneic stimulation.

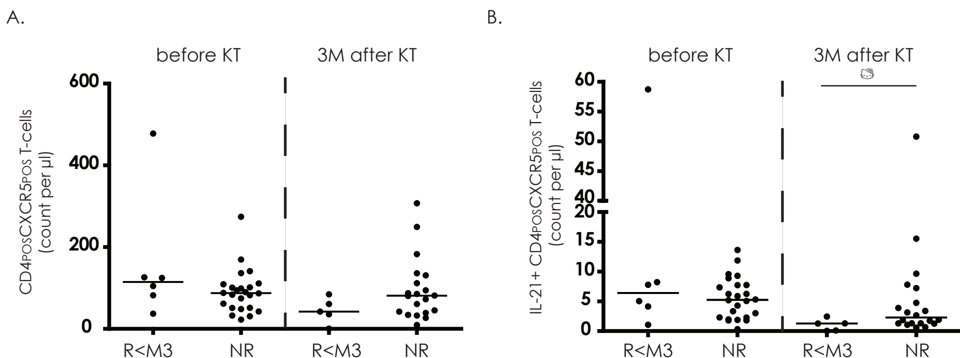


*In vivo*, the peripheral Tfh cells were also linked to humoral reactivity. The numbers of peripheral Tfh cells were associated with the presence of pretransplantation DSA measured by Luminex (Figure 3A-D). When cut-offs at MFIs ≥4000 or ≥5000 were used, identifying DSA with a low prevalence for false-positivity (34), patients with pre-existent DSA had more peripheral Tfh cells after transplantation than patients without pre-existent DSA. This can be explained by the fact that peripheral Tfh cells, including other CXCR5$^{POS}$ T cells, are memory T cells, which are less susceptible for the given immunosuppressive medication.(5, 35-37) As patients with pre-existent DSA were mostly patients with previous kidney transplants (Table 2), it is expected that HLA-specific memory T cells are present in these patients' circulations. Another explanation could be that pre-existent DSA indicates the presence of antigen-specific memory B cells, which can present donor antigen to naive T cells. These naive T cells consequently differentiate into antigen-specific Tfh cells and other subtypes of matured T cells.(5) Finally, the higher numbers of peripheral Tfh cells in patients with pre-existent DSA might be related to an underlying autoimmune disease. Two patients in the pre-existent DSA group suffered from glomerulonephritis before transplantation, which could have led to higher numbers of circulating Tfh cells.(38) The remaining five patients, however, did not have an autoimmune disease and still had higher numbers of circulating Tfh cell. Larger patient cohorts are needed to determine other confounders for the numbers of circulating Tfh cells.

All pre-existent DSA were non-complement binding, i.e. C1q-negative. The clinical relevance of non-complement binding C1q-negative DSA is under debate, and conflicting results are reported. C1q-negative DSA are often dismissed as clinically irrelevant(39, 40); however, patients with C1q-negative DSA have a more severe Banff-score during rejection(4) as well as a worse graft survival compared to patients without DSA.(4, 41) Hence, C1q-negative DSA cannot be labeled automatically as clinically irrelevant. C1q-positive DSA are known to predict graft loss.(4, 41) Despite these ongoing discussions we observed an association between peripheral Tfh cells and DSA, which suggests the involvement of Tfh cells in anti-donor B-cell mediated alloreactivity in kidney transplant patients. Interference of the Tfh-B cell interaction may be an approach to block B-cell differentiation into immunoglobulin-producing plasmablasts and plasma cells. One method of interfering in this interaction is the blockade of the IL-21 pathway, as demonstrated in our *in-vitro* studies (Figure 2G, 2H and 2I). The immunosuppressed regimen in this cohort probably partially interferes with this interaction.(29,30) The IL-21 production capacity by peripheral Tfh cells as measured in whole blood was decreased after transplantation. However, the presence of circulating DSA and the support by peripheral Tfh cells to humoral reactivity *in vitro* indicate that the interference by the current calcineurin-based immunosuppressive regimen is only partially effective.

In addition to pure humoral alloreactivity, Tfh cells are involved in B-cell alloreactivity classified as T-cell mediated rejection.(2, 3) The presence of Bcl-6 expressing Tfh cells was demonstrated in C4d-negative acute T-cell mediated rejection biopsies of kidney grafts. These findings strengthen our observations that Tfh cells contribute actively to the anti-donor response and that rejection is the result of T and B cell interactions, even when the rejection is not classified as mixed or antibody-mediated.(3, 28) As well as their effector functions, B cells still could exhibit their well-known antigen-presenting function during rejection. The Bcl-6 expressing T cells were, however, located on the T-B cell border, which is the same localization pattern of Tfh cells in lymph nodes to provide B cell help.(5) Furthermore, the presence of IgM and IgG in these follicular-like structures reflects humoral alloreactivity and thus effector function of differentiated B cells. Tfh cells may be the link between cellular and humoral reactivity in acute rejection in kidney transplantation. Whether these Tfh cells are always present in transplanted kidneys, such as their presence in stable allografts from intestinal transplant patients,(27) is yet unknown. In contrast to Tfh-cell function in the intestines, the B-cell supporting function of Tfh cells in transplanted kidneys is not desirable.

In conclusion, our blood and biopsy data on Tfh cells in kidney transplantation demonstrate that Tfh cells may mediate humoral alloreactivity, also in the immunosuppressed milieu of tacrolimus combined with MMF and steroids.




**Supplementary figure 1. Absolute numbers of CD4POSCXCR5POS T –lymphocytes (A) and IL21+CD4POSCXCR5POS T-lymphocytes (B) in patients who rejected within the first three months after transplantatie (R<M3) and patients who did not (NR).**

KT, kidney transplantation; *, $p<0.05$

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## Conflict of interest

The authors have no conflicts of interest to disclose in relation to this paper.

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# Chapter 3

## Belatacept Does Not Inhibit Follicular T Cell-Dependent B-Cell Differentiation in Kidney Transplantation

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## Abstract

Humoral alloreactivity has been recognized as a common cause of kidney transplant dysfunction. B-cell activation, differentiation and antibody production are dependent on IL-21$^+$ CXCR5$^+$ follicular T-helper (Tfh) cells. Here, we studied whether belatacept, an inhibitor of the co-stimulatory CD28-CD80/86-pathway, interrupts the crosstalk between Tfh- and B-cells more efficiently than the calcineurin inhibitor tacrolimus.

The suppressive effects of belatacept and tacrolimus on donor antigen-driven Tfh-B-cell interaction were functionally studied in peripheral blood mononuclear cells from 40 kidney transplant patients randomized to a belatacept- or tacrolimus-based immunosuppressive regimen.

No significant differences in uncultured cells or donor antigen-stimulated cells were found between belatacept and tacrolimus-treated patients in the CXCR5$^+$ Tfh cell generation and activation (upregulation of PD-1). Belatacept and tacrolimus *in vitro* minimally inhibited Tfh-cell generation (by ~6-7%) and partially prevented Tfh cell activation (by ~30-50%). The proportion of IL-21$^+$ activated Tfh-cells was partially decreased by *in vitro* addition of belatacept or tacrolimus (by ~60%). Baseline expressions and proportions of activated CD86$^+$ B-cells, plasmablasts and transitional B-cells after donor antigen-stimulation did not differ between belatacept and tacrolimus-treated patients. Donor antigen-driven CD86 upregulation on memory B-cells was not fully prevented by adding belatacept *in vitro* (~35%), even in supra-therapeutic doses. In contrast to tacrolimus, belatacept failed to inhibit donor antigen-driven plasmablast formation (~50% inhibition *vs.* no inhibition, respectively, $p<0.0001$).

In summary, donor antigen-driven Tfh-B-cell crosstalk is similar in cells obtained from belatacept and tacrolimus-treated patients. Belatacept is, however, less potent *in vitro* than tacrolimus in inhibiting Tfh-cell-dependent plasmablast formation.

**Key words:** belatacept, costimulatory blockade, follicular T-helper cells, immunoglobulins, plasmablasts, tacrolimus, transitional B-cells

## Introduction

B-cells and antibodies against the allograft are increasingly recognized to contribute to alloreactivity and subsequent graft failure after kidney transplantation under the currently used calcineurin inhibitor (CNI)-based immunosuppressive regimen.(1-7) CD4$^{+}$CXCR5$^{+}$ follicular T-helper (Tfh) cells are key mediators in B-cell activation, differentiation and antibody production.(8-12) Moreover, these cells infiltrate the allograft and co-localize with B-cells during acute rejection after kidney transplantation.(13, 14) In alloreactivity, both Tfh and B-cells are activated by the same antigen via their T and B-cell receptor, respectively.(15) The CD40-40L, CD28-CD80/86 and ICOS-ICOSL costimulatory pathways, and the cytokines IL-6 and IL-21, are important in this Tfh-B-cell interaction, and for B-cell differentiation into immunoglobulin-producing plasma cells.(16-21)

Belatacept is a selective inhibitor of the CD28-CD80/86 pathway and subsequently interrupts Tfh-B-cell interaction.(21, 22) In animal transplant models, belatacept, or the lower affinity version abatacept (CTLA4 Immunoglobulin), inhibited germinal center formation, clonal B-cell expansion, IL-21 production and the development of donor-specific anti-human leukocyte antigen antibodies (DSA).(14, 23) These findings were in line with observations from a large randomized, controlled trial in kidney transplant patients where the belatacept-based regimen resulted in a significantly lower prevalence of DSA than the cyclosporine A (CsA)-based regimen at 7 years after transplantation: 4.6% *vs.* 17.8%, respectively.(24) However, in all these clinical studies, belatacept was combined with other immunosuppressive drugs: in the BENEFIT and BENEFIT-EXT trials belatacept was combined with MMF and prednisone, and in the animal studies belatacept was combined with either sirolimus or T-cell depleting antibodies.(14, 23-25)

Contradictory effects of tacrolimus on B-cell activation, proliferation and differentiation have been reported (26-28) because tacrolimus only inhibits calcium-influx dependent, and not calcium-independent, B- and T-cell activation.(27, 29) This calcineurin-mediated activation is dependent on the type of stimulus.(26, 28, 29) B-cell activation can thus be prevented by calcineurin-inhibition in an antigen-dependent manner. The effect of tacrolimus on donor antigen-stimulated Tfh-B-cell interaction is unknown in kidney transplantation.

In addition to the *in vivo* animal studies and clinical data that suggest belatacept effectively inhibits the humoral immune response specific for donor antigen,(14, 23, 24), this class of immunosuppressive agents may also favor a more regulatory rather than effector alloreactive B-cell activity by enhancing the survival of transitional B-cells over memory B-cells in the long term.(30) Theoretically, this may reduce rejection risk.(15, 30-34)

So far no studies have been conducted which compared the effects of belatacept to tacrolimus, on Tfh-B-cell interaction in kidney transplantation. We hypothesized that belatacept more efficiently interrupts Tfh-B-cell crosstalk than tacrolimus. Therefore, we compared i) the frequencies of Tfh and B-cell subsets between belatacept- and tacrolimus-treated patients; ii) the *in vitro* donor antigen-driven Tfh-B-cell interaction in peripheral blood mononuclear cells (PBMCs) obtained from belatacept- and tacrolimus-treated kidney transplant patients; and iii) the isolated the effects of *additional* belatacept and tacrolimus *in vitro* on donor antigen-driven Tfh-B cell interaction in PBMCs obtained from the same patients.

## Material and Methods

### *Study population and materials*

Materials were collected from 40 kidney transplant patients and their donors who participated in a prospective, randomized-controlled trial (approved by the Medical Ethical Committee of the Erasmus MC, University Medical Centre Rotterdam; MEC-2012-42, EUDRACT CT # 2012-003169-16). After written informed consent, patients were included and randomized to a tacrolimus-based (control) or belatacept-based (experimental) immunosuppressive regimen. For in- and exclusion criteria, refer to Supplementary Table 1. All procedures were in accordance

with the ethical standards of the Declaration of Istanbul.(35) In short, both groups received basiliximab induction therapy (Simulect®, Novartis, Basel, Switzerland), followed by maintenance therapy with mycophenolate mofetil (MMF) and prednisolone, which was tapered to 5 mg by month 3 after transplantation. Maintenance therapy with tacrolimus (Prograf®, Astellas Pharma, Tokyo, Japan) was adjusted to pre-dose levels of 5-10 ng/mL, while belatacept (Nulojix®, Bristol-Meyers Squibb, NYC, NY) was dosed according to bodyweight (Less-Intensive regimen of the BENEFIT trials).(36)

Lithium heparin blood was collected from patients one day before transplantation, and 3 months after transplantation or during clinically suspected acute rejection before any additional anti-rejection therapy was given. All samples were processed within 24 hours of withdrawal. If patients had a biopsy-proven acute rejection (BPAR) (2) materials of that time point were used instead of their materials of 3 months after transplantation. Lithium heparinized blood from donors was collected one day before transplantation. Peripheral blood mononuclear cells (PBMCs) were isolated from blood using the Ficoll density isolation method.

***Mixed lymphocyte reactions (MLRs)***

Patients PBMCs obtained after transplantation were thawed and used in MLRs. PBMCs were obtained 3 months after transplantation in stable, non-rejecting patients or before additional anti-rejection therapy was given in rejecting patients. Live cells were counted under a light microscope and distinguished from dead cells with Trypan Blue. Per patient ~5 x $10^5$ uncultured PBMCs were stained for phenotypical analyses. 5 x $10^4$ patients' PBMCs/well (in a 96-wells plate) were stimulated for 7 days at 37°C with 5 x $10^4$ CFSE-labeled, irradiated donor PBMCs (40 Gy) in RPMI 1640 + 10% heat-inactivated fetal bovine serum. Half of patients' PBMCs were incubated for 1 hour with clinically therapeutic concentrations of belatacept (10 µg/mL) (37) or tacrolimus (10 ng/mL), dependent on the randomization group, before donor antigen was added. After the donor antigen was added, these amounts of immunosuppressive drugs remained in the culture for the whole period of 7 days. At the end of day six 100 µL supernatant per well was harvested and stored at -20°C. Subsequently, Monensin and Brefeldin (GolgiStop and GolgiPlug, BD Biosciences, Franklin Lakes, NJ) were added for 16 hours over night in a concentration of 1:1500 and 1:1000, respectively, to allow the measurement of intracellularly accumulated cytokines in PBMCs.

Refer to Figure 1 for the different comparisons made in our study. Proportions of studied cell populations (see *Flow Cytometry*) were compared between:

i) belatacept- and tacrolimus-treated patients in uncultured, unstimulated PBMCs;

ii) belatacept- and tacrolimus-treated patients in 7-day donor antigen-stimulated PBMCs;

iii) uncultured, unstimulated PBMCs and 7-day donor antigen-stimulated PBMCs in belatacept-treated patients

iv) uncultured, unstimulated PBMCs and 7-day donor antigen-stimulated PBMCs in tacrolimus-treated patients.

Patient PBMCs obtained one day before transplantation were also cultured with donor antigen in the same way to investigate whether PBMCs obtained from an immunosuppressed environment reacted differently on donor antigen compared to PBMCs before any immunosuppression was given.

Post-transplant PBMCs obtained from 3 belatacept-treated and 3 tacrolimus-treated patients were used in MLRs to study the CD40-CD40L, PD1-PDL1 and ICOS-ICOSL interaction

during costimulation blockade by belatacept 10 μg/mL or calcineurin-inhibition by tacrolimus 10 ng/mL. MLRs were conducted as described above, only with 5 x $10^4$ CD3 and CD19-depleted irradiated donor PBMCs instead of CFSE-labeled donor PBMCs. The same methods were used in 6 independent MLRs of healthy controls' PBMCs to determine free CD80/86 expression after allo-antigen stimulation in the presence of various concentrations of belatacept (0-1000 μg/mL) or tacrolimus (0-100 ng/mL).

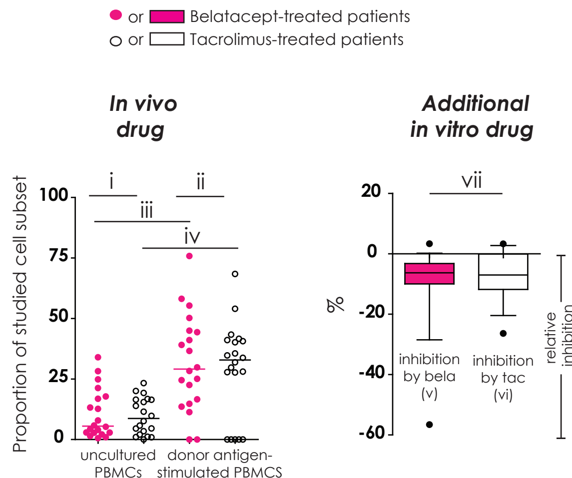


**Figure 1. Different comparisons made in conducted studies (example figure).**

In the left column ("in vivo drug"), the proportions of studied cell populations (see Flow Cytometry, Materials and Methods) were compared between i) belatacept- and tacrolimus-treated patients in uncultured, unstimulated PBMCs; ii) belatacept- and tacrolimus-treated patients in 7-day donor antigen-stimulated PBMCs; iii) uncultured, unstimulated PBMCs and 7-day donor antigen-stimulated PBMCs in belatacept-treated patients; and iv) uncultured, unstimulated PBMCs and 7-day donor antigen-stimulated PBMCs in tacrolimus-treated patients. In the right column ("Additional in vitro drug"), the relative inhibition by additional in vitro belatacept or tacrolimus is depicted by "v" and "vi", respectively, in this example figure. If the median relative inhibition is significantly smaller than zero, the in vitro drug significantly decreases the proportion of the studied cell type. Per cell type, the relative inhibitions were compared between belatacept and tacrolimus in vitro. This is depicted by "vii" in this example figure.

### *Carboxyfluorescein succinimidyl ester (CFSE) labeling of PBMCs*

To distinguish between patient and donor PBMCs in the MLRs, donor PBMCs were labeled with the cell-permeable, intracellular linker CFSE (Thermo Fisher Scientific, Waltham, MA), according to manufacturer's manual. CFSE-labeled donors' PBMCs expressed an MFI>$10^4$ on the FITC-channel.

### *Co-cultures of isolated follicular T helper (Tfh) cells and memory B-cells* (13, 38)

$CD3^+CD4^+CXCR5^+$ T-cells and $CD19^+CD27^+$ B-cells from 3 healthy controls and 3 patients before transplantation were isolated using a FACSAria II 4L SORP™ (BD Biosciences). From both populations 2 x $10^4$ cells/well were co-cultured for 7 days at 37°C with 4 x $10^4$ 40 Gy irradiated CD3/CD19-depleted, allogeneic PBMCs. Half of the wells were spiked with belatacept 10 μg/

mL or tacrolimus 10 ng/mL. After 7 days co-culture, supernatants were collected and stored at -20 °C until analysis, and the proportion of memory B-cells that differentiated into $CD27^{+}CD38^{++}$ plasmablasts was measured.

***Flow cytometry***

For a complete overview of the monoclonal antibodies used, see Supplementary Table 2. Follicular T helper (Tfh) cells were defined as $CD3^{+}CD4^{+}CXCR5^{+}$ T lymphocytes, and classified as activated or resting by their expression of the activation marker and co-inhibitor PD-1.(39, 40) Tfh cell generation was defined as an increase in the proportion of $CXCR5^{+}$ within $CD4^{+}$ T-cells; Tfh cell activation comprised the increase in the proportion of $PD\text{-}1^{+}$ within $CD4^{+}CXCR5^{+}$ T-cells; and the generation of activated Tfh-cells was equivalent to an increase in the proportion of $CXCR5^{+}PD\text{-}1^{+}$ within $CD4^{+}$ T-cells. The characteristic Tfh-cell cytokine IL-21 was determined in donor antigen-stimulated PBMCs in the presence or absence of belatacept or tacrolimus.

Within $CD19^{+}$ B-cells, we distinguished $CD27^{-}$ naïve B-cells, $CD27^{+}$ memory B-cells, $CD24^{+}CD38^{++}$ transitional B-cells, and $CD27^{+}CD38^{++}$ plasmablasts. Free CD86 expression on B-cells was measured on donor antigen-stimulated PBMCs by using an antibody that is competitive with belatacept for CD86, but binds with lower affinity.(41) Expressions of the immune regulatory cytokine IL-10 in transitional B-cells and of the aggressive effector cytokine TNFa in plasmablasts were also assessed after 7 days of donor antigen stimulation.

***ELISA for IgM and IgG3 measurements***

IgM concentrations in supernatants from all cell cultures were determined by ELISA. A calibration curve using human IgM 1.6 – 100 ng/mL (Sigma-Aldrich, St. Louis, MO) was used to quantify results. All experiments were performed *in duplo* (medians were used for end result). Supernatants were diluted, if necessary, to fit within the measurements of the calibration curve. Measurements <1.6 ng/mL were considered negative. IgG3 concentrations were measured in the same way using an ELISA-kit with a calibration curve of 4.4 – 200 ng/mL (Affymetrix/eBioscience, Santa Clara, CA).

***Single bead Luminex assay***

DSA were measured in (14-150x) concentrated culture supernatants using the Single Antigen beads mix from the LABScreen Single Antigen class II kit (Thermo Fisher, Waltham, MA).(13) Microbeads were analyzed with a Luminex LabscanTM 100 analyzer using the Luminex 100IS software and analyzed using the HLA Fusion 3.0 software. All samples fulfilled the quality criteria for reactivity of the control beads.

***Calculation of the relative inhibition***

The relative inhibition was used to account for inter-patient variability in the response to donor antigen (Figure 1, "*additional in vitro*" column). The relative inhibition by *additional in vitro* belatacept or tacrolimus was calculated for the donor antigen-driven Tfh cell generation, Tfh cell activation and the generation of activated Tfh-cells as well as for the donor antigen-driven intracellular IL-21 by activated Tfh-cells and the formation of IL21+ activated Tfh-cells. The relative inhibition by the *in vitro* drugs was also assessed for the upregulation of CD86 on naïve and memory B-cells, the formation of plasmablasts and their IgM production, and the transitional B cell survival. For these calculations the proportions of aforementioned cell subsets after donor

antigen stimulation were set to 0 by using the following equation:

***Relative inhibition (after donor antigen stimulation) =***

*[(Proportion in the presence of in vitro drug)-(Proportion without in vitro drug added)] / [Proportion without in vitro drugs added]*

If the median relative inhibition is significantly smaller than zero, the *in vitro* drug significantly decreases the proportion of the studied cell type (Figure 1, comparison “v” and “vi”). Per cell type, the relative inhibitions were compared between belatacept and tacrolimus *in vitro* (Figure 1, comparison “vii”).



### *Statistical analyses*

Proportions of cell subsets in uncultured or donor antigen-stimulated PBMCs were compared between the belatacept and tacrolimus group using the Mann-Whitney U test (Figure 1, comparisons “i” and “ii”) as well as baseline characteristics that were continuous variables. Baseline characteristics that were categorical variables were compared with the Fisher’s Exact Test. Proportions of cell subsets between uncultured and donor antigen-stimulated PBMCs were compared using the Wilcoxon Signed Rank test (Figure 1, comparisons “iii” and “iv”). The median relative inhibition was compared to a theoretical mean of zero (=no inhibition) with the Wilcoxon Signed Rank test to determine if the inhibition by the *in vitro* drug was statistically significant (Figure 1, comparisons “v” and “vi”). The relative inhibitions by *in vitro* belatacept and *in vitro* tacrolimus were compared using the Mann-Whitney U test (Figure 1, comparison “vii”).

Multivariable linear regressions were used to examine the *in vitro* effects of belatacept compared to tacrolimus on donor antigen-activated Tfh and B-cell subsets, adjusted for confounders (presence of *in vitro* added drugs [present *vs.* absent], time point [after *vs.* before transplantation] and BPAR [PBMCs obtained during rejection *vs.* 3 months after transplantation). To avoid multiple testing errors, only cell subsets in which the relative inhibition significantly differed between belatacept and tacrolimus *in vitro* were included for these analyses.

SPSS Statistics 21.0 (IBM, Armonk, NY) was used for statistical analyses. Unless mentioned otherwise, medians [+ range] are given for continuous variables, and numbers (+ proportions) are given for categorical variables. *p*-values with a 2-sided a of <0.05 were considered statistically significant.

## Results

### *1.* ***Study population***

No significant differences were observed with regard to baseline characteristics between the two treatment groups (Supplementary Table 3). Seventeen (85%) patients in the belatacept and 19 (95%) in the tacrolimus group completed the 1-year follow-up period (last patient, last visit occurred on February 19$^{th}$, 2016). The incidence of biopsy-proven acute rejection was higher among the belatacept-treated patients than in the tacrolimus-treated patients: n = 11 (55%) *vs*. n = 2 (10%), respectively; p = 0.006 (unpublished, submitted data).

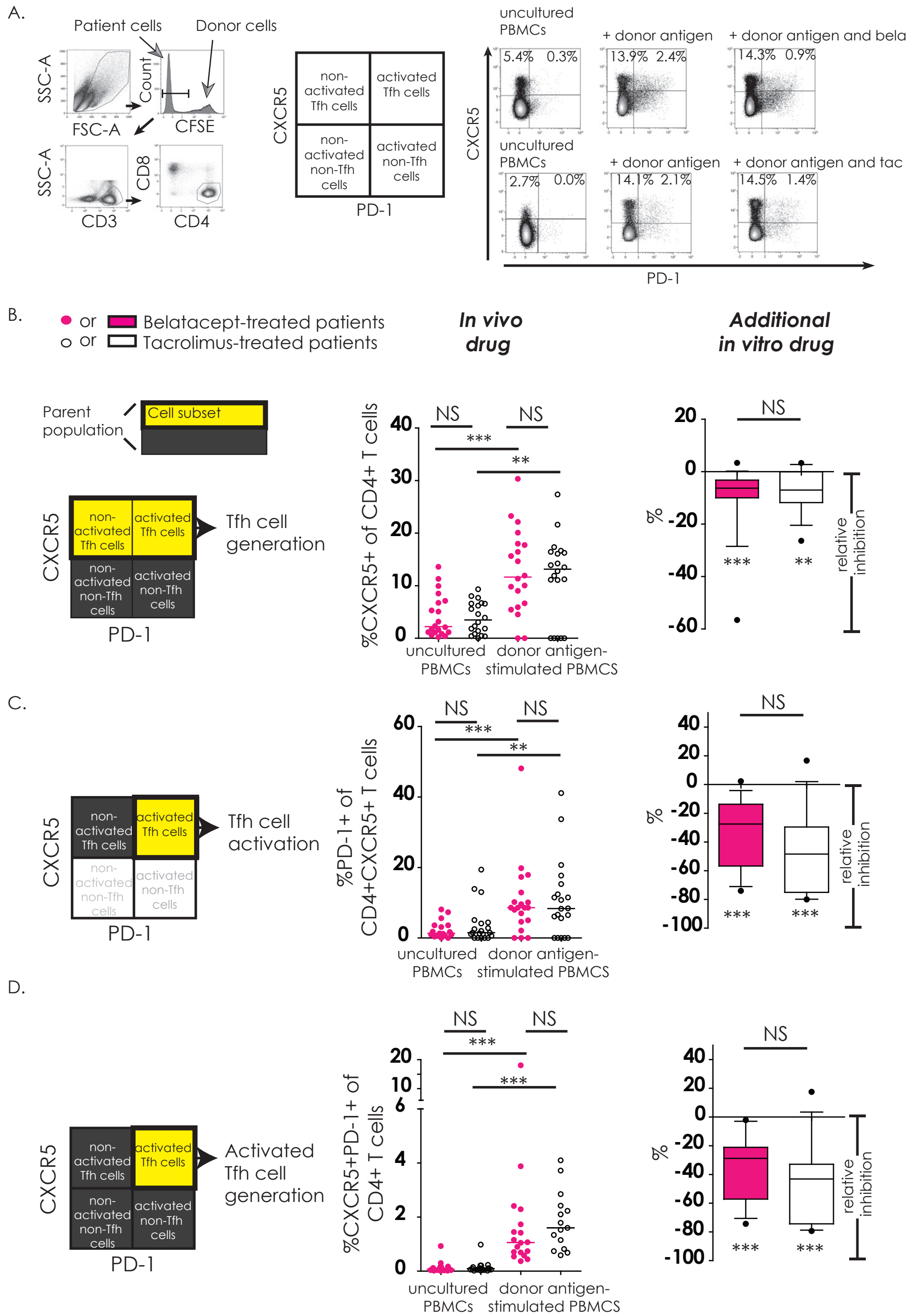


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**Figure 2. No differences between belatacept and tacrolimus in vivo or in vitro on donor antigen-driven follicular T helper cell formation and activation in cultured PBMCs**

Two typical examples are depicted for CXCR5 and PD-1 expression on T helper cells in uncultured PBMCs, and after 7 days of donor antigen-stimulation, in the presence or absence of belatacept and tacrolimus (A). Follicular T helper (Tfh)-cells were distinguished from non Tfh-cells by surface CXCR5-expression, while activated cells were defined by surface PD-1 expression. Donor PBMCs were discriminated by CFSE-labeling them prior to the mixed lymphocyte reaction and gating them out after.

The proportions are depicted of CXCR5+ within CD4+ T-cells (B); PD-1+ within CD4+CXCR5+ T-cells (C); and CXCR5+PD-1+ within CD4+ T-cells (D).

In the graphs in the "in vivo drug" column, proportions of aforementioned cell populations were compared i) between belatacept- and tacrolimus-treated patients in uncultured, unstimulated PBMCs; ii) between belatacept- and tacrolimus-treated patients in 7-day donor antigen stimulated PBMCs; iii) between uncultured, unstimulated PBMCs and 7-day donor antigen stimulated PBMCs in belatacept-treated patients; and iv) between uncultured, unstimulated PBMCs and 7-day donor antigen stimulated PBMCs in tacrolimus-treated patients. Every dot represents PBMCs of a single patient.

In the graphs in the "additional in vitro drug" column the relative inhibitions by additional in vitro belatacept and tacrolimus are depicted for aforementioned cell populations in the same belatacept- and tacrolimus-treated patients. The proportions of these cell populations after donor antigen-stimulation in the absence of in vitro drugs are set to zero. The median relative inhibitions by belatacept and tacrolimus were tested against a theoretical median of 0. Asterisks below the boxes depict the p-values of these tests. The relative inhibitions were compared between belatacept and tacrolimus in vitro. Lines in boxes represent medians, borders of boxes represent 25th and 75th percentiles, error bars present 10th and 90th percentiles. Every box represents cultures of PBMCs obtained from n = 20 belatacept-treated or n = 20 tacrolimus-treated patients.

* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, NS = not significant



## *2. The effects of belatacept and tacrolimus on follicular T helper (Tfh) cells*

### *2.1 Tfh cell generation and activation (CXCR5 and PD-1 upregulation)*

The surface expression of the Tfh marker CXCR5 and the activation marker PD-1 were determined on $CD4^+$ T-helper cells (Figure 2).

Baseline expression of CXCR5 on CD4+ T-cells in uncultured PBMCs was comparable between belatacept- and tacrolimus-treated patients (Figure 2B, "*in vivo drug*" column). Following donor antigen-stimulation, Tfh-cell generation, defined by the expression of CXCR5 on $CD4^+$ T-cells, increased ~3 to 4 fold in PBMCs obtained from belatacept- and tacrolimus-treated patients, $p$<0.001 and $p$<0.01, respectively (Figure 2B, "*in vivo drug*" column). This process was inhibited when the samples were spiked *in vitro* by adding tacrolimus and belatacept. The relative inhibition of Tfh-cell generation, however, was similar between belatacept and tacrolimus: -6.3% [-56.6 to +3.3%], $p$<0.001, by belatacept and -7.0% [-26.4 to +3.3%], $p$<0.01, by tacrolimus (Figure 2B, "*additional in vitro drug*" column).

The expression of PD-1 on $CD4^+CXCR5^+$ T-cells in uncultured PBMCs was similarly low in belatacept- and tacrolimus-treated patients (medians 1.3% and 1.5%, respectively; Figure 1C, "*in vivo drug*" column). Tfh-cells of belatacept- and tacrolimus-treated patients were significantly activated after donor antigen-stimulation, *i.e.* a significant increase of PD-1 expression on $CXCR5^+CD4^+$ T-cells was observed. The relative inhibition of Tfh cell activation was -27.5% [-74.0 to +2.3%], $p$<0.001, by belatacept and -48.4% [-80.0 to 16.7%], $p$<0.001, by tacrolimus, inhibition by belatacept *vs.* tacrolimus; $p$=0.13 (Figure 2C, "*additional in vitro drug*" column).

The proportion of $CXCR5^+PD\text{-}1^+$ double-positive $CD4^+$ T-cells was negligible in uncultured PBMCs from belatacept- and tacrolimus-treated patients (Figure 2D, "*in vivo drug*"

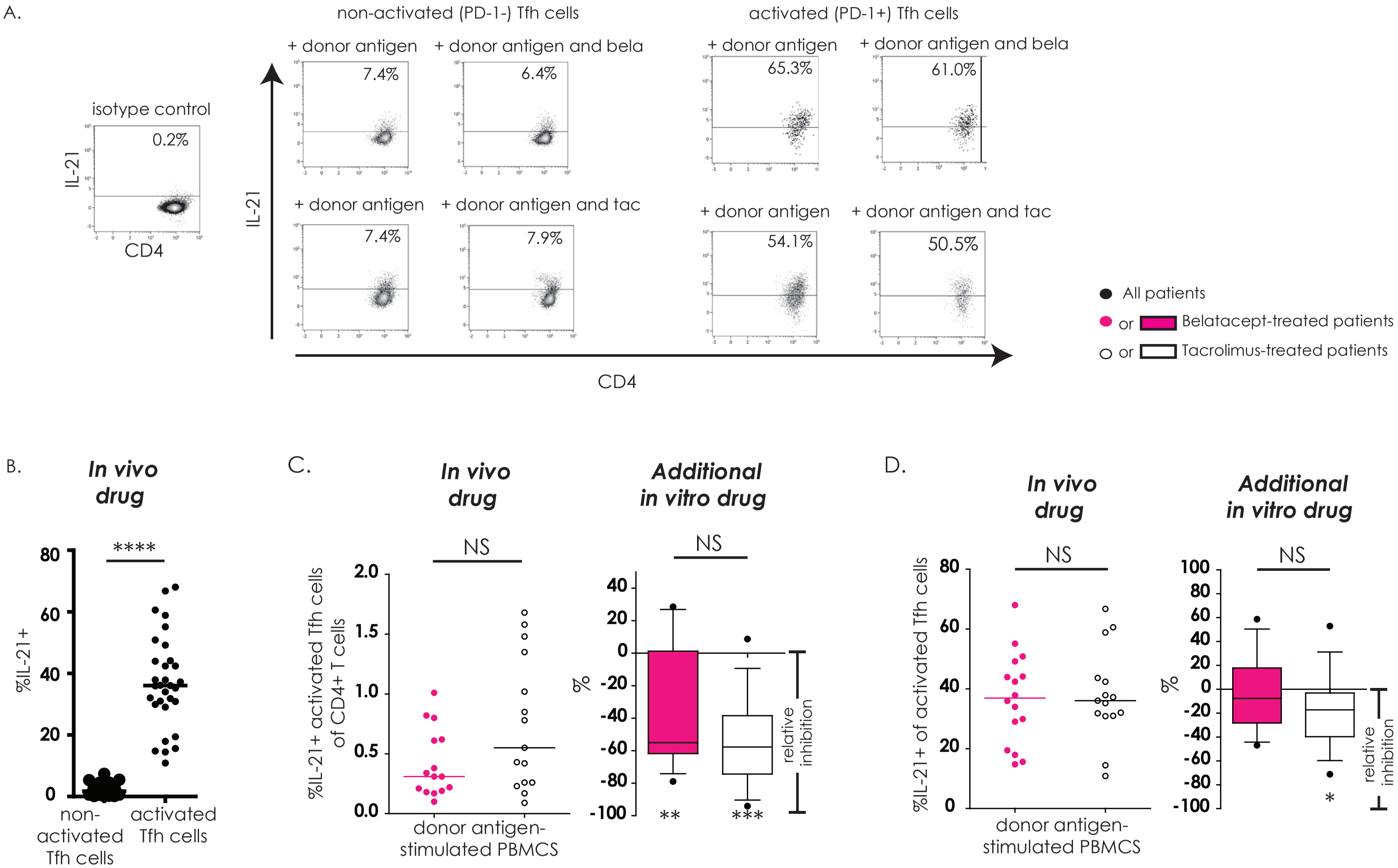


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**Figure 3. IL-21 production by remaining activated follicular T helper cells was not inhibited by belatacept in vitro**

A typical example is depicted for the intracellular IL-21 production after donor antigen-stimulation in non-activated and activated Tfh-cells (CXCR5+PD-1- and CXCR5+PD-1+ CD4+ T-cells, respectively) in the presence and absence of belatacept (A). The proportions of IL-21+ cells within non-activated and activated Tfh-cells were compared after 7 days of donor antigen-stimulation of PBMCs obtained from both belatacept- and tacrolimus-treated patients (B). The proportions of IL21+ activated Tfh-cells within CD4+ T-cells (C) and the proportions of IL-21+ cells within activated Tfh-cells (D) were compared between 7-day donor antigen-stimulated PBMCs obtained from the belatacept and tacrolimus group ("in vivo" column), as well as the relative inhibitions by in vitro addition of belatacept or tacrolimus ("additional in vitro" column).

In the graphs in the "in vivo drug" columns, every dot represents PBMCs of a single patient.

In the graphs in the "additional in vitro drug" columns the relative inhibitions by additional in vitro belatacept and tacrolimus are depicted for aforementioned cell populations in the same belatacept- and tacrolimus-treated patients. The proportions of these cell populations after donor antigen-stimulation in the absence of in vitro drugs are set to zero. The median relative inhibitions by belatacept and tacrolimus were tested against a theoretical median of 0. Asterisks below the boxes depict the p-values of these tests. The relative inhibitions were compared between belatacept and tacrolimus in vitro. Lines in boxes represent medians, borders of boxes represent 25th and 75th percentiles, error bars present 10th and 90th percentiles. Every box represents cultures of PBMCs obtained from n = 20 belatacept-treated or n = 20 tacrolimus-treated patients.

* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, NS = not significant

column). The generation of activated Tfh-cells (defined by an increase of the proportion of $CXCR5^+PD\text{-}1^+$ double-positive $CD4^+$ T-cells) was 1.1% [0.4 to 18.1%] in donor antigen-stimulated PBMCs from belatacept-treated patients and 1.6% [0.6 to 4.1%] in those from tacrolimus-treated patients. These proportions were not significantly different. The generation of activated Tfh-cells was inhibited by both belatacept and tacrolimus *in vitro* (Figure 2D, "*additional in vitro drug*" column): the relative inhibition was -28.8% [-74.3 to -2.1%], $p<0.001$ by belatacept, and -32.9% [-79.4 to +17.5%] by tacrolimus, $p<0.001$.

## *2.2 Tfh cell function (intracellular IL-21 production)*

As described previously, "activated Tfh-cells" were defined as Tfh-cells that upregulated PD-1 after donor antigen stimulation and "non-activated Tfh-cells" were defined as Tfh-cells that failed to upregulate PD-1 after donor antigen stimulation. IL-21, a key cytokine in Tfh-B-cell interaction, and subsequent B-cell differentiation into immunoglobulin-producing plasma cells, was assessed in Tfh-cells (Figure 3). The donor antigen-stimulated IL-21 production was highest in activated Tfh-cells (Figure 3B). The proportions of $IL21^+$ activated Tfh-cells within $CD4^+$ T-cells were similar between donor antigen-stimulated PBMCs from the belatacept and tacrolimus groups (Figure 3C, "*in vivo drug*" columns). The total proportion of $IL21^+$ activated Tfh-cells was partially decreased by belatacept and tacrolimus *in vitro*. The relative inhibition was -55.0% [-79.0 to +28.6%], $p<0.01$, in the presence of belatacept and -57.7% [-94.1 to +8.7%], $p<0.001$, in the presence of tacrolimus (Figure 3C, "*in vivo drug*" columns). No differences between the inhibition by belatacept and tacrolimus were observed (Figure 3C, "*additional in vitro drug*" column). When we focused on the remaining activated Tfh-cells in the presence of *in vitro* drugs, a substantial proportion could still produce IL-21. Even though the relative inhibitions of intracellular IL-21 production were not significantly different between belatacept and tacrolimus *in vitro*, only the latter (minimally) inhibited IL-21 production by activated Tfh-cells: relative inhibition -17.3% [-71.2 to +52.9%, $p<0.05$ (Figure 3D, "*additional in vitro drug*" column).

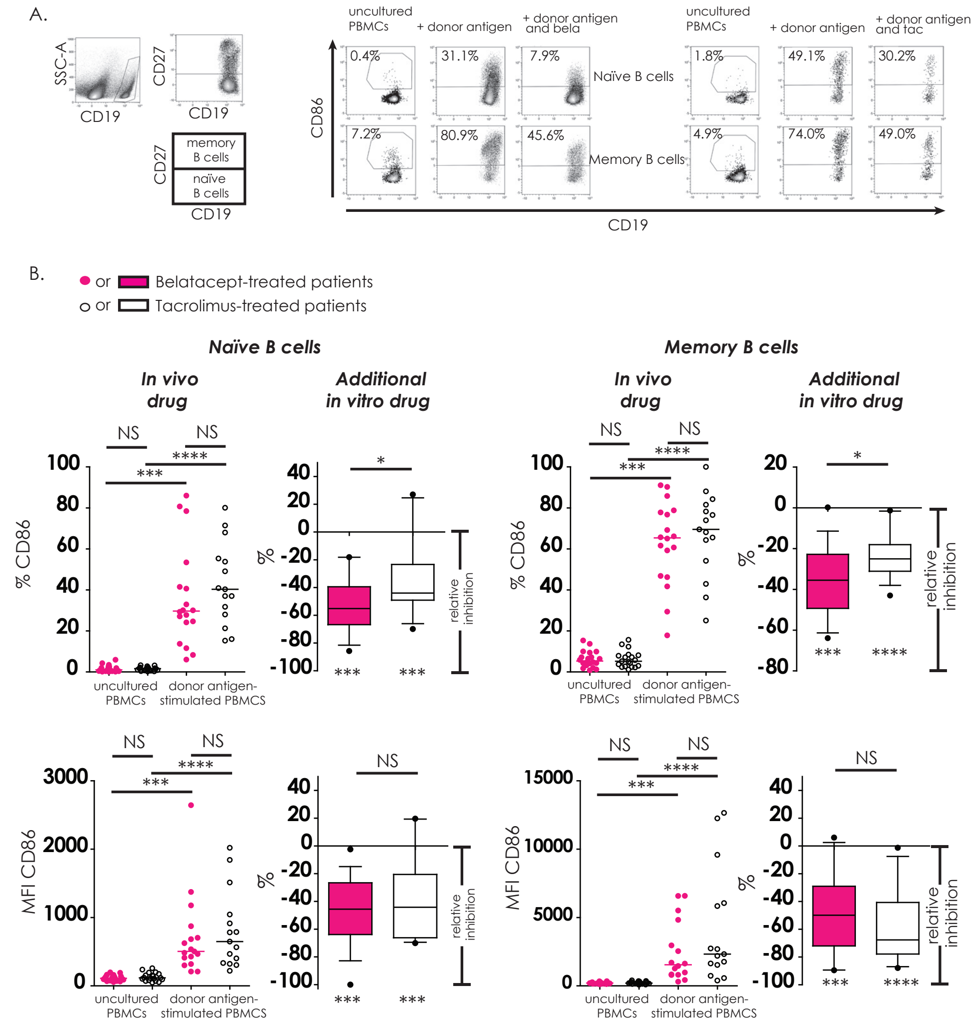


**Figure 4. Donor antigen-stimulated CD86 upregulation on B-cells is only partially blocked by belatacept in vitro**

Two typical examples are depicted for the free CD86 expression after 7 days of donor antigen-stimulation on naïve (CD27-) and memory (CD27+) CD19+ B-cells, in the presence and absence of belatacept or tacrolimus (A). The proportions are depicted of CD86+ cells within naïve and memory B-cells as well as the Median Fluorescence Intensities (MFIs) of CD86 within naïve and memory B-cells (B).

In the graphs in the "in vivo drug" columns, proportions and MFIs of aforementioned cell populations were compared i) between belatacept- and tacrolimus-treated patients in uncultured, unstimulated PBMCs; ii) between belatacept- and tacrolimus-treated patients in 7-day donor antigen stimulated PBMCs; iii) between uncultured, unstimulated PBMCs and 7-day donor antigen stimulated PBMCs in belatacept-treated patients; and iv) between uncultured, unstimulated PBMCs and 7-day donor antigen stimulated PBMCs in tacrolimus-treated patients. Every dot represents a single culture of PBMCs.

In the graphs in the "additional in vitro drug" columns the relative inhibitions by additional in vitro belatacept and tacrolimus are depicted for aforementioned cell populations in the same belatacept- and tacrolimus-treated patients. The proportions or MFIs of these cell populations after donor antigen-stimulation in the absence of in vitro drugs are set to zero. The median relative inhibitions by belatacept and tacrolimus were tested against a theoretical median of 0. Asterisks below the boxes depict the p-values of these tests. The

relative inhibitions were compared between belatacept and tacrolimus in vitro. Lines in boxes represent medians, borders of boxes represent 25th and 75th percentiles, error bars present 10th and 90th percentiles. Every box represents cultures of PBMCs obtained from n = 20 belatacept-treated or n = 20 tacrolimus-treated patients.

* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, NS = not significant

### *2.3 Summary of the effects of belatacept and tacrolimus on Tfh-cells*

Belatacept and tacrolimus minimally inhibited Tfh-cell generation and partially prevented Tfh-cell activation. The proportion of IL-21$^+$ activated Tfh-cells was not completely diminished by *in vitro* addition of belatacept or tacrolimus. Thus the remaining activated Tfh-cells have the potential capacity to provide B-cell help. Next, we tested the immunosuppressive effects of both agents on B-cell activation and functional Tfh-B-cell crosstalk.



## *3. The effects of belatacept and tacrolimus on B-cells*

### *3.1 B cell activation (CD86 upregulation)*

Part of the activation of B-cells and their ability to proliferate, differentiate and function as antigen-presenting cells is reflected by their (free) CD86-expression, but also by the expression of CD40 and ICOS-L. Here, The efficacy of belatacept was determined by means of B-cell activation, i.e. the free expression of CD86, which was measured on naïve CD19+CD27- and memory CD19$^+$CD27$^+$B-cells (in proportions and Median Fluorescence Intensities [MFIs]), using tacrolimus as control (Figure 4). The expression of CD40 and ICOS-L on B cells in the presence of belatacept is described in paragraph 4.1.

CD86 expression was almost absent on naïve B-cells and low on memory B-cells in unstimulated uncultured PBMCs (Figure 4B, "*in vivo*" columns). No differences were observed between belatacept- or tacrolimus-treated patients. After donor antigen-stimulation both the proportions of CD86$^+$ B-cells, as well as the expression of CD86 (MFIs) significantly increased on both naïve and memory B-cells (Figure 4B, "*in vivo*" columns). These were not different between the belatacept and tacrolimus group.

Despite the selective binding of belatacept to CD86,(22) the upregulation of CD86 was not completely blocked by the *in vitro* addition of belatacept. The relative inhibition of CD86 upregulation (proportion) by belatacept was -55.2% [-85.7 to -18.8%], $p$<0.001, on naïve B-cells, and -35.5% [-63.9 to +0.2%], $p$<0.001, on memory B-cells (Figure 4B, "*additional in vitro drug*"). The relative inhibition of CD86 upregulation on naïve and memory B-cells was significantly more by belatacept than by tacrolimus *in vitro*, $p$<0.05. MFIs of CD86 on naïve and memory B-cells were significantly decreased by both belatacept and tacrolimus *in vitro* (Figure 4B, "*additional in vitro drug*"). The relative inhibitions of CD86 MFIs were comparable between belatacept and tacrolimus.

To determine if the residual B-cell activation in the presence of immunosuppressive drugs was dose-dependent, the relative inhibitions by belatacept and tacrolimus were measured in the presence of supra-therapeutic concentrations. Even in the presence of supra-therapeutic concentrations of belatacept, membrane CD86 expression on allo-antigen-stimulated B-cells was still detectable (Supplementary Figure 1): The relative inhibition by 1000 µg/mL belatacept (100 x higher than the therapeutic concentration) was -72.4% [-86.5 to -19.7%], $p$<0.05, in naïve B-cells and -43.2% [-53.9 to -7.4%], $p$<0.05, in memory B-cells.

Since belatacept binds CD80 with much higher affinity than CD86,(22, 41) the residual surface expression of CD80 was low on activated naïve and memory B-cells in the presence of the different doses of belatacept (Supplementary Figure 1): The relative inhibition by 1000 µg/

mL belatacept was -90.2% [-97.5 to -75.4%], $p<0.05$, in naïve B-cells and -85.0% [-95.1 to -57.3%], $p<0.05$, in memory B-cells. CD80 expression on B-cells was not significantly decreased in the presence of tacrolimus.

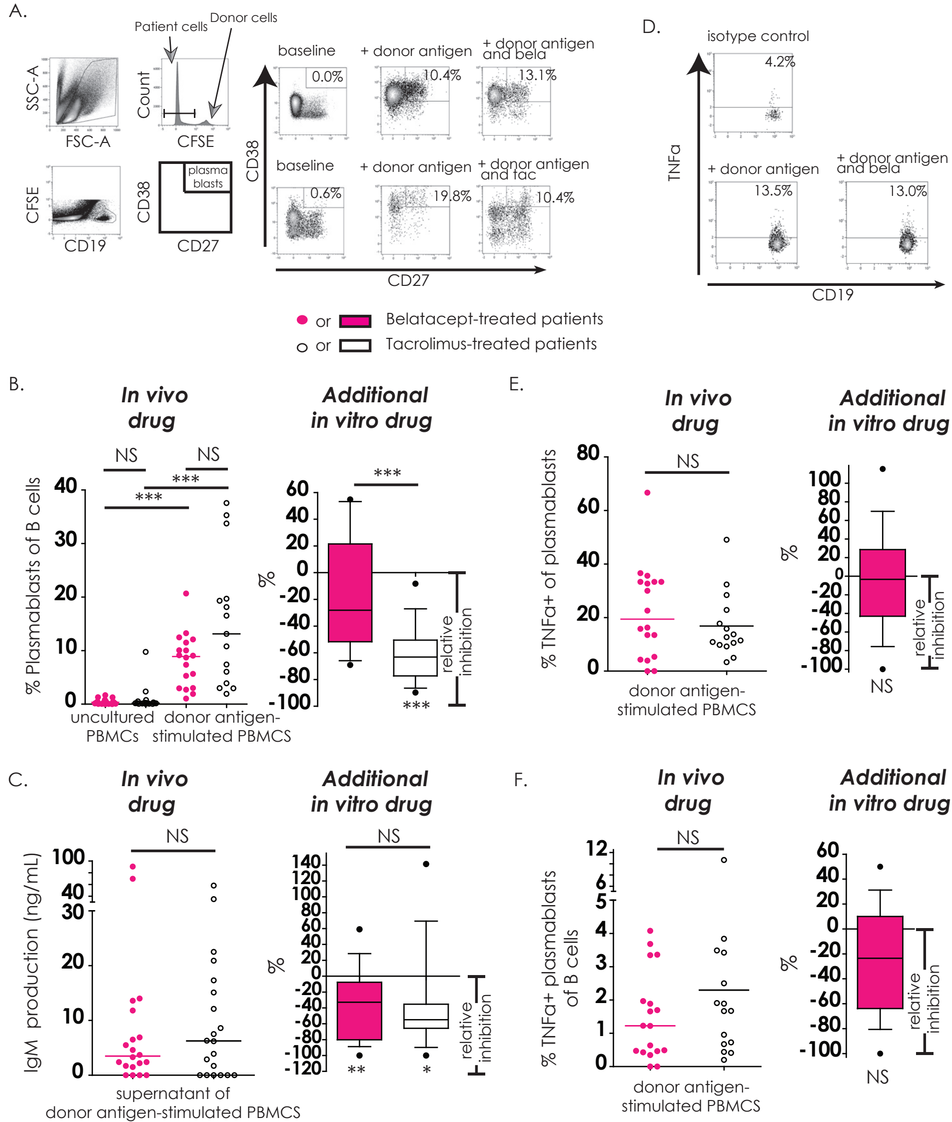


**Figure 5. Belatacept *in vitro* did not inhibit donor antigen-driven plasmablast formation or TNFa production in a PBMC-based assay, but suppressed IgM production**

The gating strategy is depicted for plasmablasts (CD19+CD27+CD38++) after 7 days of donor antigen-stimulation, in the presence or absence of belatacept and tacrolimus (A). Donor PBMCs were discriminated by CFSE-labeling them prior to the mixed lymphocyte reaction and gating them out after. The proportions of plasmablasts are shown for 7-day donor antigen-stimulated PBMCs obtained from the belatacept or tacrolimus group ("in vivo" column), as well as the relative inhibitions by in vitro addition of belatacept or tacrolimus

("additional in vitro" column) (B). The IgM concentrations in the supernatants are shown for the same cultures as previously mentioned ("in vivo" column), as well as the relative inhibitions by in vitro addition of belatacept or tacrolimus ("additional in vitro" column) (C). A typical example is depicted for intracellular TNFa-production by plasmablasts after 7 days of donor antigen-stimulation, in the presence or absence of belatacept (D). The proportions of TNFa+ cells within plasmablasts (E) and the proportions of TNFa+ plasmablasts within B-cells (F) are shown for 7-day donor antigen-stimulated PBMCs obtained from the belatacept or tacrolimus group ("in vivo" column), as well as the relative inhibitions by in vitro addition of belatacept ("additional in vitro" column). The proportions of TNFa+ plasmablasts could not be reliably determined in the presence of tacrolimus in vitro, because of the strong inhibition of plasmablast formation by tacrolimus. In the graphs in the "in vivo drug" columns, every dot represents PBMCs of a single patient.

In the graphs in the "additional in vitro drug" columns the relative inhibitions by additional in vitro belatacept and tacrolimus are depicted for aforementioned cell populations in the same belatacept- and tacrolimus-treated patients. The proportions of these cell populations after donor antigen-stimulation in the absence of in vitro drugs are set to zero. The median relative inhibitions by belatacept and tacrolimus were tested against a theoretical median of 0. Asterisks below boxes depict the p-values of these tests. The relative inhibitions were compared between in vitro belatacept and tacrolimus. Lines in boxes represent medians, borders of boxes represent 25th and 75th percentiles, error bars present 10th and 90th percentiles. Every box represents cultures of PBMCs obtained from n = 20 belatacept-treated or n = 20 tacrolimus-treated patients.

* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, NS = not significant



### *3.2 B-cell differentiation (plasmablast formation)*

To study the effect of belatacept and tacrolimus on the antigen dependent Tfh-B-cell interaction, differentiation of B-cells into plasmablasts was measured in donor antigen-activated PBMCs obtained after transplantation (Figure 5).

The proportions of plasmablasts were equally low in PBMCs from belatacept- and tacrolimus-treated patients (Figure 4B, "*in vivo drug*" column). Plasmablast formation was significant after donor antigen-stimulation in PBMCs from the belatacept group (8.8% [1.0 to 20.7%], $p<0.001$) and from the tacrolimus group (13.1% [1.9 to 37.6%], $p<0.001$), belatacept *vs.* tacrolimus group, $p=0.10$). Only tacrolimus significantly inhibited plasmablast formation with a relative inhibition of -50.5% [-89.7 to -8.2%], $p<0.0001$ (Figure 5B, "*additional in vitro drug*" column). Belatacept failed to inhibit this alloreactive process in PBMCs, and its relative inhibition (-28.1% [-69.1 to +54.8%]) was significantly less than the inhibition by tacrolimus, $p<0.001$.

### *3.3 Plasmablast function (IgM production and intracellular TNFa production)*

IgM production by donor antigen-stimulated PBMCs was not significantly different in PBMCs obtained from the belatacept-treated patients compared to the tacrolimus-treated patients (Figure 5C, "*in vivo drug*" column). The relative inhibitions of IgM production by belatacept and tacrolimus *in* vitro were -32.9% [-100.0 to +59.1%], $p<0.01$, and -54.9% [-100.0 to +141.4%), $p<0.05$, respectively (Figure 4C, "*additional in vitro drug*" column). Even though tacrolimus more efficiently inhibited plasmablast formation than belatacept, the inhibition of IgM production did not significantly differ between these two drugs.

Since belatacept is a fusion protein consisting of the Fc-fragment of IgG1,(22) total human IgG could not be determined by ELISA. No IgG DSA were detected by Luminex in supernatants of the MLRs. Total IgG3 could be detected in 8 cultures with donor-antigen stimulated PBMCs (median 6.6 [4.8 -34.3] ng/mL; 5x from tacrolimus-treated and 3x from belatacept-treated patients), and was -12.3% [-79.3 to +33.5%] inhibited in these samples by tacrolimus or belatacept, p=0.01 (Supplementary Figure 2). Because of the limited amount of $IgG3^+$ supernatants no subgroup analysis per treatment arm was performed.

Of the B-cells that differentiated into plasmablasts 19.4% [0.0 to 66.7%] expressed intracellular TNFa in the PBMCs obtained from belatacept-treated patients, and 12.7% [3.4 to

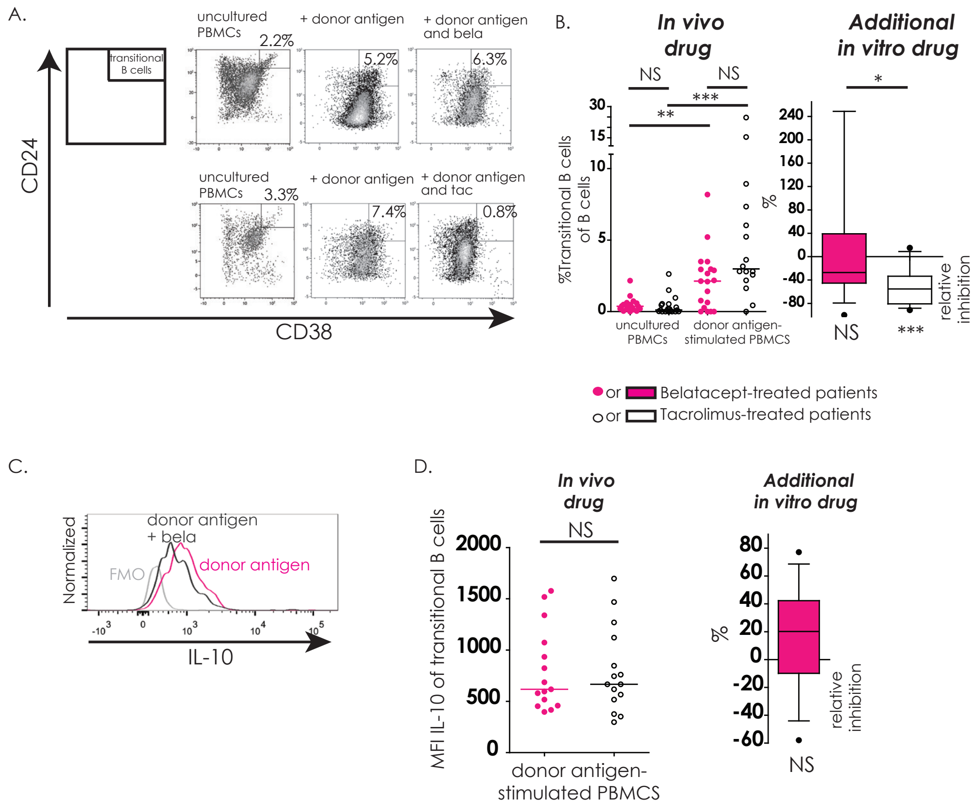


**Figure 6. Transitional B-cells and their donor antigen-driven IL-10 production were conserved by belatacept *in vitro*, but inhibited by tacrolimus**

The gating strategy is depicted for transitional B-cells (CD24+CD38++) after donor antigen-stimulation (A). Cells were gated from CD19+ B-cells like depicted in Figure 4A. The proportions of transitional B-cells are shown for 7-day donor antigen-stimulated PBMCs obtained from the belatacept or tacrolimus group ("in vivo" column), as well as the relative inhibitions by in vitro addition of belatacept or tacrolimus ("additional in vitro" column) (B). A typical example is depicted for intracellular IL-10 expression (Median Fluorescence Intensity [MFI]) by transitional B-cells after 7 days of donor antigen-stimulation, in the presence or absence of belatacept, including a Fluorescence-Minus-One control (FMO) (C). The MFIs of IL-10 within transitional B-cells are shown for 7-day donor antigen-stimulated PBMCs obtained from the belatacept or tacrolimus group ("in vivo" column), as well as the relative inhibitions by in vitro addition of belatacept ("additional in vitro" column) (D). The MFI of IL-10 within transitional B-cells could not be reliably determined in the presence of tacrolimus in vitro, because of the decreased transitional B-cells survival in the presence of tacrolimus. In the graph in the "In vivo drug" column in (B), proportions of transitional B cell populations were compared i) between belatacept- and tacrolimus-treated patients in uncultured, unstimulated PBMCs; ii) between belatacept- and tacrolimus-treated patients in 7-day donor antigen stimulated PBMCs; iii) between uncultured, unstimulated PBMCs and 7-day donor antigen stimulated PBMCs in belatacept-treated patients; and iv) between uncultured, unstimulated PBMCs and 7-day donor antigen stimulated PBMCs in tacrolimus-treated patients. Every dot represents PBMCs of a single patient. In the graphs in the "Additional in vitro drug" column the relative inhibitions by additional in vitro belatacept and tacrolimus are depicted for aforementioned cell populations in the same belatacept- and tacrolimus-treated patients. The proportions of these cell populations after donor antigen-stimulation in the absence of in vitro drugs are set to zero. The median relative inhibitions by belatacept and tacrolimus were tested against a theoretical median of 0. Asterisks below boxes depict the p-values of these tests. The relative inhibitions were compared between in vitro belatacept and tacrolimus. Lines in boxes represent medians, borders of boxes represent 25th and 75th percentiles, error bars present 10th and 90th percentiles. Every box represents cultures of PBMCs obtained from n = 20 belatacept-treated or n = 20 tacrolimus-treated patients.

* = p<0.05, ** = p<0.01, *** = p<0.001, **** = p<0.0001, NS = not significant

49.1%] in those from the tacrolimus-treated patients, $p=0.34$ (Figure 5E, "*in vivo*" column). The proportions of $TNFa^+$ plasmablasts within total B-cells were also similar in PBMCs from belatacept- and tacrolimus-treated patients: 1.2% [0.0 to 4.1%] and 1.7% [0.2 to 10.1%], respectively, $p=0.34$ (Figure 5F, "*in vivo*" column). Belatacept did not affect the proportion of TNFa+ within plasmablasts nor the proportions of TNFa+ plasmablasts within total B-cells (Figure 5E-F, "*additional in vitro*" column). The proportions of TNFa+ plasmablasts could not be reliably determined in the presence of tacrolimus, because of the strong inhibition of plasmablast formation by tacrolimus *in vitro*.

### *3.4 B-cell differentiation and plasmablast function in an isolated co-culture system*

To eliminate the effects of other cell types and cytokines present in the PBMC-based assay, we tested the effects of belatacept and tacrolimus in an isolated system of antigen-activated $CXCR5^+$ Tfh and $CD19^+CD27^+$ memory B-cells (Supplementary Figure 3). The differentiation of memory B-cells into IgM producing plasmablasts was used as read out. Plasmablast formation of 13.2% [2.1-44.9%] was decreased by the addition of belatacept to 1.7% [1.3-4.2%], and by tacrolimus to 0.5% [0.1-0.9%], both $p<0.05$ (Supplementary Figure 3D). Tacrolimus more potently inhibited the plasmablast formation than belatacept, $p<0.05$. The same pattern was seen in the IgM production of 253.8 ng/mL [86.5-541.5 ng/mL] (Supplementary Figure 3E). Belatacept decreased IgM production to 18.1 ng/mL [13.7-68.4 ng/mL], and tacrolimus to 6.2 ng/mL [2.2-8.6 ng/mL] (both $p<0.05$; tacrolimus *vs.* belatacept $p<0.05$).

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### *3.5 Immune regulatory phenotype (IL-10+ transitional B cell survival)*

The presence of B-cells with a regulatory phenotype, *i.e.* $IL10^+$ transitional B-cells, was assessed after donor antigen-stimulation in the presence or absence of belatacept or tacrolimus (Figure 6).

In unstimulated, uncultured PBMCs the proportions of transitional B-cells were below 3% in both treatment groups (Figure 5B, "*in vivo drug*" column). After donor antigen-stimulation, an increase in the proportion of transitional B-cells was observed in PBMCs from belatacept- and tacrolimus-treated patients, to 2.1% [0.0 to 8.2%], $p<0.01$, and 3.0% [0.0 to 24.7%], $p<0.001$, respectively (Figure 5B, "*in vivo drug*" column). The survival of these transitional B-cells was not different between the belatacept and tacrolimus groups. The *in vitro* addition of tacrolimus, however, diminished transitional B-cell survival (relative inhibition: -55.4% [-91.4 to +15.1%], $p<0.001$), while belatacept did not affect the survival of these potentially regulatory B-cells (relative inhibition: -27.2% [-100.0 to +247.4%], $p=0.54$), tacrolimus *vs.* belatacept, $p<0.05$.

Both the transitional B-cells in the PBMCs obtained from belatacept-treated patients as those from tacrolimus-treated patients expressed IL-10 after donor antigen-stimulation: MFI 617 [397 to 1577] and MFI 666 [297 to 1697], respectively, $p=1.00$ (Figure 6D, "*in vivo*" column). Belatacept *in vitro* did not decrease IL-10 production in transitional B-cells (Figure 6D, "*additional in vitro*" column). The MFI of IL-10 within transitional B-cells could not be reliably determined in the presence of tacrolimus, because of the decreased transitional B-cells survival in the presence of tacrolimus *in vitro*.

### *3.6 Summary of the effects of belatacept and tacrolimus on B-cells*

Donor antigen-driven CD86 upregulation was not fully inhibited by belatacept, especially on memory B-cells, even by supra-therapeutic doses of belatacept. In contrast to tacrolimus, belatacept could not inhibit donor antigen-driven plasmablast formation in a PBMC-based assay, but only in an isolated Tfh-B-cell co-culture. Also in the latter, belatacept was less effective than tacrolimus. The survival of the potentially immune regulatory $IL-10^+$ transitional

B-cells was, however, not affected by belatacept, while this was diminished by tacrolimus.

## *4. Redundancy of the immune system*

### *4.1 The effect of belatacept and tacrolimus on redundant co-stimulatory pathways*

To explain why belatacept did not inhibit plasmablast formation in our PBMC studies, and because patients' cellular interactions are influenced by redundant and pleiotropic mechanisms of immune cells, surface receptors of other costimulatory pathways were measured (Supplementary Figure 4).

In PBMCs from 3 belatacept-treated and 3 tacrolimus-treated patients, the ICOS-ICOSL, PD-1-PD-L1, and the CD40L-CD40 pathways were studied after donor antigen stimulation Expressions of all surface molecules, except for CD28 and ICOSL, were increased on Tfh and B-cells after donor antigen-stimulation (Supplementary Figures 4A and C). The upregulation of the costimulatory molecules on Tfh-cells was not fully suppressed by belatacept, and to a lesser extent than by tacrolimus (Supplementary Figure 4).

## *5. Multivariable regression analyses*

### *5.1 The effect of belatacept and tacrolimus in vitro on Tfh and B-cells*

The effect of belatacept *in vitro* was compared to the effect of tacrolimus *in vitro* in multivariable regression analyses for proportions of $CD86^+$ naïve and memory B-cells, plasmablast formation and transitional B-cell survival (adjusted for the variables as stated in Table 1). These cell subsets were chosen, because they significantly differed when belatacept was added compared to tacrolimus *in vitro* (Figures 4, 5 and 6). Multivariable analyses confirmed that belatacept and tacrolimus differed in inhibition of plasmablast formation: plasmablast formation was 4.5% (SE 1.3) higher in the presence of belatacept than in the presence of tacrolimus *in vitro*, $p$=0.001 (Table 1). In the multivariable analysis, transitional B-cell survival (defined as the proportion of transitional B-cells of total B-cells) was not significantly higher in the presence of belatacept compared to *in vitro* addition of tacrolimus ($p$=0.91). Finally, the free CD86 expression on $CD27^+$ memory B-cells was 9.3% (SE 2.8) lower when the donor antigen-stimulated PBMCs were spiked with belatacept *in vitro* compared to tacrolimus *in vitro*, $p$=0.001, but no significant difference was found for free CD86-expression on the surface of naïve B-cells ($p$=0.12).

PBMCs were obtained 3 months after transplantation in non-rejectors, and during rejection before additional anti-rejection therapy was given in rejectors. Eleven of twenty belatacept-treated patients and two of twenty tacrolimus-treated patients developed a biopsy-proven acute rejection. Obtaining PBMCs during acute rejection did not alter the *in vitro* reaction to donor antigen or drug (Table 1).

The effects of belatacept and tacrolimus *in vitro* on the different Tfh and B-cell subsets are summarized in Table 2.

**Table 1:** The effect of belatacept *in vitro* on free CD86 expression, plasmablast formation and transitional B-cell survival

| Dependent variable | Independent variables | Beta | SE | p |
|---|---|---|---|---|
| CD86 expressing naïve B-cells (% $CD86^+$ of $CD19^+CD27^-$ B-cells) | Belatacept added *in vitro* (*vs.* tacrolimus added *in vitro*) | -4.53 | 2.85 | 0.12 |
| | PBMCs obtained after transplantation (*vs.* before transplantation) | 1.76 | 2.50 | 0.48 |
| | PBMCs obtained from rejector (*vs.* non-rejector) | -0.09 | 3.02 | 0.98 |
| | Proportion of cell subset without *in vitro* drugs added | 0.55 | 0.06 | 0.00 |
| CD86 expressing memory B-cells (% $CD86^+$ of $CD19^+CD27^+$ B-cells) | Belatacept added *in vitro* (*vs.* tacrolimus added *in vitro*) | -9.34 | 2.84 | 0.02 |
| | PBMCs obtained after transplantation (*vs.* before transplantation) | -1.15 | 2.49 | 0.65 |
| | PBMCs obtained from rejector (*vs.* non-rejector) | 2.10 | 3.01 | 0.49 |
| | Proportion of cell subset without *in vitro* drugs added | 0.61 | 0.07 | 0.00 |
| Plasmablasts (% $CD27^+CD38^{++}$ of $CD19^+$ B-cells) | Belatacept added *in vitro* (*vs.* tacrolimus added *in vitro*) | 4.45 | 1.28 | 0.00 |
| | PBMCs obtained after transplantation (*vs.* before transplantation) | -1.57 | 1.08 | 0.15 |
| | PBMCs obtained from rejector (*vs.* non-rejector) | 2.34 | 1.32 | 0.08 |
| | Proportion of cell subset without *in vitro* drugs added | 0.66 | 0.07 | 0.00 |
| Transitional B-cells (% of $CD19^+$ B-cells) | Belatacept added in vitro (vs. tacrolimus added in vitro) | -0.09 | -0.01 | 0.91 |
| | PBMCs obtained after transplantation (vs. before transplantation) | -0.38 | 0.71 | 0.59 |
| | PBMCs obtained from rejector (vs. non-rejector) | 0.40 | 0.86 | 0.65 |
| | Proportion of cell subset without in vitro drugs added | 0.24 | 0.07 | 0.00 |

A multivariable regression analysis was performed per cell subset with as dependent variable the value after donor antigen-stimulation plus in vitro drugs (belatacept or tacrolimus) and as independent variables addition of in vitro belatacept (vs. in vitro tacrolimus), the time point PBMCs were obtained (after vs. before transplantation), PBMCs obtained during rejection (rejector vs. non-rejector), and the value after donor antigen-stimulation without in vitro drugs added. Statistically significant p-values (<0.05) are underscored.

Proportions of the different donor antigen-stimulated subsets without in vitro drugs added were most predictive for the proportions in the presence of in vitro drugs. In 11 belatacept-treated patients and 2 tacrolimus-treated patients the PBMCs were obtained during acute rejection, before additional anti-rejection therapy was given. Obtaining PBMCs from patients who rejected or time point the PBMCs were obtained (before or after transplantation) were not predictive for the proportions of these cells.

PBMCs, peripheral blood mononuclear cells; Rejector vs. non-rejector, patients who rejected versus patients who did not reject within 12 months after transplantation (biopsy-proven); SE, standard error of beta

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**Table 2:** Effects of belatacept and tacrolimus *in vitro* on donor antigen-activated follicular T (Tfh) helper and B-cells *(Table continues on next page)*

| **Immunological reaction** | **Defined by** | **Effect by belatacept *in vitro*** | **Effect by tacrolimus *in vitro*** | **Comparison belatacept *vs* tacrolimus*** |
|---|---|---|---|---|
| Tfh cell generation | CXCR5 expression ↑ on $CD4^+$ T-cells | Inhibition (minimal) | Inhibition (minimal) | bela = tac |
| Tfh cell activation | PD-1 expression ↑ on $CD4^+CXCR5^+$ T-cellS | Inhibition (partial) | Inhibition (partial) | bela = tac |
| Activated Tfh cell generation | $CXCR5^+PD\text{-}1^+$ double expression ↑ on $CD4^+$ T-cells | Inhibition (partial) | Inhibition (partial) | bela = tac |
| IL-$21^+$ activated Tfh cell generation | The proportion of IL-$21^+$ $CXCR5^+PD\text{-}1^+$ ↑ within $CD4^+$ T-cells | Inhibition (partial) | Inhibition (partial) | bela = tac |
| IL-21 production by activated Tfh-cells | Intracellular IL-21 expression of $CXCR5^+PD\text{-}1^+$ $CD4^+$ T-cells | None | Inhibition (minimal) | bela = tac |
| B-cell activation | CD86 expression ↑ on B-cells | Inhibition (partial) | Inhibition (partial) | bela is more efficient than tac** |
| Transitional B-cell survival | The proportion of $CD24^+CD38^{++}$ B-cells | None | Inhibition (partial) | bela is more benificial than tac*** |
| Plasmablast formation | Proportion of B-cells differentiated into $CD27^+CD38^{++}$ B-cells | *In PBMCs:* None | *In PBMCs:* Inhibition (partial) | bela is less efficient than tac**** |
| | | *In isolated system with Tfh and B-cells:* Inhibition (almost completely) | *In isolated system with Tfh and B-cells:* Inhibition (completely) | bela is slightly less efficient than tac |

| | | | | |
|---|---|---|---|---|
| IgM production | Total IgM in supernatant of co-cultures | *In PBMCs:* Inhibition (partial) | *In PBMCs:* Inhibition (partial) | bela = tac |
| | | *In isolated system with Tfh and B-cells:* Inhibition (almost completely) | *In isolated system with Tfh and B-cells:* Inhibition (completely) | bela is slightly less efficient than tac |

bela, belatacept; IgM, immunoglobulin M; PBMCs, peripheral blood mononuclear cells; tac, tacrolimus

* Comparison of the relative inhibition by belatacept in vitro and tacrolimus in vitro; ** These observations were confirmed in a multivariable regression analysis for memory B-cells, but not for naïve B-cells (Table 1); *** These observations were not confirmed in a multivariable regression analysis (Table 1); **** These observations were confirmed in a multivariable regression analysis (Table 1).



## Discussion

In this study, the effects of belatacept on Tfh-B-cell interaction were compared to those of tacrolimus for the first time in kidney transplant patients. No differences were observed in unstimulated uncultured PBMCs or donor antigen-stimulated PBMCs obtained from belatacept- or tacrolimus-treated patients, which may be explained by the predominant effects by mycophenolate mofetil and prednisone in both regimens. Therefore the isolated effects of *in vitro* belatacept and tacrolimus were compared. *In vitro* addition of both drugs only minimally inhibited Tfh-cell generation and partially decreased activation of Tfh-cells (defined by PD-1 upregulation). Activated Tfh-cells produced the highest levels of IL-21, and the total proportion of IL-21$^+$ activated Tfh-cells in the presence of *in vitro* immunosuppression was also partially reduced. Still, IL-21 production and B-cell help by remaining Tfh-cells was sufficient in the presence of *in vitro* belatacept, because the donor antigen-driven formation of plasmablasts in our MLR-based PBMC assay was not inhibited by the co-stimulation blocker, in contrast to *in vivo* observations in animal studies.(14, 23) These newly-formed TNFα$^+$ plasmablasts, that have been associated with aggressive reactivity in autoimmunity,(42) were suppressed in the presence of tacrolimus.

A first explanation for these findings are the differences between our study and previous work.(14, 23, 24) Belatacept has always been compared to CsA and not with the more potent tacrolimus, and used in combination with other types of immunosuppressive agents, like T-cell depleting therapy or mTOR inhibition in the animal studies,(14, 23) or mycophenolate mofetil and prednisone in the BENEFIT trial.(24) The study presented here reports on the isolated effects of belatacept and tacrolimus on the functional interaction of patient-derived Tfh and B-cells. These differences might have led to an overestimation of the inhibition of Tfh-B-cell interaction by belatacept, not taking into account the effects of other immunosuppressive agents.

A second reason could be the significant residual expression of CD86 on donor antigen-activated B-cells, even in the presence of supra-therapeutic concentrations of belatacept. This might be explained by 1) a lower affinity of belatacept for donor antigen-activated CD86 molecules on B-cells; 2) a higher turnover of CD86 by B-cells; or 3) degradation of belatacept during the 7-day cultures. The latter is unlikely, since CD80 was efficiently blocked by belatacept. Until the study presented here, the efficacy of belatacept on occupying CD86 had only been studied on monocyte-derived dendritic cells, and not on B-cells.(41) As a result of the incomplete blockade of CD86 on B-cells, activation and consequently differentiation of B-cells

were not prevented by costimulation blockade. The production of IgM was, however, inhibited by belatacept (median ~50%), possibly because CD80 blockade or partial CD86 blockade also leads to impaired immunoglobulin responses.(43, 44) Nonetheless, belatacept was not more efficient than tacrolimus in preventing IgM production, and even slightly less efficient in an isolated system. The lower percentage of DSA-positive patients in the belatacept than in the CsA group in the BENEFIT trial (24) could be 1) a reflection of better compliance in the first group,(45, 46) 2) the lower potency of CsA compared to tacrolimus,(25) and 3) higher concentrations of mycophenolate acid in the first group.(47, 48)

A third answer can be found in redundant costimulatory pathways taking over during costimulation blockade of the CD28-CD80/86 pathway. Because belatacept affects only this pathway,(22) other costimulatory pathways, like CD40-CD40L and ICOS-ICOSL, may "bypass" blockade of CD28-CD80/86. In our small cohort study of n = 6 independent experiments, the upregulation of CD40L and ICOS on Tfh-cells were less reduced by belatacept than by tacrolimus, making these cells more capable of helping B-cells. Since tacrolimus has a direct effect on T and B-cells by inhibiting calcineurin downstream the surface receptors (27, 29, 49), its effect is not dependent on costimulation blockade. Further studies that test the combination of belatacept with CD40- or ICOS-blockade could confirm this hypothesis, but were beyond the scope of the study presented here.

A final possibility is that belatacept less effectively inhibits dendritic cells (DCs) and their interaction with Tfh and B-cells than the interaction between Tfh and B-cells, especially in an *in vitro* setting in the absence of a germinal center.(50-52) Unlike in donor antigen-stimulated PBMCs, in an isolated system of pure CXCR5$^+$ Tfh and memory B-cells, belatacept successfully inhibited plasmablast formation. A big difference between PBMCs and isolated Tfh and memory B-cells is the absence of patient DCs and their antigen-presenting function in the isolated system, *i.e.* the absence of the indirect and semi-direct pathways of antigen presentation.(53) The effect of belatacept on human dendritic cells is not yet studied and a lack hereof could explain the less efficient inhibition by belatacept on Tfh-B-cell interaction. Nevertheless, donor DCs, facilitating the direct pathway for antigen presentation, were still present in the isolated system. This suggests belatacept effectively inhibits the direct, but not the indirect or semi-direct pathways of antigen presentation. Absence of other cells, like natural killer cells, could also be an explanation for the successful inhibition by belatacept in the isolated system. We believe a PBMC-based assay is more similar to the milieu in patients than an isolated cell assay, because in the first system multiple cell types and redundant pathways are of importance.

It should be noted almost no IgG3 by ELISA and no anti-HLA IgG by Luminex were detected in the culture supernatants. Possibly because only materials of immunologically low risk patients were used. Since belatacept is an IgG1, it cannot be ruled out IgG1 antibodies were present in the co-cultures' supernatants. Another limitation of this study is that anti-CD86 monoclonal antibodies that are non-competitive to belatacept, and bind to another epitope than belatacept, are not commercially available.(41) The total CD86 expression, irrespective of saturated CD86 by belatacept in the co-cultures, could therefore not be determined.

A possible beneficial consequence of the incomplete inhibition of B-cells by costimulation blockade, is that belatacept favored the (potentially regulatory) IL-10$^+$ transitional B-cell survival, while this was diminished by tacrolimus.(30-32) However, these findings were not confirmed by multivariable regression analyses. Therefore, the clinical relevance of these observations need to be validated in a larger population. Even more, since 1) most rejections occur within the first months after transplantation, when glucocorticoids are still used and negatively influence transitional B-cell survival,(30) and 2) the regulatory capacities of antigen-specific transitional B-cells have not yet been confirmed in functional studies in humans. Another favorable outcome of incomplete Tfh-B-cell inhibition by belatacept could be a lower infection risk and more potent vaccine responses in belatacept-treated patients than in tacrolimus-treated patients. So far no evidence for this emerged from previous studies, nor has it been confirmed in randomized-controlled trials comparing belatacept and CNI-treated patients.(24, 54-56)

In this functional study, belatacept was less potent than tacrolimus in inhibiting donor antigen-driven plasmablast formation.

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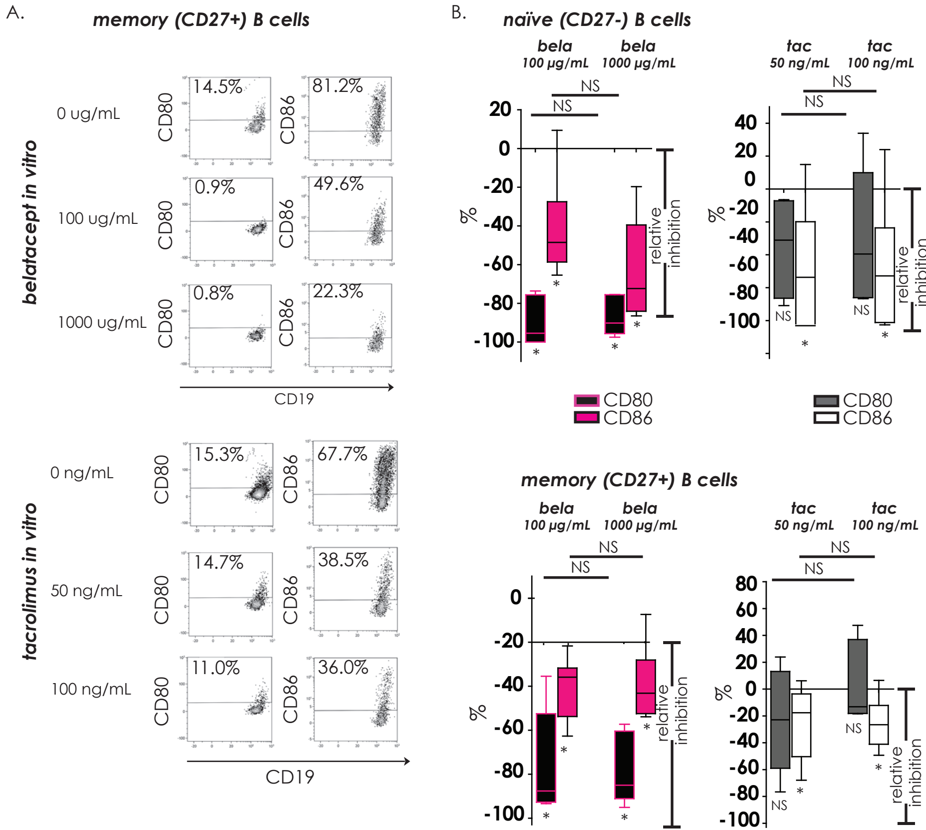


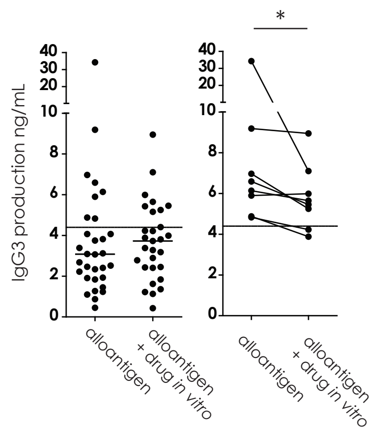



**Supplementary Figure 1. CD86 is not fully blocked by belatacept on alloantigen-activated B-cells, using 100x higher than therapeutic concentrations.**

A typical example is shown for free CD80 and CD86 expression on memory B-cells after allo-antigen stimulation in the presence of two supra-therapeutic concentrations of belatacept and tacrolimus (A). The expression on naïve B-cells was gated in the same way. The relative inihibitions of CD80 and CD86 on naïve ($CD27^-$) and memory ($CD27^+$) $CD19^+$ B-cells after allo-antigen stimulation is depicted for 6 independent mixed lymphocyte reactions using peripheral blood mononuclear cells of healthy controls (B). Allogeneic PBMCs were CD19-depleted before adding them to the cultures. The expression of CD80 and CD86 without drugs is set to zero.

N.B.: Lines in boxes represent medians, borders of boxes represent 25th and 75th percentiles, error bars present 10th and 90th percentiles. Every box represents cultures of PBMCs obtained from n = 6 healthy controls. Using the Wilcoxon Signed Rank test, the median relative inhibitions by belatacept and tacrolimus were tested against a theoretical median of 0. Asterisks below boxes depict the p-values of these tests. The relative inhibitions between the different concentrations were also compared using the Wilcoxon Signed Rank test.

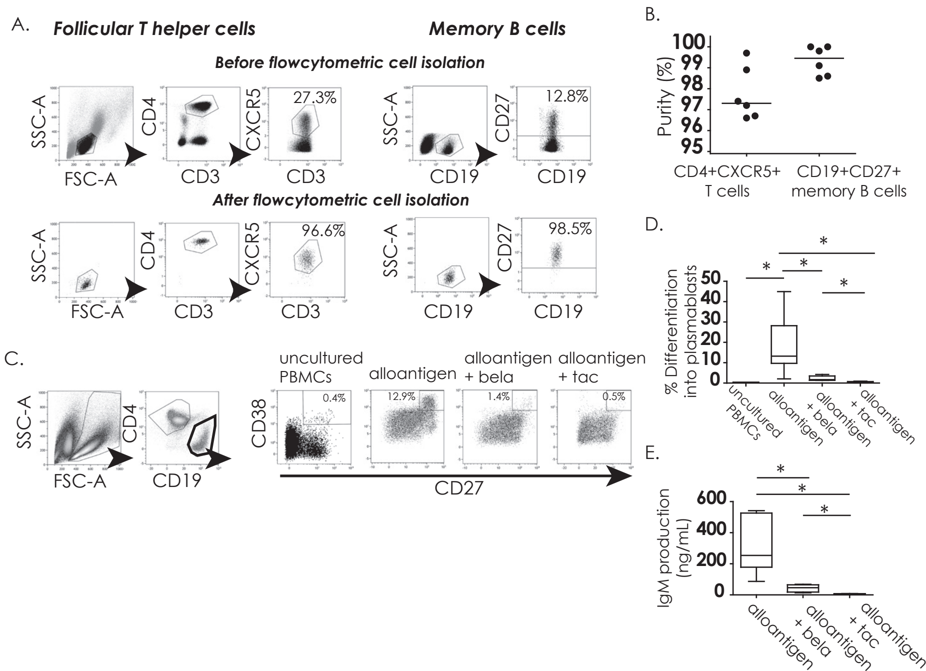
* = p<0.05 / ** = p<0.01 / *** = p<0.001 / **** = p<0.0001 / NS = not significant




**Supplementary Figure 2. IgG3 production after 7 days of donor-antigen stimulation of PBMCs**

obtained after kidney transplantation. The calibration curve of the IgG3 ELISA started at 4.4 ng/mL (dotted line). IgG3 concentration was above this cut-off point in 8 cultures with donor-antigen stimulated PBMCs: 5x from tacrolimus-treated and 3x from belatacept-treated patients), and was inhibited in all these samples by tacrolimus or belatacept (right column). Because of the limited amount of IgG3+ supernatants no subgroup analysis per treatment arm was performed.

N.B.: * $p<0.05$

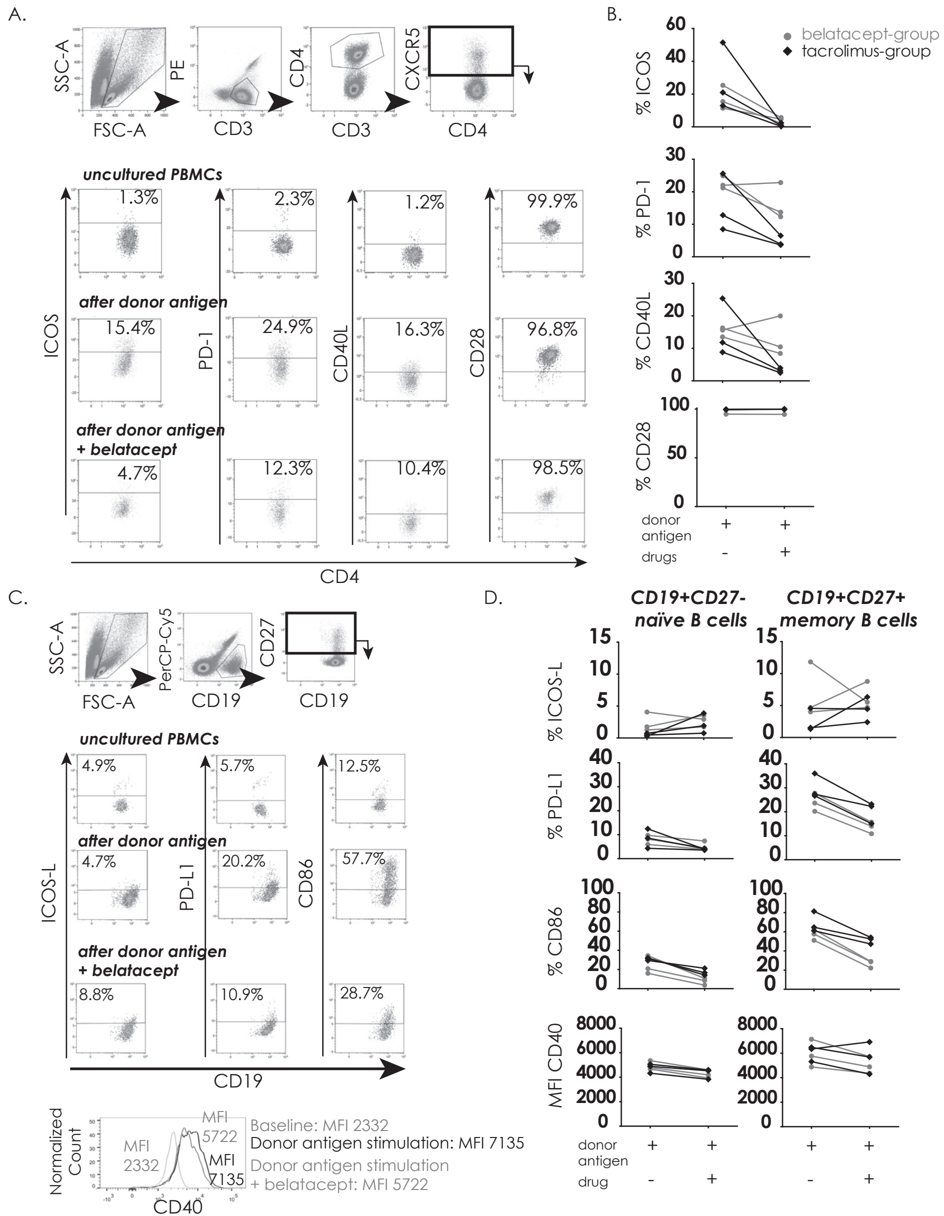



**Supplementary Figure 3. Belatacept inhibited plasmablast formation and IgM production in an allo-antigen activated co-culture of isolated follicular T helper (Tfh) cells and memory B-cells**

A typical example of Tfh cells and memory B-cells is depicted before and after flow cytometric cell isolation (A). The purities are depicted of the isolated CD4+CXCR5+ T-cells and CD19+CD27+ memory B-cells in 6 independent experiments, using materials of 3 healthy controls and 3 patients before transplantation (B). A typical example is shown for CD27+CD38++ plasmablasts after 7 days of alloantigen stimulation in a co-culture of isolated CD4+CXCR5+ T helper cells and CD19+CD27+ memory B-cells, in the presence or absence of belatacept 10 µg/mL or tacrolimus 10 ng/mL (C). Allogeneic PBMCs were CD3/19-depleted and irradiated (40 Gy) before adding them to the cultures. The proportions of plasmablasts are shown for uncultured PBMCs, and alloantigen-stimulated isolated CD19+CD27+ memory B-cells and CD4+CXCR5+ T helper cells, in the presence or absence of belatacept 10 µg/mL or tacrolimus 10 ng/mL (D). For the experiments with belatacept and tacrolimus the same materials were used, namely of 3 healthy controls and 3 pre-transplant patients. The IgM concentration in the supernatants are shown for above mentioned co-cultures (E).

N.B.: The black lines in the boxes represent the medians. The upper and lower borders of the boxes represent the 25th and 75th percentile. The error lines represent the 10th and 90th percentiles. bela=belatacept 10 µg/mL, tac=tacrolimus 10 ng/mL

* = $p<0.05$ / ** = $p<0.01$ / *** = $p<0.001$ / **** = $p<0.0001$




**Supplementary Figure 4. Redundant co-stimulatory pathways in follicular T helper and B cells.**

A typical example is shown for the expression of different co-stimulatory molecules on CD4⁺CXCR5⁺ T helper cells obtained from a belatacept-treated patient (A). PBMCs from tacrolimus-treated patients were gated the same way. The proportions of ICOS⁺, PD-1⁺, CD40L⁺ and CD28⁺ within CD4⁺CXCR5⁺ T helper cells are depicted for n=3 belatacept-treated patients (dots) and n=3 tacrolimus-treated patients (diamonds) in the presence or absence of belatacept 10 µg/mL or tacrolimus 10 ng/mL, respectively (B). A typical example is shown for the expression of different co-stimulatory molecules on memory (CD27⁺) B-cells obtained from a belatacept-treated patient 3 months after transplantation (C). Naïve (CD27⁻) B cells and B cells from tacrolimus-treated patients were gated the same way. The proportions of ICOS-L⁺, PD-L1⁺, and CD86⁺, and the Median Fluorescence Intensity (MFI) for CD40 within CD19⁺CD27⁻ naïve and CD19⁺CD27⁺ memory B-cells

> *Continuation of Supplementary Figure 3 legend*

are depicted for n=3 belatacept-treated patients (dots) and n=3 tacrolimus-treated patients (diamonds) in the presence or absence of belatacept 10 µg/mL or tacrolimus 10 ng/mL, respectively (D).

N.B.: All materials were obtained in stable, non-rejecting patients 3 months after transplantation. No statistical analyses were conducted.

**Supplementary Table 1:** Inclusion and exclusion criteria

| *Inclusion criteria:* |
|---|
| • Recipient of a first or second renal allograft |
| • Recipient of a living donor (related or unrelated) |
| • Current or historical panel-reactive antibodies (PRA) < 30% |
| • ≥1 HLA-DR mismatch |
| *Exclusion criteria:* |
| • Recipient <18 years of age at time of transplantation |
| • Recipient of a deceased donor |
| • Recipient of a third (or more) renal allograft |
| • Recipient of a non-renal organ transplant (present, past or near-future) |
| • Recipient of an AB0-incompatible allograft |
| • Recipient with a historically positive cytotoxicity-dependent cross-match |
| • Recipient with a history of lymphoma |
| • Recipient with a seronegative or unknown EBV status |
| • Recipient with HIV, hepatitis B or C, and/or untreated latent tuberculosis |
| • Recipient with a high risk for polyoma virus-associated nephropathy, which is mostly due to BK virus infection |
| • Recipient who already uses tacrolimus pre-transplantation |
| • Pregnancy |

**Supplementary Table 2:** Monoclonal antibodies (*Table continues on next page*)

| Experiment | Markers | Monoclonal antibodies | Firms | Added (μL)* |
|---|---|---|---|---|
| MLRs using PBMCs for Tfh-B-cell interaction (*n*=40 patients before and after transplantation) | CD3 | Brilliant Violet 510 | BioLegend, San Diego, CA | 0.25 |
| | CD4 | Brilliant Violet 421 | BioLegend, San Diego, CA | 2 |
| | CXCR5 | Alexa Fluor 647 | BD Pharmingen, San Diego, CA | 0.25 |
| | PD-1 (CD279) | APC-Cy7 | BioLegend, San Diego, CA | 10 |
| | CD8 | PerCP | BD Biosciences, Franklin Lakes, NJ | 5 |
| | IL-21 | PE | eBioscience, San Diego, CA | 0.5 |
| | CD19 | Brilliant Violet 510 | BioLegend, San Diego, CA | 5 |
| | CD24 | APC | eBioscience, San Diego, CA | 5 |
| | CD27 | PE-Cy7 | eBioscience, San Diego, CA | 1 |
| | CD38 | Brilliant Violet 421 | BioLegend, San Diego, CA | 1 |
| | IgD | APC-Cy7 | BioLegend, San Diego, CA | 5 |
| | CD86 | PE | Beckman Coulter, Brea, CA | 10 |
| | IL-10 | PE | BD Biosciences, Franklin Lakes, NJ | 0.5 |
| | TNFa | PerCP-Cy5.5 | BD Biosciences, Franklin Lakes, NJ | 2 |

| | | | | |
|---|---|---|---|---|
| MLRs using PBMCs for redundant costimulatory pathways (*n*=6 patients after transplantation) | CD3 | Brilliant Violet 510 | BioLegend, San Diego, CA | 0.25 |
| | CD4 | Brilliant Violet 421 | BioLegend, San Diego, CA | 2 |
| | CXCR5 | Alexa Fluor 647 | BD Pharmingen, San Diego, CA | 0.25 |
| | PD-1 (CD279) | APC-Cy7 | BioLegend, San Diego, CA | 10 |
| | CD40-Ligand (CD154) | FITC | BioLegend, San Diego, CA | 5 |
| | ICOS (CD278) | PE-Cy7 | BioLegend, San Diego, CA | 0.5 |
| | CD28 | PerCP-Cy5.5 | BD Biosciences, Franklin Lakes, NJ | 10 |
| | CD19 | Brilliant Violet 510 | BioLegend, San Diego, CA | 5 |
| | CD27 | PE-Cy7 | eBioscience, San Diego, CA | 1 |
| | PD-Ligand 1 (CD274) | Brilliant Violet 421 | BioLegend, San Diego, CA | 2 |
| | CD40 | FITC | BioLegend, San Diego, CA | 1 |
| | ICOS-Ligand (CD275) | APC | BioLegend, San Diego, CA | 1 |
| | CD86 | PE | Beckman Coulter, Brea, CA | 10 |
| MLRs in the presence of various concentrations of belatacept or tacrolimus (*n*=6 independent experiments) | CD19 | Brilliant Violet 510 | BioLegend, San Diego, CA | 5 |
| | CD27 | PE-Cy7 | eBioscience, San Diego, CA | 1 |
| | CD80 | APC | BioLegend, San Diego, CA | 5 |
| | CD86 | PE | Beckman Coulter, Brea, CA | 10 |
| Co-cultures of isolated Tfh and memory B-cells (*n*=6 independent experiments) | CD4 | PerCP | BD Biosciences, Franklin Lakes, NJ | 10 |
| | CXCR5 | Alexa Fluor 647 | BD Pharmingen, San Diego, CA | 0.25 |
| | PD-1 (CD279) | APC-Cy7 | BioLegend, San Diego, CA | 10 |
| | CD19 | Brilliant Violet 510 | BioLegend, San Diego, CA | 5 |
| | CD27 | PE-Cy7 | eBioscience, San Diego, CA | 1 |
| | CD38 | Brilliant Violet 421 | BioLegend, San Diego, CA | 1 |
| | CD86 | PE | Beckman Coulter, Brea, CA | 10 |

< *Table on previous page*

* The monoclonal antibodies were titrated to the optimal concentrations to discriminate between the positive and negative fraction for the tested marker. The depicted amounts of monoclonal antibodies were added to 100 µL cell suspension.

**Supplementary Table 3** Characteristics of study subjects

| Characteristics | Total randomized (*n*=40) | Randomized | | p |
|---|---|---|---|---|
| | | belatacept (*n*=20) | tacrolimus (*n*=20) | |
| Age at transplantation (years) | 55 (21-76) | 57 (25-76) | 55 (21-76) | 0.88 |
| Gender (female) | 10 (25%) | 6 (30%) | 4 (20%) | 0.72 |
| HLA A mismatch (mean ± SD) | 1.2 (±0.6) | 1.1 (±0.7) | 1.4 (±0.5) | 0.13 |
| HLA B mismatch (mean ± SD) | 1.4 (±0.5) | 1.3 (±0.5) | 1.5 (±0.5) | 0.51 |
| HLA DR mismatch (mean ± SD) | 1.2 (±0.4) | 1.1 (±0.4) | 1.3 (±0.4) | 0.70 |
| HLA total mismatch (mean ± SD) | 4.0 (±1.1) | 3.5 (±1.1) | 4.1 (±1.1) | 0.07 |
| current PRA | 0% (0-17%) | 0% (0-5%) | 0% (0-17%) | 0.30 |
| highest PRA | 4% (0-21%) | 4% (0-6%) | 4% (0-21%) | 0.78 |
| CMV seropositivity at transplantation | 22 (55%) | 10 (50%) | 12 (60%) | 0.75 |

Data represent medians (plus ranges) for continuous variables and numbers (plus percentages) for categorical variables, unless otherwise specified. Two-sided p values comparing the two treatment arms result from the Mann-Whitney U test for comparing continuous variables or the Fisher's exact tests for comparing categorical variables. Two-sided $p<0.05$ was considered statistically significant.

CMV, cytomegalovirus; HLA, human leukocyte antigen; PRA, panel reactive antibodies; SD, standard deviation.

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# Chapter 4

## Down-regulation of surface CD28 under belatacept treatment: an escape mechanism for antigen-reactive T-cells

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## Abstract

### Background

The co-stimulatory inhibitor of the CD28-CD80/86-pathway, belatacept, allows calcineurin-inhibitor-free immunosuppression in kidney transplantation. However, aggressive T-cell mediated allogeneic responses have been observed in belatacept-treated patients, which could be explained by effector-memory T-cells that lack membrane expression of CD28, *i.e.* CD28-negative ($CD28^{NULL}$) T-cells. CD28-positive ($CD28^{POS}$) T-cells that down regulate their surface CD28 after allogeneic stimulation could also pose a threat against the renal graft. The aim of this study was to investigate this potential escape mechanism for $CD28^{POS}$ T-cells under belatacept treatment.

### Materials & Methods

PBMCs, isolated T-cell memory subsets and isolated $CD28^{POS}$ T-cells were obtained from end-stage renal disease (ESRD) patients and co-cultured with allo-antigen in the presence of belatacept to mimic allogeneic reactions in kidney transplant patients under belatacept treatment. As a control, IgG was used in the absence of belatacept.

### Results

Despite high *in vitro* belatacept concentrations, a residual T-cell growth of ±30% was observed compared to the IgG control after allogeneic stimulation. Of the alloreactive T-cells, the majority expressed an effector-memory phenotype. This predominance for effector-memory T-cells within the proliferated cells was even larger when a higher dose of belatacept was added. Contrary to isolated naïve and central-memory T cells, isolated effector-memory T cells could not be inhibited by belatacept in differentiation or allogeneic IFNγ production. The proportion of CD28-positive T cells was lower within the proliferated T cell population, but was still substantial. A fair number of the isolated initially $CD28^{POS}$ T-cells differentiated into $CD28^{NULL}$ T-cells, which made them not targetable by belatacept. These induced $CD28^{NULL}$ T-cells were not anergic as they produced high amounts of IFNγ upon allogeneic stimulation. The majority of the proliferated isolated originally $CD28^{POS}$ T-cells, however, still expressed CD28 and also expressed IFNγ.

### Conclusion

This study provides evidence that, apart from $CD28^{NULL}$ T-cells, also $CD28^{POS}$, mostly effector-memory T-cells can mediate allogeneic responses despite belatacept treatment.

## Introduction

The co-stimulatory inhibitor of the CD28-CD80/86-pathway, belatacept, is a promising alternative for calcineurin-inhibitors in kidney transplantation.(1-3) This co-stimulatory inhibitor does not directly down-regulate or block CD28 on T-cells, but induces T-cell anergy by depriving T-cells from the necessary co-stimulatory signal from CD80/86 on antigen-presenting cells.(4) Aggressive T cell-mediated allogeneic responses have been observed in belatacept-treated patients.(1) This phenomenon can be explained by the actions of memory T-cells that are less or not susceptible to co-stimulatory blockade of the CD28-CD80/86 pathway.(5, 6) *In vitro* studies demonstrated that, despite the presence of belatacept, effector-memory T-cells which lack membrane expression of CD28, *i.e.*CD28-negative ($CD28^{NULL}$) T-cells, produce high levels of effector cytokines upon allogeneic stimulation.(6-8) $CD28^{POS}$ T-cells can down regulate their surface CD28 when the transcriptional initiator element of CD28 is disrupted,(9) which occurs after repeated antigen-stimulation (*e.g.* as the result of physiological aging, chronic viral infection, malignancy, auto-immunity, and transplantation).(10) So, in belatacept-treated patients, in addition to pre-existing $CD28^{NULL}$ T-cells, $CD28^{POS}$ T-cells that down regulate their surface CD28 after allogeneic stimulation could also pose a threat to the renal graft. In solid organ transplantation, seemingly opposing functions of $CD28^{NULL}$ T-cells have been reported. These cells can have immunoregulatory functions(11, 12), show features of immunoquiescence(10), as well as mediate allogeneic or anti-viral immune responses.(5-7, 13, 14) One study reported $CD4^{POS}CD28^{NULL}$ T-cells could play an important role in glucocorticoid-resistant rejection occurring during belatacept treatment.(8) No studies on peripheral blood mononuclear cells (PBMCs) derived from end-stage renal disease (ESRD) patients have been conducted to determine the ability of their $CD28^{POS}$ T-cells to down regulate surface CD28 in the presence of belatacept, making them resistant to blockade of the CD28-CD80/86-pathway.

The aim of this study was to investigate a potential escape mechanism for $CD28^{POS}$ T-cells under belatacept treatment, *i.e.* the down regulation of surface CD28 by these cells after allogeneic stimulation. PBMCs, isolated T-cell memory subsets and isolated $CD28^{POS}$ T-cells were obtained from ESRD patients (one day before kidney transplantation) and co-cultured with donor antigen in the presence or absence of belatacept to mimic allogeneic reactions in kidney transplant patients under belatacept treatment, and therefore explain the aggressive T cell-mediated responses in these patients.(1)

## Materials and methods

*Study population and materials*

Defrosted PBMCs from patients sampled one day before kidney transplantation were analyzed. This study was approved by the Medical Ethical Committee of the Erasmus MC University Medical Center in Rotterdam, the Netherlands (MEC-2007-228, MEC-2010-022). All patients gave written consent to collect their biomaterial as part of the ongoing transplant biobank programs. None of the transplant donors were from a vulnerable population and all donors or next of kin provided written informed consent that was freely given. Samples were randomly selected when enough patient and donor material were available, and when patient and donor were not identical for HLA class II. The patient characteristics are depicted in Supplementary Table

1. Materials of 61 patients were used for the PBMC study (*n*=33), for the isolated T-cell memory subset study (*n*=4) and for the isolated CD28$^{POS}$ T-cell study (*n*=24).

*Flow cytometric isolation of recipients' PBMCs*

By use of an AriaII FACS sorter™ (Becton Dickinson, BD, Franklin Lakes, NJ), pure CD28$^{POS}$ cells (purity 98% [95-100%]) were isolated. PBMCs were stained with CD3 Brilliant Violet 510 (BioLegend, San Diego, CA), CD4 Pacific Blue (BD, Franklin Lakes, NJ), CD8 APC-Cy7 (BD Pharm, San Diego, CA), CD28 APC (BD), and the viability dye 7-AAD PerCP (BD). Pure memory subsets (≥95% pure) were isolated using CD3 Brilliant Violet 510 (BioLegend), CD45RO PE-Cy7 (BD) and CCR7 PE (BD): naïve ($T_N$ cells: CCR7+CD45RO-), central-memory ($T_{CM}$ cells: CCR7+, CD45RO+), effector-memory ($T_{EM}$ cells: CCR7-, CD45RO+), and end-stage terminally-differentiated EMRA ($T_{EMRA}$ cells: CCR7-CD45RO-) T-cells.

*Mixed lymphocyte reactions (MLRs)*

The $IC_{50}$ for belatacept was determined in 6 independent MLR assays with PBMCs of healthy volunteers (Figure 1). PBMCs were washed in serum-free medium and suspended in PKH67 FITC or PKH26 PE 1:50 in 1 mL Diluent C per 10 million cells (Membrane Dye Kit by Sigma-Aldrich, St. Louis, MO). After incubation of 4 minutes at room temperature, fetal bovine serum (FBS) was added to stop the incorporation of the PKH dye. Subsequently, PBMCs were washed twice in RPMI + 10% heat-inactivated FBS. Finally $5x10^4$ PKH-26 PE or PKH-67 FITC labeled (MFI>10,000) responders' PBMCs were incubated for 1 hour with 15 different concentrations of belatacept (Bristol-Myers Squib, NYC, NY, kindly provided by the manufacturer) ranging from 0-5 mg/mL before the stimulator cells were added for 7 days. A lower concentration (100 ng/mL) and a higher concentration (500 ng/mL) for belatacept were used in further experiments.

$5x10^4$ PKH-26 PE or PKH-67 FITC labeled (MFI>10,000) patients' PBMCs, FACS-isolated T-cell memory subsets or FACS-isolated CD28$^{POS}$ cells were incubated for 1 hour in 100 ng/mL or 500 ng/mL belatacept (Bristol-Myers Squibb, New York City, NY) or 100 or 500 ng/mL IgG (human IgG, Sigma-Aldrich, St. Louis, MO) as control. Hereafter $5x10^4$ CD3-depleted and irradiated (total dose of 40 Gy) stimulator PBMCs were added to the culture. Subsequently, the cells were incubated for 1 week at 37°C and analyzed by flow cytometry (BD FACSDiva 8.0.1, BD, Franklin Lakes, NJ).

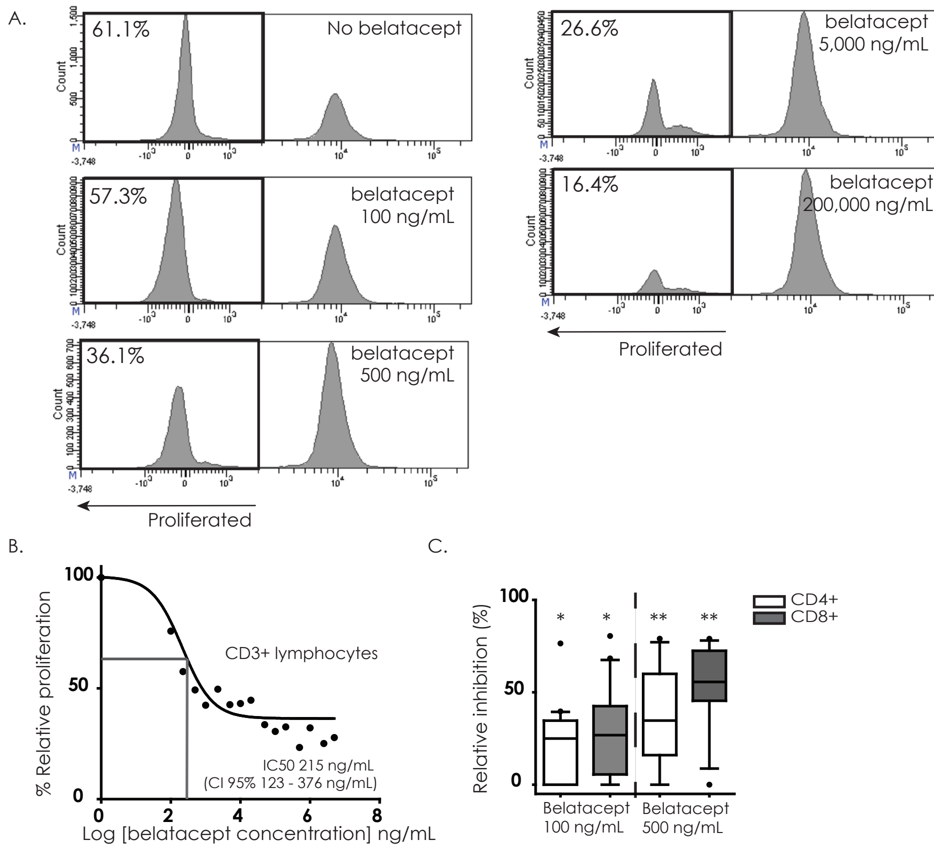



**Figure 1. Despite the dose-dependent inhibition by belatacept of T-cell proliferation, residual T-cell proliferation is present despite high doses of belatacept**

Experiments were performed with PBMCs derived from healthy volunteers (n=6). A representative sample is shown (A). The $IC_{50}$ was calculated using a logarithmic transformation of belatacept concentrations (log [inhibitor]- versus- response curve) (B). The relative inhibition by belatacept in MLRs with patients' PBMCs is given for $CD4^{POS}$ and $CD8^{POS}$ T-cells. The human IgG control has been set at the zero-line (C).

N.B.: In (C) black lines represent the medians. The upper and lower borders of the boxes represent the 25th and 75th percentiles. The error lines represent the10th and 90th percentiles. Values above 1.5 quartile distances of the box are considered outliers and are represented by a dot. Twenty independent experiments were conducted using the lower dose of belatacept (100 ng/ml) and 13 using the higher dose of belatacept (500 ng/mL).

*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001

*Flow cytometry*

PBMCs were characterized (*n*=33) before and at day 7 of the MLR. Memory subsets were defined by CCR7 and CD45RA surface expression: naïve ($T_N$ cells: CCR7+CD45RA+), central-memory ($T_{CM}$ cells: CCR7+, CD45RA-), effector-memory ($T_{EM}$ cells: CCR7-, CD45RA-), and end-stage terminally-differentiated EMRA ($T_{EMRA}$ cells: CCR7-CD45RA+) T-cells. At day 7 the cells were

plugged with brefeldin A (Golgiplug, BD Pharm; 1 μg/mL) for 4 hours. Thereafter, the allogeneic intracellular IFNγ-production was measured. Also the intracellular IFNγ production capacity was assessed, by re-stimulating part of the cells at day 7 with phorbolmyrisate acetate (PMA) 0.05 μg/mL and ionomycin 1 μg/mL (Sigma-Aldrich, St Louis, MO). FACS- isolated memory subsets of recipient cells (*n*=4 independent experiments) were stained before and after MLR using CD3 Brilliant Violet 510 (BioLegend), CD4 APC (BD), CD8 APC-Cy7 (BioLegend), CCR7 PE (BD) and CD45RO PE-Cy7 (BD). Intracellular staining was done using IFNγ Brilliant Violet 421 (BioLegend). The proportion of PKH67-FITC negative cells was assessed as measurement for proliferation. FACS-sorted CD28$^{POS}$ cells (*n*=24 independent experiments) were stained for CD28-expression and IFNγ-production after 7 days of antigen stimulation. Monoclonal antibodies used for surface marker staining and intracellular cytokine staining for PBMCs and isolated CD28$^{POS}$ cells were CD3 Brilliant Violet 510 (BioLegend, San Diego, CA), CD4 PerCP (B DBiosciences, Frankin Lakes, NJ), CD4 APC-Cy7 (BioLegend), CD8 APC (BD), CD8 APC-Cy7 (BD Pharmingen, San Diego, CA), CCR7 PE (BD Pharmingen), CD45RA brilliant violet 421 (BioLegend), CD28 APC (BD), CD28 PerCP-Cy5 (BD) and IFNγ BV421 (BioLegend) or IFNγ APC (BD Pharmingen). The proportion of PKH-26 PE or PKH-67 FITC-negative cells was also assessed as measurement for proliferation.

*Calculating the relative inhibition by belatacept*

The relative inhibition by belatacept was expressed as the proliferation of T-cells in the presence of belatacept compared to the proliferation in the presence of IgG control, which was set to 100%:

*Statistical analyses*

The differences between measurements before and after 7 days of MLR, and between IgG control and belatacept, were analyzed using the Wilcoxon signed-rank test. The differences between belatacept 100 ng/mL and belatacept 500 ng/mL were analyzed using the Mann-Whitney-U test.

Graph pad prism 5.01 (GraphPad Software, San Diego, CA) was used for statistical analyses. *P*-values with a 2-sided a of 0.05 were considered statistically significant. When not otherwise specified, medians [range] are presented.

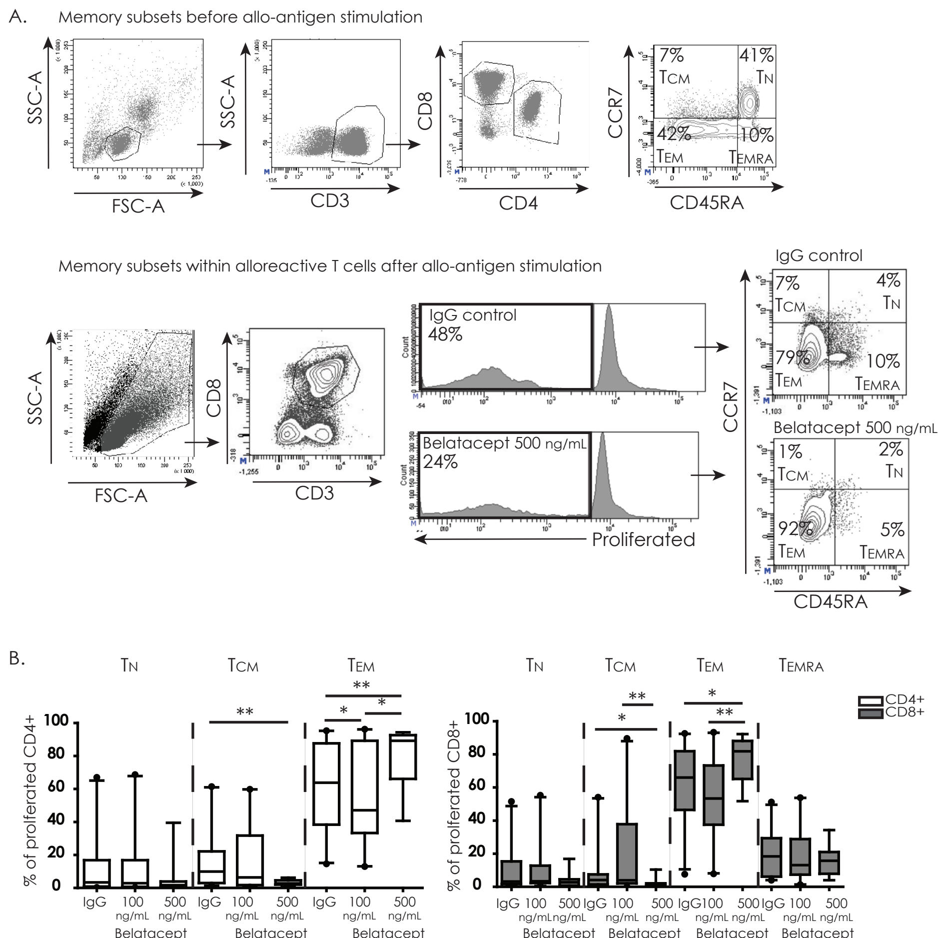



**Figure 2. Predominance of effector-memory T-cells after allogeneic stimulation was enhanced by belatacept**

A representative sample of 33 measurements is depicted for the gating strategy of the memory subsets within the proliferated, alloreactive CD8$^{POS}$ T-cells after 7 days of allo-antigen stimulation, in the presence and abcense of belatacept, as well as the memory subsets before allo-antigen stimulation (A). Memory subsets were gated the same way for CD4$^{POS}$ T-cells. The percentages of naïve, central memory, effector-memory and terminally-differentiated EMRA T-cells are given before allogeneic stimulation within CD4$^{POS}$ and CD8$^{POS}$ T cells, and after allogeneic stimulation in the presence of human IgG control, 100 ng/mL or 500 ng/mL belatacept for proliferated CD4$^{POS}$ T-cells and for CD8$^{POS}$ T-cells (B).

N.B.: Black lines represent the medians. The upper and lower borders of the boxes represent the 25th and 75th percentiles. The error lines represent the10th and 90th percentiles. Values above 1.5 quartile distances of the box are considered outliers and are represented by a dot. Twenty independent experiments were conducted using the lower dose of belatacept (100 ng/ml) and 13 using the higher dose of belatacept (500 ng/mL).

*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, $T_N$=naïve T-cells, $T_{CM}$=central-memory T-cells, $T_{EM}$=effector-memory T-cells, $T_{EMRA}$=terminally differentiated EMRA T-cells

## Results

*Despite the dose-dependent inhibition by belatacept of T-cell proliferation, residual T-cell proliferation was still present when high doses of belatacept were added*

Belatacept inhibited T-cell proliferation in a dose-dependent manner (Figure 1).The $IC_{50}$ of belatacept for T-cell proliferation was 215 ng/mL [CI95% 123-376 ng/mL) in MLRs of healthy volunteers' PBMCs. Remarkably, even at very high concentrations, belatacept could not inhibit T-cell proliferation more than ±70%, resulting in a residual proliferation of ±30%.

A concentration lower and a concentration higher than the IC50 of belatacept were used in further experiments (100 ng/mL and 500 ng/mL), to ensure the inhibitory effects of belatacept were dose-dependent.

In MLRs of PBMCs of ESRD patients stimulated with allo-antigen, both $CD4^{POS}$ and $CD8^{POS}$ T-cells were significantly inhibited in proliferation by the lower and higher dose of belatacept, in a dose-dependent manner (Figure 1C).

*Predominance of effector-memory T-cells after allogeneic stimulation was enhanced by belatacept*

The T-cells that proliferated upon allogeneic stimulation were analyzed in the presence and absence of belatacept to gain more insight into the alloreactive T-cells that were less susceptible to belatacept (Figure 2A). A predominance of effector-memory T-cells was seen within the proliferated $CD4^{POS}$ and $CD8^{POS}$ T-cells after allo-antigen stimulation in the presence of the higher dose of belatacept (500 ng/mL). 89% [41-94%] of the alloreactive $CD4^{POS}$ T cells expressed an effector-memory phenotype in the presence of 500 ng/mL belatacept *vs*. 64% [15-95%] for the IgG control, $p<0.01$. Similar observations were made for $CD8^{POS}$ T cells: 82% [52-92%] in the presence of 500 ng/mL belatacept *vs*. 66% [8-93%] for the IgG control, $p<0.01$. In parallel with the proportional increase of effector-memory T cells amongst alloreactive T cells, the proportion of naïve and central-memory T cells decreased. The predominance for effector-memory T cells was not enhanced when the lower dose of belatacept was used.

*Phenotyping and function of alloreactive effector-memory T-cells*

Since the allo-reactive proliferated T-cells mostly consisted of effector-memory T-cells, these cells were further analyzed for CD28-expression and allogeneic IFNγ-production (Figure 3). The proliferated effector-memory T-cells were compared to the total effector-memory T-cell population (both proliferated and non-proliferated cells). Within the proliferated effector-memory $CD4^{POS}$ T-cell population, the percentage of $CD28^{POS}$ T-cells (85% [41-98%]) was lower than in the total $CD4^{POS}$ effector-memory population (93% [76-98%]), $p=0.01$, but still substantial (Figure 3B). No selection of $CD28^{NULL}$ T-cells occurred in the presence of belatacept, since similar proportions of $CD28^{POS}$ cells within the proliferated $CD4^{POS}$ effector-memory T-cells were observed in the presence of the IgG control, belatacept 100 ng/mL or 500 ng/mL. In the proliferated $CD8^{POS}$ effector-memory T-cell population 45% [1-85%] of the cells were $CD28^{POS}$ compared to 63% [30-

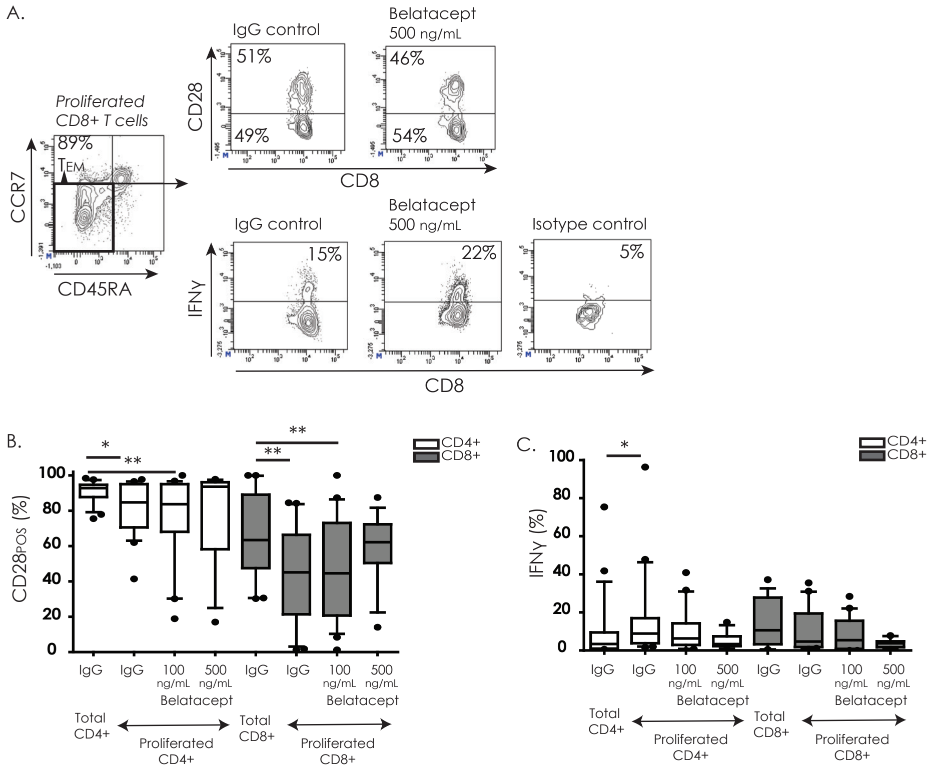



**Figure 3. A large proportion of alloreactive T cells is CD28$^{POS}$, and allogeneic IFNγ production is not inhibited by belatacept**

A representative sample of 33 experiments is depicted for the gating strategy of CD4POSand CD8POS effector-memory T-cells and their CD28 expression and the intracellular IFNγ production (A). The percentage of CD28POSeffector-memory T-cells, (B) and the percentage of IFNγ producing cells (C) was assessed in the total and in the proliferated effector-memory CD4$^{POS}$ and CD8$^{POS}$ populations upon 7-day allogeneic stimulation by means of MLR. The percentages are given in the presence of human IgG control, 100 ng/mL or 500 ng/mL belatacept.

N.B.: Black lines represent the medians. The upper and lower borders of the boxes represent the 25th and 75th percentiles. The error lines represent the10th and 90th percentiles. Values above 1.5 quartile distances of the box are considered outliers and represented are by a dot. Twenty samples were used for the lower dose of belatacept (100 ng/ml) and 13 samples for the higher dose of belatacept (500 ng/mL).

*=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001

100%] in the total CD8$^{POS}$ effector-memory T-cell population, $p$=0.01 (Figure 3B). Despite adding the lower or higher dose of belatacept, similar proportions of CD28$^{POS}$ cells were observed among the proliferated CD8$^{POS}$ effector-memory T cells as in the IgG control (Figure 3B).

9% [2-96%] of the proliferated CD4$^{POS}$ effector-memory T-cells and 5% [0-36%] of the proliferated CD8$^{POS}$ effector-memory T-cells produced IFNγ upon allogeneic stimulation. The allogeneic production of this important cytokine by CD4$^{POS}$ and CD8$^{POS}$ effector-memory T-cells was not significantly inhibited by the lower or higher dose of belatacept. The allogeneic IFNγ

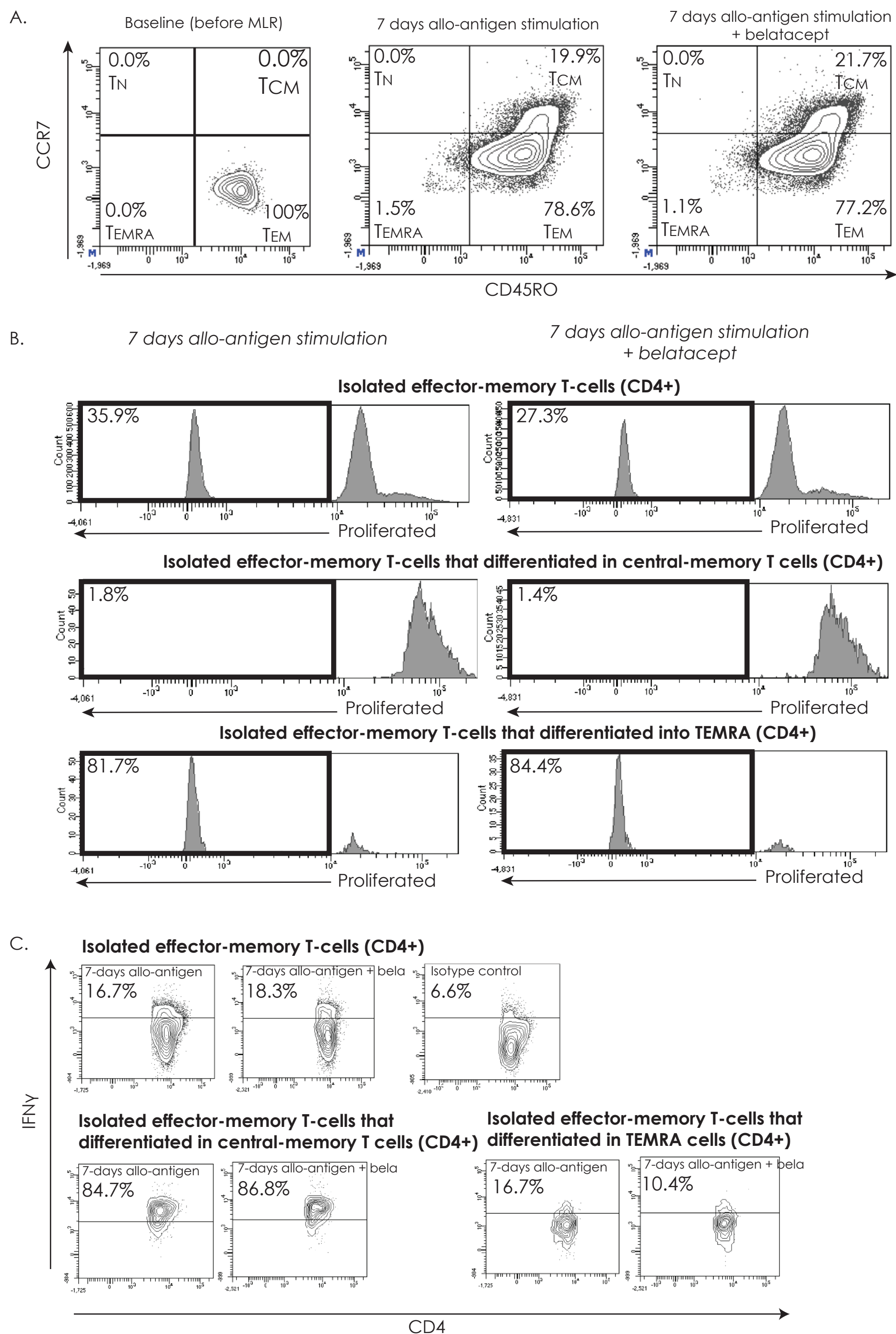
production by proliferated CD4$^{POS}$ effector-memory T-cells (9% [2-96%]) was significantly higher than the production by the total CD4$^{POS}$ effector-memory T-cell population (3% [1-75%]), $p$=0.03 (Figure 3C). This difference was not observed between the proliferated and total CD8$^{POS}$ effector-memory T-cells. A fair proportion of CD4$^{POS}$ and CD8$^{POS}$ T cells had the capacity to produce IFNγ after re-stimulation with PMA/ionomycin (Supplementary Figure 1).

*Isolated effector-memory T cells were not inhibited in differentiation or IFNγ production by belatacept*

For detailed information regarding the experiments using isolated memory subsets, see Supplementary Table 2. Sufficient cell numbers were not available for all test conditions. The differentiation of isolated effector-memory T-cells into central-memory or end-stage differentiated effector-memory T-cells was not inhibited by belatacept 500 ng/mL (Figure 4A and Supplementary Table 3). The differentiation of isolated naïve or central-memory T-cells into other subsets was successfully suppressed by belatacept (Supplemenary Figure 2A and Supplementary Table 3). Each subset, except for $T_{EMRA}$ cells, was successfully inhibited in proliferation by 500 ng/mL belatacept (Figure 4B, Supplemenary Figure 2B and Supplementary Table 4). The proliferation within the newly formed memory subsets, however, was high and not inhibited by belatacept, especially in the newly formed effector-memory and $T_{EMRA}$ cells (Figure 4B and Supplementary Table 4). Intracellular IFNγ concentrations were highest in the newly formed memory subsets differentiated from isolated naïve, effector-memory and $T_{EMRA}$ cells (Supplementary Table 5). Belatacept 500 ng/mL could not prevent the IFNγ production by these induced subsets (Supplementary Table 5). IFNγ production by isolated effector-memory T-cells was also not blocked by belatacept (Figure 4C and Supplementary Table 5). Belatacept could, however, inhibit IFNγ production by the isolated naïve and central-memory T-cells (Supplemenary Figure 2C), as well as in the newly formed subsets differentiated from central-memory T-cells (Supplementary Table 5).

*The indirect target of belatacept, CD28, can be down-regulated by T-cells upon allogeneic stimulation, resulting in IFNγ-producing CD28$^{NULL}$ T-cells*

To study the dynamics of CD28-expression on T-cells, pure CD28$^{POS}$ and CD28$^{NULL}$ cell populations were studied after allogeneic stimulation (Figure 5). A fair proportion of the isolated CD28$^{NULL}$ T-cells up-regulated CD28, but more importantly, also a proportion of the CD28$^{POS}$ T-cells down-regulated CD28. (Figure 5A-B) Therefore, CD28-expression was also studied within proliferated isolated CD28$^{POS}$ T-cells, in the presence or absence of belatacept (Figure 5C). The inhibitory effect on isolated CD28$^{POS}$ T-cell proliferation is depicted in Supplementary Figure 3. After allogeneic stimulation, in the presence of belatacept 100 ng/mL, 6% [1-49%] of the CD4$^{POS}$ T-cells did not express CD28 anymore and 5% [1-53%]of the CD8$^{POS}$ population. These percentages were similar to the percentages of CD28$^{NULL}$ T-cells in the absence of belatacept (Figure 5D). Similar proportions of CD28$^{NULL}$ T-cells were found for cultures with the higher concentration of belatacept.

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Chapter 4

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**Figure 4. Isolated effector-memory T cells are not inhibited in differentiation or IFNγ production by belatacept**

FACS-sorted effector-memory T-cells (CD3+CCR7-CD45RO+) were stimulated for 7 days with allo-antigen (purities≥99%). One of in total four independent experiments is shown in this figure. The surface expression of CCR7 and CD45RO was assessed on $CD4^{POS}$ and $CD8^{POS}$ T cells after 7 days of MLR to determine the differentiation of effector-memory T cells into other memory subsets; an example of $CD4^{POS}$ isolated effector-memory T cells is shown in (A). The proliferation by the isolated effector-memory T cells, and by the cells differentiated into central-memory and end-stage terminally-differentiated effector-memory T-cells in particular, is depicted (B), as well as the allogeneic IFNγ production by these cells (C). bela=belatacept 500 ng/mL, $T_N$=naïve T-cells, $T_{CM}$=central-memory T-cells, $T_{EM}$=effector-memory T-cells, $T_{EMRA}$=terminally differentiated EMRA T-cells

The allogeneic IFNγ production was compared between T-cells that remained $CD28^{POS}$ and T-cells that had down-regulated their CD28 surface molecules (Figure 5C for typical examples). In the presence of 100 ng/mL belatacept, the proportion of $CD4^{POS}CD28^{NULL}$ T-cells that produced IFNγ (39% [4-93%]) was comparable to cells that remained $CD28^{POS}$ (15% [2-68%]), *p*=0.08. (Figure 5E) The same was seen when 500 ng/mL belatacept was added. Within the $CD8^{POS}$ T-cells, in the presence of 100 ng/mL belatacept, slightly more $CD28^{POS}$ T-cells produced IFNγ than the cells that turned $CD28^{NULL}$: 7%[0-96% ] *vs.* 4% [0-66%], respectively, *p*=0.003. This difference was not observed when 500 ng/mL belatacept was added to the cultures. Similar percentages of IFN-γ production were found for the IgG control samples, *i.e.* in the absence of belatacept. (Figure 5E)

## Discussion

Here, the ability of ESRD patients' $CD28^{POS}$ T-cells to down-regulate surface CD28 upon allogeneic stimulation was studied after belatacept was added *ex vivo*. Kidney transplantation was mimicked to explain the severe T-cell-mediated immune responses that have been observed in belatacept-treated patients.(1) Although the overall allogeneic proliferation by T-cells was inhibited by belatacept, this inhibition never reached 100% (Figure 1). Moreover, a part of the antigen-reactive T-cells down-regulated surface CD28 molecules without becoming anergic (*i.e.* their capacity to produce intracellular IFNγ production upon allogeneic stimulation remained intact; Figure 5E). Even in the presence of belatacept, the co-stimulatory inhibitor of the CD80/86-CD28 pathway, these originally $CD28^{POS}$ T-cells were not susceptible to co-stimulatory blockade and are therefore a selection of the most dangerous immune cells for the allograft.(6, 7) In addition, amongst the antigen-reactive proliferated T-cells, a large proportion remained $CD28^{POS}$ and also produced intracellular IFNγ. Explanations for the severe alloreactivity in belatacept-treated patients include the possibility that belatacept inhibits negative regulators of the immune system(15, 16); ineffectively permeate lymph nodes and kidney tissue(17); or the alloreactivity is the result of heterologous immunity, like EBV positive memory T cells may cross-react with donor HLA expressed on the transplanted kidney.(18) Based on our research presented here, we postulate three additional mechanisms for the severe alloreactivity in belatacept-treated patients(1): (i) proliferation is not inhibited in all T-cells; (ii) naïve and central-memory T-cells differentiate into effector-memory T-cells, which are less susceptible to

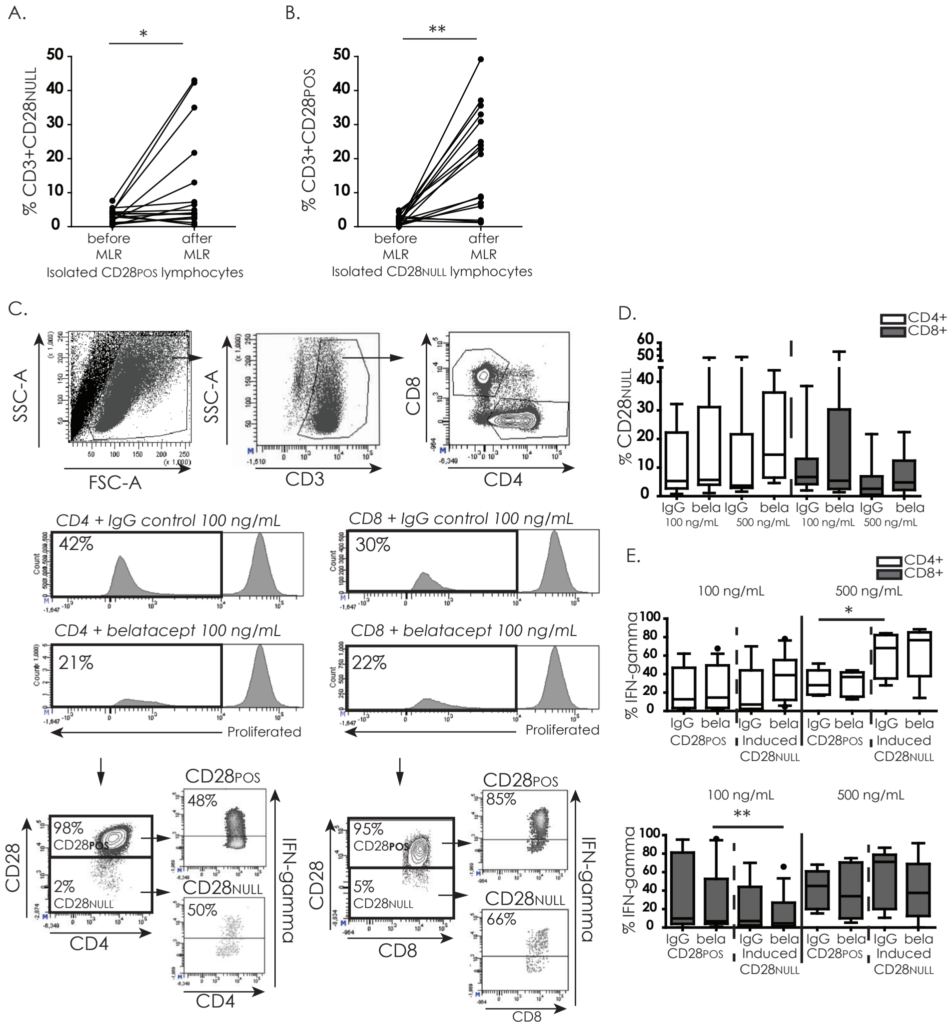


**Figure 5. The indirect target of belatacept, CD28, can be down regulated by T-cells upon allogeneic stimulation, resulting in IFNγ-producing CD28$^{NULL}$ T-cells**

FACS-sorted CD28$^{POS}$ T-cells (A) and FACS-sorted CD28$^{NULL}$ T-cells (B) from n=16 kidney-transplant candidates were stimulated for 7 days with allo-antigen (purities ≥95%). CD28-expression was assessed after 7 days of MLR and compared to the CD28-expression of the pure starting populations. Gating strategies to determine proliferated CD4$^{POS}$ and CD8$^{POS}$ T-cells are depicted, as well as a typical example for proliferation with and without belatacept. The gating strategy for CD28 expression within proliferated CD4$^{POS}$ and CD8$^{POS}$ T-cells, including allogeneic IFNγ production, is depicted in a typical example (C). IFNγ expression is depicted in this typical example in the presence of 100 ng/mL belatacept. The proportion of CD4$^{POS}$ and CD8$^{POS}$ T-cells which lost their CD28-molecules are shown for the study population (D). The percentage of IFNγ producing cells within T-cells that remained CD28$^{POS}$ and within T-cells that differentiated into CD28$^{NULL}$ are shown, in the presence and absence of belatacept (E).

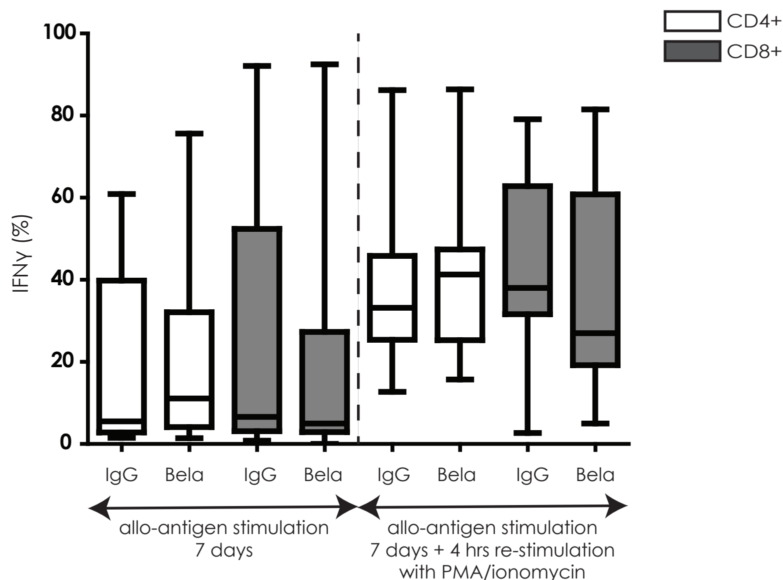
N.B.: Black lines represent the medians. The upper and lower border of the boxes represent the 10th and 90th percentile. The error lines represent the10th and 90th percentiles. Values above 1.5 quartile distances of the box are considered outliers and are represented by a dot. Levels of significance in (B) were given for the difference between proliferation in the presence of belatacept and the IgG control. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001, $T_N$=naïve T-cells, $T_{CM}$=central-memory T-cells, $T_{EM}$=effector-memory T-cells, $TE_{MRA}$=terminally differentiated EMRA T-cells

immunosuppressive drugs(19, 20); and (iii) T-cells can down-regulate their cell surface CD28 molecule and consequently become independent of co-stimulatory signals from CD80/86.

The log [inhibitor]- *versus* - response curve of belatacept (Figure 1B) demonstrated that a plateau phase is reached for its inhibitory capacity. Even when high doses of belatacept (>1 mg/mL) were added *in vitro*, the maximum inhibition was ±70%. In the BENEFIT study, serum belatacept concentrations were not higher than 10 μg/mL(1, 21), suggesting that T-cell proliferation may also be incompletely blocked *in vivo*. The $IC_{50}$ of belatacept found in our *in vitro* experiments (0.22 μg/mL, 95% CI 0.12-0.38 μg/mL) was similar to the serum belatacept concentrations of stable patients 2-5 years after kidney transplantation that received belatacept every 8 weeks (0.13 - 0.21 μg/mL).(2) Because the volume of distribution of belatacept is low(22), the concentration in lymph nodes or graft tissue is presumably even lower, which could result in even more proliferation of allo-reactive T-cells.

It is known that $CD28^{NULL}$ T-cells are not susceptible to belatacept and can produce high amounts of effector cytokines.(5-7) When adding belatacept to patients' PBMCs *ex vivo*, a smaller proportion of $CD28^{POS}$ T-cells, thus a larger proportion of $CD28^{NULL}$ T-cells was observed within the cells proliferated upon allogeneic stimulation (Figs.3B and 5D). This can be explained by a selection of $CD28^{NULL}$ T-cells, because these cells are not susceptible to belatacept. Another explanation is that not all $CD28^{POS}$ T-cells are inhibited by belatacept and that their CD28 co-stimulatory molecule is down-regulated, since $CD28^{NULL}$ T-cells were present in cultures of isolated $CD28^{POS}$ T-cells after one week of MLR. (Figure 5A and 5D) When adding the higher dose of belatacept to MLRs with patients' PBMCs (Figure 3), the predominance of $CD28^{NULL}$ T-cells was not observed, possibly because belatacept at this concentration sufficiently inhibited the activation of $CD28^{POS}$ T-cells and subsequently prevented the differentiation into $CD28^{NULL}$ T-cells. Another possibility could be that equal numbers of $CD28^{NULL}$ T-cells upregulated CD28 as the number of $CD28^{POS}$ T-cells that down-regulated CD28, and therefore the net-result was no increase of $CD28^{NULL}$ T-cells. Nevertheless, apart from $CD28^{NULL}$ T-cells, a large proportion of allo-reactive, proliferated T cells was $CD28^{POS}$, which means that despite their surface CD28 molecules these cells were not susceptible for belatacept.

To accurately establish the dynamics of CD28-expression by alloreactive T-cells of ESRD patients in the presence of belatacept, the proportion of $CD28^{NULL}$ T-cells was measured after one week of allogeneic stimulated pure $CD28^{POS}$ T-cells. (Figure 5) Indeed, even in the presence of belatacept, a proportion of T-cells lost their CD28 surface molecules upon allogeneic stimulation, making them not susceptible to inhibition of the CD28-pathway. These $CD28^{NULL}$ T-cells did not become anergic, since they remained capable of producing intracellular IFNγ upon allogeneic stimulation. For $CD4^{POS}$ and $CD8^{POS}$ T-cells, both $CD28^{POS}$ or newly-formed $CD28^{NULL}$ T-cells produced comparable large amounts of allogeneic IFNγ (Figure 5E). The differentiation of



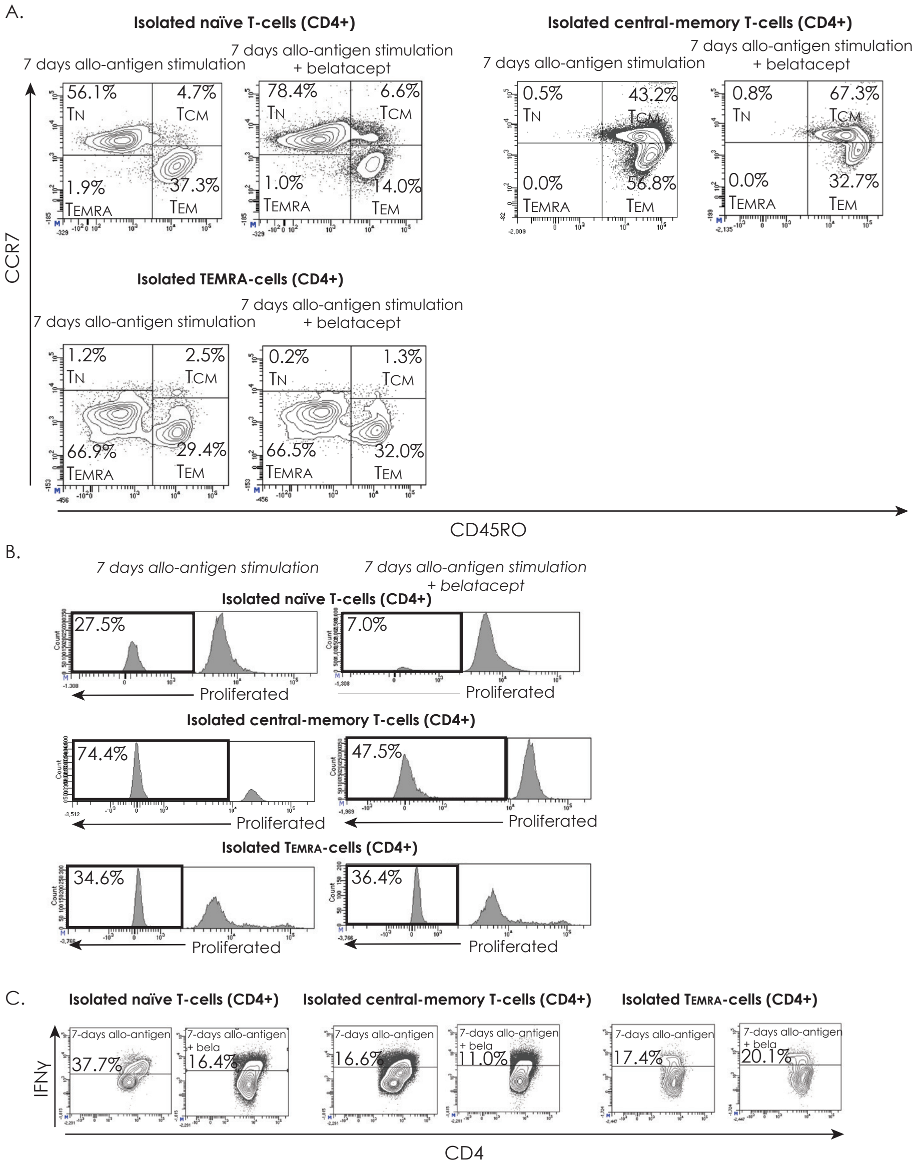

**Supplementary Figure 1. CD4POS and CD8POS T-cells from end-stage renal disease patients have a high IFNγ production capacity**

The intracellular IFNγ production is depicted for both CD4POS and CD8POS T-cells after 7 days of allo-antigen stimulation with and without 4 hours PMA/ionomycin re-stimulation. bela=belatacept 100 ng/mL



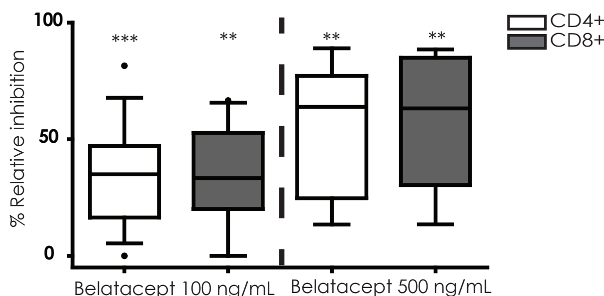
CD28POS T-cells into IFNγ–producing CD28NULL T-cells reflects the absence of belatacept-induced anergy of these T-cells (Figure 5), possibly because of alternative routes for co-stimulation.(23)

In the present study, using PBMCs of kidney transplant candidates, the alloreactive cells mostly had effector-memory T-cell features, especially after allogeneic stimulation in the presence of belatacept (Figure 2). However, the absolute numbers of such responding cells were lower because belatacept inhibits T-cell proliferation (Figure 1). The predominance of effector-memory T cells is in line with previous findings from studies using animals(5) or PBMCs from healthy volunteers. (6, 7) These effector memory T-cells are less susceptible to the currently prescribed immunosuppressive drugs, like tacrolimus(19), and are especially less subject to co-stimulation blockade.(23) The predominance of effector-memory T-cells could be the result of less affected proliferation of the pre-existing effector-memory T-cells. Isolated effector-memory T cells could, however, be inhibited in proliferation by belatacept (Figure 4B and Supplementary Table 4), but not in differentiation into central-memory and $T_{EMRA}$ T cells or in IFNγ production (Figure 4B, Supplementary Tables 3 and 5). The selection of effector-memory T-cells was evident when the higher dose of belatacept was added, since naïve and central-memory T-cells are then more sufficiently inhibited (Figs. 1 and 2). In addition, the predominance of effector-memory T-cells could also




**Supplementary Figure 2. Belatacept inhibits differentiation, proliferation and allogeneic IFNγ production by naïve and central-memory T-cells**

For detailed information about these experiments refer to Supplementary Tables B-E. The differentiation of isolated T-cell memory subsets (naïve, central-memory and end-stage terminally-differentiated effector-memory T-cells) into other memory subsets is depicted in the presence and absence of 500 ng/mL belatacept (A). All starting population were ≥97% pure. The proliferation of these isolated memory subsets was assessed in the presence and absence of 500 ng/mL belatacept (B), as well as the allogeneic IFNγ production (C). bela=belatacept 500 ng/mL, $T_N$=naïve T-cells, $T_{CM}$=central-memory T-cells, $T_{EM}$=effector-memory T-cells, $T_{EMRA}$=terminally differentiated EMRA T-cells




**Supplementary Figure 3. The proliferation of isolated CD28^POS T-cells is inhibited by belatacept**

The relative inhibition of kidney-transplant candidates' CD28^POS-isolated CD4^POS and CD8^POS T-cells in the presence of 100 (n=16) or 500 ng/mL (n=8) belatacept is shown (B). The human IgG control has been set at the zero-line.

be the result of differentiation of naïve and central-memory T-cells into the effector-memory phenotype upon allogeneic stimulation (Supplementary Figure 2.).(24)

A limitation of our study is the low availability of patient materials, which makes it difficult to test multiple conditions, *e.g.* the distinction between allorecognition via the direct or indirect pathway. Also, the difference between patients and healthy controls would be an interesting question, but does not address to our initial study purpose.

In conclusion, CD28-positive, mostly effector-memory T-cells can become resistant to belatacept by down-regulating their surface CD28 molecules, indicating differentiation into highly allo-reactive CD28^NULL T-cells. This study provides evidence that not only CD28^NULL T-cells but also CD28^POS T-cells can mediate anti-donor responses despite belatacept treatment.

**Supplementary Table 1:** Patient characteristics

| | **PBMC study (*n* = 33)** | **Isolated T-cell memory subsets study (*n*=4)** | **Isolated CD28^POS cells study (*n* = 24)** |
|---|---|---|---|
| Age in years (median + range) | 49 (20-75) | 70 (53-74) | 55 (19-71) |
| Gender (female) | 33% | 0% | 25% |
| HLA mismatches with allo-antigen (mean ± SD) | 4.0 (±1.3) | 3.8 (±1.5) | 4.1 (±1.4) |
| CMV seropositive | 70% | 50% | 71% |
| Renal replacement therapy | 67% | 0% | 55% |
| • Time on renal replacement therapy in months (median + range) | 21 (2-71) | - | 6 (1-73) |

CD28^POS, CD28-positive; HLA, human leukocyte antigen; MLR, mixed lymphocyte reaction; PBMCs, peripheral blood mononuclear cells; SD, standard deviation

**Supplementary Table 2:** Detailed information about the experiments using isolated T-cell memory subsets.

| No. | | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|
| PBMCs | # | 39 * 10^6 | 51 *10^6 | 62 * 10^6 | 72 * 10^6 |
| Isolated TN | # | 0,07 * 10^6 | 2,8 * 10^6 | 1,4 * 10^6 | 0,3 * 10^6 |
| | No bela | - | + | + | + |
| | bela | - | + | + | + |
| | ics | - | + | + | - |
| Isolated TCM | # | 0,14 * 10^6 | 1,8 *10^6 | 0,7 * 10^6 | 0,12 * 10^6 |
| | No bela | + | + | + | + |
| | bela | + | + | + | - |
| | ics | - | + | + | - |
| Isolated TEM | # | 2,7 * 10^6 | 1,5 * 10^6 | 2,7 * 10^6 | 0,8 * 10^6 |
| | No bela | + | + | + | + |
| | bela | + | + | + | + |
| | ics | + | + | + | + |
| Isolated TEMRA | # | 0,12 * 10^6 | 0,3 * 10^6 | 1,6 * 10^6 | 0,05 * 10^6 |
| | No bela | + | + | + | - |
| | bela | - | - | + | - |
| | ics | - | - | + | - |

#, number of cells; +, experiment performed; -, experiment not performed due to too low or no cell numbers; No bela, no belatacept added to culture; bela, belatacept 500 ng/mL added to culture; ics, intracellular staining for IFNγ; No., Number of experiment performed (total n=4 independent experiments); PBMCs, peripheral blood mononuclear cells (number of cells before FACS isolation); $T_{CM}$, central-memory T cells; $T_{EM}$, effector-memory T cells; $T_{EMRA}$, end-stage terminally-differentiated effector-memory T cells; $T_N$, naïve T cells

**Supplementary Table 3:** Differentiation by isolated T-cell memory subsets upon allo-antigen stimulation (*Table continues on next page*)

| No. | Starting subset | Purity starting subset | Bela | CD4+ T cells after 7 days allo-antigen stimulation | | | | CD8+ T cells after 7 days allo-antigen stimulation | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | % within CD3+ cells | ng/mL | TN (%) | TCM (%) | TEM (%) | TEMRA (%) | TN (%) | TCM (%) | TEM (%) | TEMRA (%) |
| 1 | TN | 99,3 | 0 | - | - | - | - | - | - | - | - |
| | | | 500 | - | - | - | - | - | - | - | - |
| 2 | | 99,9 | 0 | 56,1 | 4,7 | 37,3 | 1,9 | 75,6 | 2,7 | 20,9 | 1,2 |
| | | | 500 | 78,4 | 6,6 | 14,0 | 1,0 | 93,7 | 2,3 | 3,9 | 0,2 |
| 3 | | 99,6 | 0 | 95,7 | 1,1 | 2,6 | 0,7 | 97,1 | 0,8 | 1,8 | 0,4 |
| | | | 500 | 95,8 | 1 | 2,5 | 0,8 | 98,8 | 0,6 | 0,4 | 0,2 |
| 4 | | 99,9 | 0 | 98,9 | 0,7 | 0,5 | 0 | 97,8 | 1 | 1,2 | 0,2 |
| | | | 500 | 99,3 | 0,6 | 0,1 | 0,3 | 99,2 | 0,8 | 0,1 | 0 |
| 1 | TCM | 98,4 | 0 | 0,0 | 43,2 | 56,8 | 0 | 0,3 | 75,7 | 24,4 | 0 |
| | | | 500 | 0,0 | 67,3 | 32,7 | 0 | 0,4 | 95,5 | 4,2 | 0 |
| 2 | | 95,2 | 0 | 0,3 | 56,5 | 43,4 | 0,1 | 1,5 | 83,9 | 14,7 | 0,2 |
| | | | 500 | 0,3 | 91 | 4 | 0,1 | 2,3 | 95,5 | 2,3 | 0,2 |
| 3 | | 97,9 | 0 | 0,9 | 91,4 | 7,8 | 0,2 | 4,3 | 94,3 | 1,7 | 0,3 |
| | | | 500 | 0,8 | 97,7 | 1,4 | 0,2 | 4,3 | 92,3 | 2,9 | 0,7 |
| 4 | | 98,7 | 0 | 1,9 | 98,1 | 0,1 | 0 | 4 | 93,8 | 2,3 | 0 |
| | | | 500 | - | - | - | - | - | - | - | - |
| 1 | TEM | 99,5 | 0 | 0 | 10,6 | 86,7 | 3,3 | 0 | 1,6 | 97 | 1,8 |
| | | | 500 | 0 | 16,3 | 81,3 | 3,5 | 0 | 2,9 | 96,4 | 1,1 |
| 2 | | 99,8 | 0 | 0,2 | 56,5 | 43,4 | 0,1 | 1,5 | 83,9 | 14,7 | 0,2 |
| | | | 500 | 0,1 | 96 | 4 | 0,1 | 0,8 | 98,8 | 0,6 | 0 |
| 3 | | 99,4 | 0 | 0 | 5,8 | 94,2 | 0,3 | 0 | 1,3 | 98,1 | 0,7 |
| | | | 500 | 0 | 5,5 | 94,5 | 0,2 | 0 | 1,5 | 98,2 | 0,6 |
| 4 | | 100 | 0 | 0 | 19,9 | 78,6 | 1,5 | 0,1 | 4,5 | 91,9 | 4 |
| | | | 500 | 0 | 21,7 | 77,2 | 1,1 | 0,1 | 5,8 | 91,5 | 3,4 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | TEMRA | 99,9 | 0 | 2,1 | 0,5 | 26,1 | 71,5 | 2 | 0,3 | 4,6 | 93,3 |
| | | | 500 | - | - | - | - | - | - | - | - |
| 2 | | 99,8 | 0 | 0,7 | 0,1 | 6,8 | 92,4 | 2,6 | 0,2 | 8,6 | 89 |
| | | | 500 | - | - | - | - | - | - | - | - |
| 3 | | 99,8 | 0 | 1,2 | 2,5 | 29,4 | 66,9 | 3,5 | 1,9 | 2 | 93 |
| | | | 500 | 0,2 | 1,3 | 32 | 66,5 | 3,6 | 2,4 | 2,4 | 91,9 |
| 4 | | 97,4 | 0 | - | - | - | - | - | - | - | - |
| | | | 500 | - | - | - | - | - | - | - | - |

The percentage of every memory subsets is depicted within the total $CD4^{POS}$ and $CD8^{POS}$ T cell population after 7 days allo-stimulation.

-, experiment not conducted; bela, belatacept; No., Number of experiment performed (total n=4 independent experiments); $T_{CM}$, central-memory T cells; $T_{EM}$, effector-memory T cells; $T_{EMRA}$, end-stage terminally-differentiated effector-memory T cells; $T_N$, naïve T cells

**Supplementary Table 4:** Proliferation by isolated T-cell memory subsets upon allo-antigen stimulation (*Table continues on next page*)

| No. | Start-ing subset | Bela | CD4+ T cells after 7 days allo-antigen stimulation | | | | | CD8+ T cells after 7 days allo-antigen stimulation | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ng/mL | Total (%) | TN (%) | TCM (%) | TEM (%) | TEMRA (%) | Total (%) | TN (%) | TCM (%) | TEM (%) | TEM-RA (%) |
| 1 | TN | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 500 | - | - | - | - | - | - | - | - | - | - |
| 2 | | 0 | 27,5 | 1,1 | 38,4 | 99,8 | 94,8 | 21 | 0,9 | 41,2 | 99,6 | 85,3 |
| | | 500 | 7 | 0,2 | 29,3 | 98,8 | 85,7 | 2,8 | 0 | 16,3 | 99,4 | 38 |
| 3 | | 0 | 3,5 | 0,1 | 32,3 | 99 | 78,2 | 2 | 0 | 13,8 | 98,5 | 39,8 |
| | | 500 | 3,6 | 0,1 | 35,3 | 99,2 | 91,8 | 0,5 | 0 | 7,6 | 88,2 | 33,7 |
| 4 | | 0 | 0,9 | 0 | 37,9 | 91,2 | 28 | 1,2 | 0 | 24,1 | 91,7 | 0 |
| | | 500 | 0,2 | 0 | 8 | 84,6 | 66,7 | 0 | 0 | 0 | 0 | 0 |
| 1 | TCM | 0 | 74,4 | 1,4 | 46,2 | 99,9 | *N.A.* | 31,2 | 0 | 9,5 | 99,7 | *N.A.* |
| | | 500 | 47,5 | 1,2 | 27 | 99,5 | *N.A.* | 6,1 | 0 | 2 | 96,4 | *N.A.* |
| 2 | | 0 | 43,3 | 0,8 | 10,1 | 99,8 | 100 | 14,6 | 0 | 4,4 | 99,5 | *N.A.* |
| | | 500 | 9,7 | 0,8 | 2,2 | 99,5 | 99,2 | 1,6 | 1,9 | 0,6 | 98,6 | *N.A.* |
| 3 | | 0 | 8,3 | 0,8 | 2,3 | 97,9 | 95 | 0,9 | 0 | 0,2 | 75 | 0 |
| | | 500 | 1,8 | 0,9 | 0,3 | 94,8 | 98,5 | 2,5 | 0 | 0,8 | 84,2 | *N.A.* |
| 4 | | 0 | 0,4 | 0 | 0,3 | 54,8 | *N.A.* | 3,9 | 0 | 0,6 | 100 | *N.A.* |
| | | 500 | - | - | - | - | - | - | - | - | - | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | TEM | 0 | 35,9 | 0 | 1,8 | 37,7 | 81,7 | 3,4 | 0 | 0,4 | 3,2 | 12,4 |
| | | 500 | 27,3 | 0 | 1,4 | 28,6 | 84,4 | 0,9 | N.A. | 0 | 0,7 | 18,8 |
| 2 | | 0 | 10,4 | 7,2 | 3,7 | 98,2 | N.A. | 3,3 | 0 | 0,9 | 98 | 100 |
| | | 500 | 7,4 | 13,4 | 3,8 | 98,3 | N.A. | 0,4 | 0 | 0,2 | 74 | 33,3 |
| 3 | | 0 | 2,6 | 20 | 0,1 | 2,6 | 41,1 | 0,3 | 0 | 0 | 0,3 | 11,5 |
| | | 500 | 1,9 | 0 | 0,1 | 1,8 | 50,5 | 0,7 | N.A. | 0,6 | 0,7 | 17,1 |
| 4 | | 0 | 4,1 | 0 | 2,6 | 4,1 | 32,2 | 0,3 | N.A. | 1,2 | 0,3 | 1,3 |
| | | 500 | 3,1 | N.A. | 1,4 | 3,3 | 25,6 | 0,3 | 0 | 0 | 0,2 | 4,7 |
| 1 | TEMRA | 0 | 22,2 | 0 | | 87 | 3,6 | 3 | 0 | N.A. | 55,9 | 0,2 |
| | | 500 | - | - | - | - | - | - | - | - | - | - |
| 2 | | 0 | 22,4 | 0 | N.A. | 94,2 | 1,5 | 7,9 | 0 | N.A. | 87,5 | 0,5 |
| | | 500 | - | - | - | - | - | - | - | - | - | - |
| 3 | | 0 | 34,6 | 0 | 0 | 79,1 | 10,1 | 0,4 | 0 | 0 | 23,4 | 0,2 |
| | | 500 | 36,4 | 0 | 0 | 79,6 | 10,8 | 0,6 | 0 | 0 | 31,4 | 0,2 |
| 4 | | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 500 | - | - | - | - | - | - | - | - | - | - |

The percentage of proliferation is given within the total CD4$^{POS}$ and CD8$^{POS}$ T cell population, and within the CD4$^{POS}$ and CD8$^{POS}$ memory subsets after 7 days allo-stimulation.

-, experiment not conducted; bela, belatacept; No., Number of experiment performed (total n, 4 independent experiments); N.A., not applicable (not enough cells to assess this measurement); $T_{CM}$, central-memory T cells; $T_{EM}$, effector-memory T cells; $T_{EMRA}$, end-stage terminally-differentiated effector-memory T cells; $T_N$, naïve T cells

**Supplementary Table 5:** Intracellular IFNγ expression by isolated T-cell memory subsets upon allo-antigen stimulation (*Table continues on next page*)

| No. | Starting subset | Bela | CD4+ T cells after 7 days allo-antigen stimulation | | | | | CD8+ T cells after 7 days allo-antigen stimulation | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ng/mL | Total (%) | TN (%) | TCM (%) | TEM (%) | TEMRA (%) | Total (%) | TN (%) | TCM (%) | TEM (%) | TEMRA (%) |
| 1 | TN | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 500 | - | - | - | - | - | - | - | - | - | - |
| 2 | | 0 | 37,7 | 15,8 | 83,3 | 24,8 | 14,7 | 27,1 | 16,7 | 94,1 | 43 | 8,8 |
| | | 500 | 16,4 | 16,7 | 94,5 | 32,3 | 8,3 | 12,7 | 22,9 | 93,9 | 51,8 | 22,3 |
| 3 | | 0 | 2,7 | 2,9 | 74,3 | 5,4 | 9,7 | 3,4 | 3,9 | 84,8 | 13,7 | 18,1 |
| | | 500 | 2,5 | 2,7 | 63,8 | 5,1 | 6,3 | 2,7 | 4,1 | 64,4 | 49 | 12 |
| 4 | | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 500 | - | - | - | - | - | - | - | - | - | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | TCM | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 500 | - | - | - | - | - | - | - | - | - | - |
| 2 | | 0 | 16,6 | N.A. | 12,1 | 12,3 | 12,3 | 13,8 | 11,6 | 10,3 | 36,9 | 16,7 |
| | | 500 | 11 | N.A. | 8,5 | 14 | 4,2 | 8,3 | 11,1 | 7,7 | 21,1 | 25 |
| 3 | | 0 | 8,2 | N.A. | 7,2 | 6,4 | 1,7 | 5,2 | 3,2 | 7 | 0 | 0 |
| | | 500 | 4,1 | N.A. | 4,1 | 1,5 | 0,7 | 2,6 | 2,4 | 2,7 | 5,3 | 0 |
| 4 | | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 500 | - | - | - | - | - | - | - | - | - | - |
| 1 | TEM | 0 | 16,7 | N.A. | 84,7 | 10 | 10,7 | 4,9 | N.A. | 76,2 | 2 | 6,6 |
| | | 500 | 18,3 | N.A. | 86,8 | 10,4 | 10,4 | 5,9 | N.A. | 79,9 | 2,2 | 8,7 |
| 2 | | 0 | 9,6 | N.A. | 8 | 5,3 | 8,3 | 5,6 | N.A. | 5,9 | 25,3 | 0 |
| | | 500 | 10 | N.A. | 8,8 | 2,3 | 5,5 | 4,1 | N.A. | 6 | 4 | 0 |
| 3 | | 0 | 3,3 | N.A. | 57,2 | 2,2 | 2,9 | 2 | N.A. | 60,3 | 1,2 | 0 |
| | | 500 | 3,7 | N.A. | 58,3 | 2,7 | 3,5 | 3,1 | N.A. | 59,9 | 2,1 | 1,4 |
| 4 | | 0 | 20,9 | N.A. | 82,3 | 3,8 | 1,7 | 2,3 | N.A. | 53,6 | 1 | 1,3 |
| | | 500 | 24,3 | N.A. | 85,5 | 4,3 | 1,5 | 2,8 | N.A. | 59 | 1,1 | 1,6 |
| 1 | TEMRA | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 500 | - | - | - | - | - | - | - | - | - | - |
| 2 | | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 500 | - | - | - | - | - | - | - | - | - | - |
| 3 | | 0 | 17,4 | N.A. | N.A. | 26,5 | 16,2 | 4,6 | N.A. | N.A. | 21,9 | 0,9 |
| | | 500 | 20,1 | N.A. | N.A. | 28,4 | 19,3 | 5,9 | N.A. | N.A. | 20,6 | 1,3 |
| 4 | | 0 | - | - | - | - | - | - | - | - | - | - |
| | | 500 | - | - | - | - | - | - | - | - | - | - |

The percentage of IFNγ production is given within the total $CD4^{POS}$ and $CD8^{POS}$ T cell population, and within the $CD4^{POS}$ and $CD8^{POS}$ memory subsets after 7 days allo-stimulation.

-, experiment not conducted; bela, belatacept; No., Number of experiment performed (total n, 4 independent experiments); N.A., not applicable (not enough cells to assess this measurement); $T_{CM}$, central-memory T cells; $T_{EM}$, effector-memory T cells; $T_{EMRA}$, end-stage terminally-differentiated effector-memory T cells; $T_N$, naïve T cells

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# Chapter 5

## Belatacept causes a higher incidence of acute rejection compared with tacrolimus after de novo kidney transplantation: A randomized controlled trial

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**Clinical Trial Notation:**

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## Abstract

### Background

Belatacept, an inhibitor of the CD28-CD80/86 co-stimulatory pathway, allows for calcineurin-inhibitor free immunosuppressive therapy in kidney transplantation but is associated with a higher acute rejection risk than ciclosporin. Thus far, no biomarker for belatacept-resistant rejection has been validated. In this randomized controlled trial, acute rejection-rate was compared between belatacept- and tacrolimus-treated patients and various immunological biomarkers for acute rejection were investigated.

### Methods

Forty kidney-transplant recipients were 1:1 randomized to belatacept or tacrolimus combined with basiliximab, mycophenolate mofetil and prednisolone. The 1-year incidence of biopsy-proven acute rejection was monitored. Potential biomarkers, namely CD8+CD28-, CD4+CD57+PD1- and CD8+CD28++ EMRA T-cells were measured pre- and post-transplantation and correlated to rejection. Pharmacodynamic monitoring of belatacept was performed by measuring free CD86 on monocytes.

### Results

The rejection incidence was higher in belatacept-treated than tacrolimus-treated patients: 55% vs. 10%; $p = 0.006$. All three graft losses, due to rejection, occurred in the belatacept group. Although 4 of 5 belatacept-treated patients with >35 cells CD8+CD28++ EMRA T-cells/µL rejected, median pre-transplant values of the biomarkers did not differ between belatacept-treated rejectors and non-rejectors. In univariable Cox regressions, the studied cell subsets were not associated with rejection-risk. CD86 molecules on circulating monocytes in belatacept-treated patients were saturated at all time points.

### Conclusions

Belatacept-based immunosuppressive therapy resulted in higher and more severe acute rejection compared to tacrolimus-based therapy. This hypothesis-generating study did not identify cellular biomarkers predictive of rejection. In addition, the CD28-CD80/86 co-stimulatory pathway appeared to be sufficiently blocked by belatacept and also did not predict rejection.

## Introduction

Belatacept, an inhibitor of the CD28-CD80/86 co-stimulatory pathway, has the potential to improve long-term outcomes of kidney transplantation.(1-5) Seven year follow-up of the BENEFIT study demonstrated a higher patient and graft survival, as well as better graft function in patients who were treated with belatacept as compared to ciclosporin.(1) Nonetheless, the higher incidence and severity grade of acute rejection (AR) that have been observed among belatacept-treated patients remain a concern.(6-9) Up until now, belatacept has not been compared head-to-head with tacrolimus in randomized controlled trials (RCTs) in kidney transplantation without the use of lymphocyte-depleting therapy.(10-12) Observations made in uncontrolled studies suggest that the performance of belatacept in terms of preventing acute rejection as compared with tacrolimus may be inferior.(13, 14)

Identification of patients pre-transplantation who will develop AR during belatacept treatment would greatly help to personalize immunosuppressive therapy and maximize the potential of the drug. Experimental studies in rhesus macaques and *ex vivo* studies using human lymphocytes have demonstrated that antigen-experienced, cytotoxic $CD28^-CD8^+$ T-cells are not dependent on co-stimulatory signaling via CD80/86 and are therefore less susceptible to the immunosuppressive effects of belatacept.(15-17) Recently, Espinosa and colleagues suggested that patients with a high frequency of cytotoxic $CD57^+PD1^-CD4^+$ T-cells were at increased risk of AR during belatacept treatment.(18) A preliminary study in non-human primates suggested another biomarker for AR under belatacept, namely $CD28^{++}$ end-stage differentiated (EMRA) $CD8^+$ T-cells that rapidly downregulate CD28 after kidney transplantation.(19) Biomarkers such as these may help in risk stratification and a more rational use of belatacept, but require prospective validation.

Alternatively, therapeutic drug monitoring (TDM) of belatacept therapy may improve outcomes. Because serum belatacept concentrations tend to vary little between individual patients, pharmacokinetic TDM is currently not recommended.(5, 20) However, pharmacodynamic TDM of belatacept is feasible. *Ex vivo* flow cytometric measurement of CD86 occupancy on monocytes by belatacept reflects effector T-cell function,(21) demonstrating the potential of TDM to improve outcomes of belatacept therapy. However, no data from prospective clinical trials is available to provide guidance in this respect.

Here, the results of a RCT are reported in which forty patients were randomized to receive either belatacept- or tacrolimus-based immunosuppressive therapy after *de novo* kidney transplantation. The primary aims of this RCT were to compare the AR rate between belatacept and tacrolimus-treated patients and to identify biomarkers that were predictive of AR.

## Materials and Methods

Refer to Supplemental Digital Content (SDC), Materials and Methods for additional and detailed information.

### *Study design*

This was an investigator-initiated, prospective, randomized controlled, parallel group, open-label, single-center, clinical trial. Adult patients (≥18 years) who were scheduled to receive a single-organ, blood group AB0-compatible kidney from a living donor at the Erasmus MC, Rotterdam, the Netherlands, were eligible for participation. Historical and current cross-match-dependent cytotoxicity tests were negative. Table 1 lists the inclusion and exclusion criteria in detail. The study was approved by the institutional review board of the Erasmus MC (Medical Ethical Review Board number 2012-421) and was registered in the Dutch national trial registry (http://www.trialregister.nl/trialreg/index.asp; number NTR4242, registered October 2013). Written informed consent was obtained from all patients before inclusion and randomization. The study was carried out in compliance with the Good Clinical Practice guidelines (http://apps.who.int/prequal/info_general/documents/gcp/gcp1.pdf) and the Declaration of Istanbul.(22)

### *Randomization procedure and intervention*

Enrolled patients were randomly assigned on a 1:1 basis by one of the coordinating investigators (G.N.G. or D.A.H.) to either receive tacrolimus (Prograf®; Astellas Pharma, Leiden, the Netherlands) or belatacept (Nulojix®; Bristol Myers-Squibb, New York City, NY). Randomization was performed by use of 40 sealed, opaque, sequentially numbered envelopes containing treatment allocation. The random allocation sequence was generated by an independent biostatistician by use of a random number generator. Before the start of the study, it was determined that 20 patients would be allocated to each treatment arm. Data were collected and monitored by the coordinating investigators in a hospital-based electronic study database.

Tacrolimus was dosed based on bodyweight (a dose of 0.2 mg/kg/day in two equally divided doses, rounded off to the nearest 0.5 mg) according to the package insert (https://www.astellas.us/docs/prograf.pdf). Thereafter, the tacrolimus dose was adjusted based on whole-blood predose concentrations. The tacrolimus target predose concentrations were as follows: 10-15 ng/mL (weeks 1 and 2), 8-12 ng/mL (weeks 3 and 4) and 5-10 ng/mL from week 5 onwards. Belatacept was dosed according to the Less-Intensive (LI) regimen as described previously.(6, 7) Belatacept was administered intravenously in a dose of 10 mg/kg on the day of transplantation (day 0) and on days 4, 15, 30, 60 and 90 after transplantation. Thereafter, the dose was reduced to 5 mg/kg and given as monthly infusions up until month 12 after transplantation (end of study). Additional treatment is discussed in the SDC.

### *Safety*

Refer to SDC, Material and Methods for data collection on (serious) adverse events.

**Table 1:** Inclusion and exclusion criteria

*Inclusion criteria:*

- Recipient of a first or second renal allograft
- Recipient of a living donor (related or unrelated)
- Current or historical panel-reactive antibodies (PRA) < 30%
- ≥1 HLA-DR mismatch

*Exclusion criteria:*

- Recipient <18 years of age at time of transplantation
- Recipient of a deceased donor
- Recipient of a third (or more) renal allograft
- Recipient of a non-renal organ transplant (present, past or near-future)
- Recipient of an AB0-incompatible allograft
- Recipient with a historically positive cytotoxicity-dependent cross-match
- Recipient with a history of lymphoma
- Recipient with a seronegative or unknown EBV status
- Recipient with HIV, hepatitis B or C, and/or untreated latent tuberculosis
- Recipient with a high risk for polyoma virus-associated nephropathy, which is mostly due to BK virus infection
- Recipient who already uses tacrolimus pre-transplantation
- Pregnancy



***Primary end points***

The overall aim of this trial was to determine the effect of belatacept and tacrolimus-based immunosuppressive regimens on alloreactivity after kidney transplantation. The primary end point of the study presented here was the incidence of biopsy-proven acute rejection (BPAR) within the first year after transplantation. BPAR-rates were compared between belatacept- and tacrolimus-treated patients. We postulated that the incidence of BPAR would be higher among belatacept-treated patients(7) and that BPAR-biomarkers could be identified. All kidney transplant biopsies were obtained for cause and no protocol biopsies were obtained. Refer to SDC, Materials and Methods for BPAR scoring system.

Pre-transplant circulating frequencies of $CD8^{+}CD28^{-}$, $CD4^{+}CD57^{+}PD1^{-}$ and end-stage terminally differentiated memory (EMRA) $CD8^{+}CD28^{++}$ T-cells, as well as their intracellular expression of a Granzyme B (GrB: an important cytotoxic protease during acute rejection) were measured as immunological primary end points.(19, 23, 24) These cell subsets were also measured post-transplantation, during acute rejection before additional anti-rejection therapy was given, or 3 months after transplantation in non-rejecting belatacept-treated patients. Free CD86 expression on circulating $CD14^{+}$ monocytes was determined pre-transplantation as a predictor for rejection; and before every dose of belatacept administered after transplantation as a pharmacodynamic drug monitoring tool. A for belatacept competitive monoclonal

antibody was used (clone HA5.2B7, Beckman Coulter, Brea, CA). In patients who rejected, the free CD*6 expression was also assessed before additional anti-rejection therapy was given. Refer to SDC, Material and Methods for detailed information about our laboratory studies, including detection methods for DSA.

No formal statistical power calculation for the present study was performed, because 1) when the study was designed, it was unclear what the difference would be between belatacept and tacrolimus-treated patients in terms of BPAR, as only data from the BENEFIT and BENEFIT-EXT, in which the comparator was ciclosporin, were available at the time;(6, 7) 2) there were no published data available regarding the studied biomarkers and their association with BPAR that could serve for such a power calculation; and 3) because of financial constraints, we chose to conduct the present randomized controlled clinical trial with a limited number of patients in both arms.(25) In our view, the present trial should therefore be regarded as a hypothesis-generating, pilot study. It may serve as the basis for a larger study by providing the data needed to perform a statistical power calculation.

***Statistical analyses***

Additional information is depicted in SDC, Materials and Methods. Percentages and counts are given for categorical variables, and medians plus ranges for continuous variables, unless otherwise specified. Continuous variables were compared between the belatacept and the tacrolimus group or between belatacept-treated rejectors and non-rejectors using the Mann-Whitney U test, and categorical variables using the Fisher's exact test. Patient and death-censored graft survival, as well as death-censored BPAR-free survival were compared between the belatacept and tacrolimus group using the log-rank test. All included patients were analyzed according to the intention-to-treat principle.

To determine if high numbers of cytotoxic $CD4^{+}CD57^{+}PD\text{-}1^{+}$, $CD8^{+}CD28^{-}$, or $CD8^{+}CD28^{++}$ EMRA T-cells, as well as CD86 molecules/monocyte were risk factors for BPAR, univariable Cox regression analyses were performed with death-censored BPAR-free survival as the dependent variable. Independent variables included the aforementioned cell types after log transformation (to ensure approximately normal distribution of these variables), treatment arm, age, gender, ethnicity, HLA mismatches, HLA-DR mismatches, highest PRA, and cytomegalovirus (CMV) serostatus. Independent variables with a $p<0.10$ in the univariable analyses were intended to be included in a multivariable Cox regression analysis to predict BPAR.

Repeated measurements of CD86 occupancy on monocytes over time were compared between the study groups using a linear mixed model. To ensure a normal distribution of the model residuals, the dependent variable in the model was log transformed. Predictors were the values of CD86 molecules/monocyte pre-transplantation, time point after transplantation (coded as categorical variable), treatment arm (belatacept or tacrolimus) and an interaction effect of time point and treatment arm to account for different trends over time between groups. The dependent variable was the value of CD86 molecules/monocyte after transplantation at a given time point. A random intercept was included in the linear mixed model to account for the within-subject correlations.

All tests were two-tailed and statistical significance was defined as a p-value <0.05. Bonferroni's correction for multiple testing was applied when necessary.(26) Statistical analyses were performed using IBM SPSS version 21 (SPSS Inc., Chicago, IL).

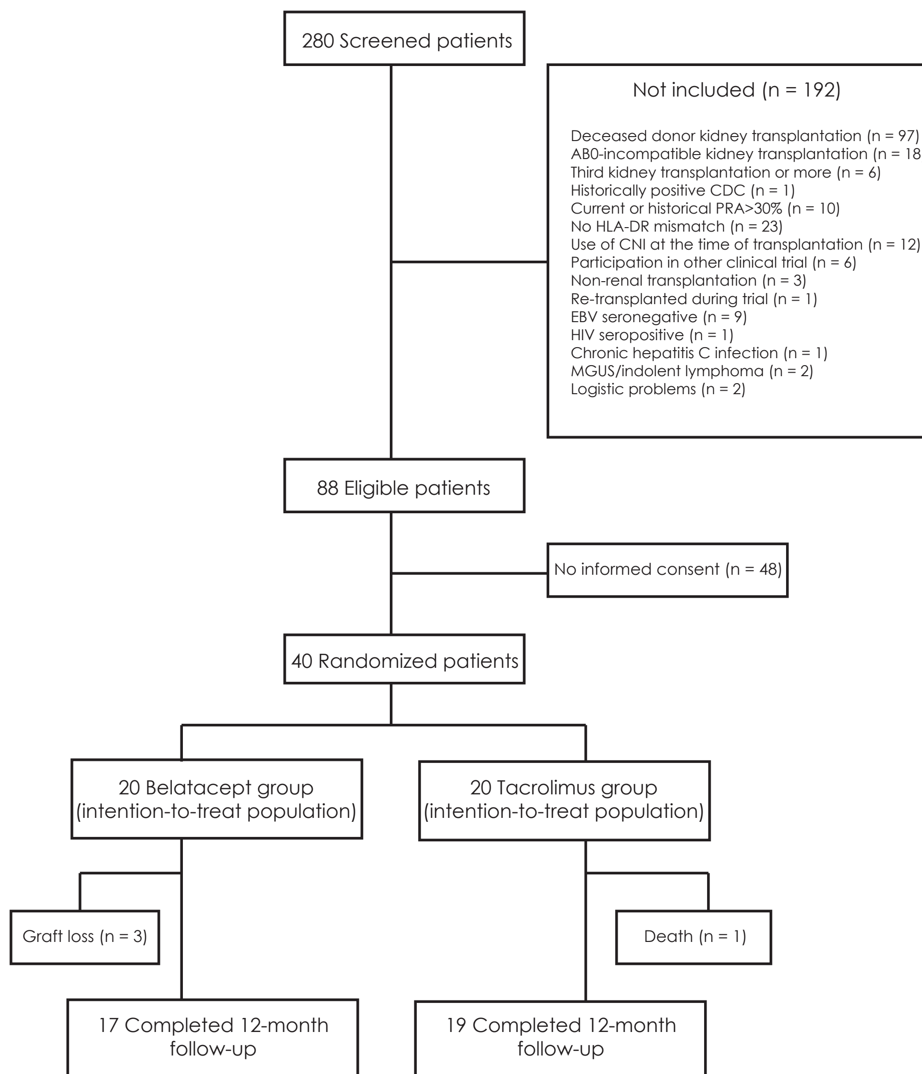



**Figure 1: Trial flowchart.**

All patients who were included in the study were randomized, underwent transplantation and received at least one dose of belatacept or tacrolimus.

CDC, cytotoxicity dependent cross-match; CNI, calcineurin inhibitor; EBV, Epstein Barr Virus; HIV, human immunodeficiency virus; HLA, human leukocyte antigen; MGUS, monoclonal gammopathy of unknown significance; PRA, panel reactive antibodies

**Table 2:** Baseline characteristics at time of transplantation

| | Belatacept group (n = 20) | Tacrolimus group (n = 20) | p |
|---|---|---|---|
| Age (years) | 57 (25-76) | 55 (21-76) | 0.88 |
| Male / female | 14 (70%) / 6 (30%) | 16 (80%) / 4 (20%) | 0.72 |
| Ethnicity | | | 1.00 |
| • Caucasian | 17 (85%) | 16 (80%) | |
| • African | 2 (10%) | 2 (10%) | |
| • Asian | 1 (5%) | 2 (10%) | |
| Body weight (kg) | 79.0 (56.6-111.4) | 93.6 (51.4-120.0) | 0.06 |
| HLA A mismatch (mean ± SD) | 1.1 (± 0.7) | 1.4 (± 0.5) | 0.13 |
| HLA B mismatch (mean ± SD) | 1.3 (± 0.5) | 1.5 (± 0.5) | 0.51 |
| HLA DR mismatch (mean ± SD) | 1.1 (± 0.4) | 1.3 (± 0.4) | 0.70 |
| Current PRA (%) | 0 (0-5) | 0 (0-17) | 0.30 |
| Peak PRA (%) | 4 (0-6) | 4 (0-21) | 0.78 |
| CMV status | | | 0.80 |
| • Donor + / Recipient - | 3 (15%) | 2 (10%) | |
| • Donor + / Recipient + | 4 (20%) | 7 (35%) | |
| • Donor - / Recipient - | 7 (35%) | 6 (30%) | |
| • Donor - / Recipient + | 6 (30%) | 5 (25%) | |
| Donor age (years) | 59 (24-71) | 51 (22-80) | 0.18 |
| Related / unrelated donor | 6 (30%) / 14 (70%) | 5 (25%) / 15 (75%) | 1.00 |
| Cause of end-stage renal disease | | | 0.09 |
| • Diabetes mellitus | 3 (15%) | 7 (35%) | |
| • Hypertension | 2 (10%) | 5 (25%) | |
| • IgA nephropathy | 1 (5%) | 3 (15%) | |
| • Polycystic kidney disease | 3 (15%) | 3 (15%) | |
| • Obstructive nephropathy | 3 (15%) | 1 (5%) | |
| • Unknown | 5 (25%) | 0 (0%) | |
| • Other | 3 (15%) | 1 (5%) | |
| Renal replacement therapy | | | 0.91 |
| • None (pre-emptive) | 10 (50%) | 12 (60%) | |
| • Hemodialysis | 7 (35%) | 6 (30%) | |
| • Peritoneal dialysis | 3 (15%) | 2 (10%) | |
| Time on dialysis (days) | 425 (123-2782) | 605 (465-1519) | 0.41 |

| | | | |
|---|---|---|---|
| Number of kidney transplantation | | | 1.00 |
| • First | 19 (95%) | 20 (100%) | 1.00 |
| • Second | 1 (5%) | - | |

Continuous variables are presented as medians (plus ranges) and categorical variables as numbers (plus percentages), unless otherwise specified

BPAR, biopsy-proven acute rejection; CMV, cytomegalovirus; HLA, human leukocyte antigen; PRA, panel reactive antibodies (current = PRA at time of transplantation, peak = historically highest measured PRA); SD, standard deviation.

## Results

*Patients*

Between October 1st, 2013 (first patient, first visit) and February 26th, 2015 (last patient, first visit) 280 patients were screened, of whom 88 were eligible for participation (Figure 1). Forty-eight patients did not wish to participate. Major reasons were fear of acute rejection and inconvenience of the monthly belatacept infusions. Forty patients were randomized and included in the intention-to-treat analysis. The baseline characteristics of these patients are described in Table 2. Seventeen (85%) patients in the belatacept and 19 (95%) in the tacrolimus group completed the 1-year follow-up period (last patient, last visit occurred on February 19th, 2016).



*Patient and graft survival*

Patient survival was 95% in the tacrolimus group and 100% in the belatacept group (p = 0.32). One patient, randomized to the tacrolimus group, died 294 days after transplantation as a result of traumatic head injury. Three graft losses, all in the belatacept group, occurred on days 12, 59 and 161 after transplantation, resulting in a 1-year death-censored graft-survival of 85% in the belatacept group *vs.* 100% in the tacrolimus group (p = 0.08). All three graft losses were the result of glucocorticoid-resistant acute rejection (Banff type IIB in two cases and type III in the third patient(23)).

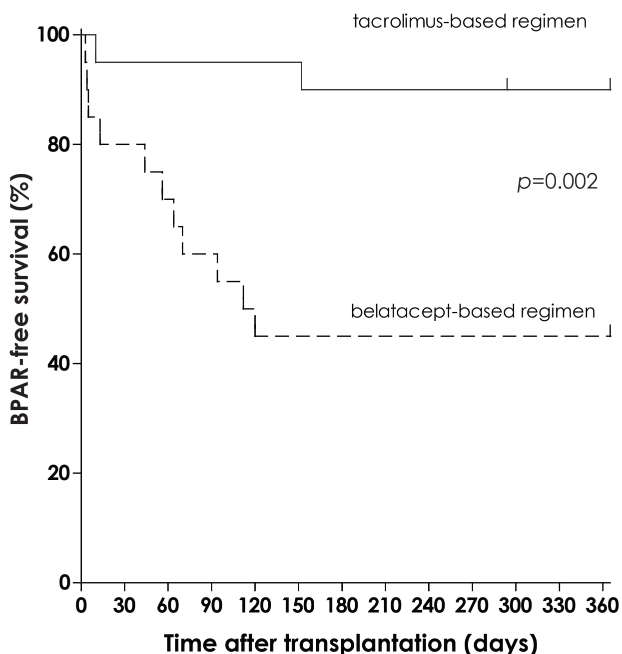
*Biopsy-proven acute rejection*

In total, 29 for cause biopsies were performed in the belatacept group and 10 in the tacrolimus group in 14 and 6 patients, respectively, p = 0.015. The incidence of BPAR was higher among the belatacept-treated patients than in the tacrolimus-treated patients: n = 11 (55%) vs. n = 2 (10%), respectively; p = 0.006 (Table 3). The death-censored BPAR-free survival was significantly lower in the belatacept-treated patients than in the tacrolimus-treated patients, (p = 0.002; Figure 2). Median time to rejection of patients who experienced AR was 56 (3–120) days in the belatacept group and 81 (10–152) days in the tacrolimus group. BPAR was of a more

severe histological grade in the belatacept than in the tacrolimus group (p = 0.003; Table 3).

A detailed overview of the clinical course of the individual patients is depicted in Figure 3. In the belatacept group, n = 10 patients (50%) were treated for BPAR with pulse methylprednisolone therapy. Six patients (30%) received additional treatment with alemtuzumab, which is the preferred T-cell depleting antibody in our center.(27) In retrospect, and after revision by the second pathologist, one more patient in the belatacept group (case no. 13) was diagnosed as suffering from rejection but he was not treated with additional anti-rejection therapy. This patient had a so-called isolated v-lesion and despite not treating him, his graft function has remained excellent to the present day. After exclusion of this particular case, the BPAR rate was still significantly higher in the belatacept group than in the tacrolimus group. Nine patients (45%), all suffering from BPAR, were converted from belatacept to tacrolimus.

In the tacrolimus group, n = 2 patients were treated for BPAR: in one case with methylprednisolone pulse therapy only, in the other, additional treatment with alemtuzumab was given. Five patients (2 in the belatacept and 3 in the tacrolimus arm) received methylprednisolone for suspected rejection (For details see Figure 3 legend).




| Days after transplantation | 0 | 3 | 4 | 5 | 10 | 13 | 44 | 56 | 64 | 70 | 94 | 112 | 120 | 152 | 294 | 365 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Belatacept-based regimen (No. at risk) | 20 | 20 | 19 | 18 | 18 | 17 | 16 | 15 | 14 | 13 | 12 | 11 | 10 | 10 | 10 | 9 |
| Tacrolimus-based regimen (No. at risk) | 20 | 20 | 20 | 20 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 19 | 18 | 17 |

**Figure 2: Biopsy-proven acute rejection (BPAR)-free survival.**

The time to first BPAR is depicted for the belatacept (dotted line) and the tacrolimus (solid line) group. In the tacrolimus group one patient died 294 days after transplantation due to traumatic head injury.

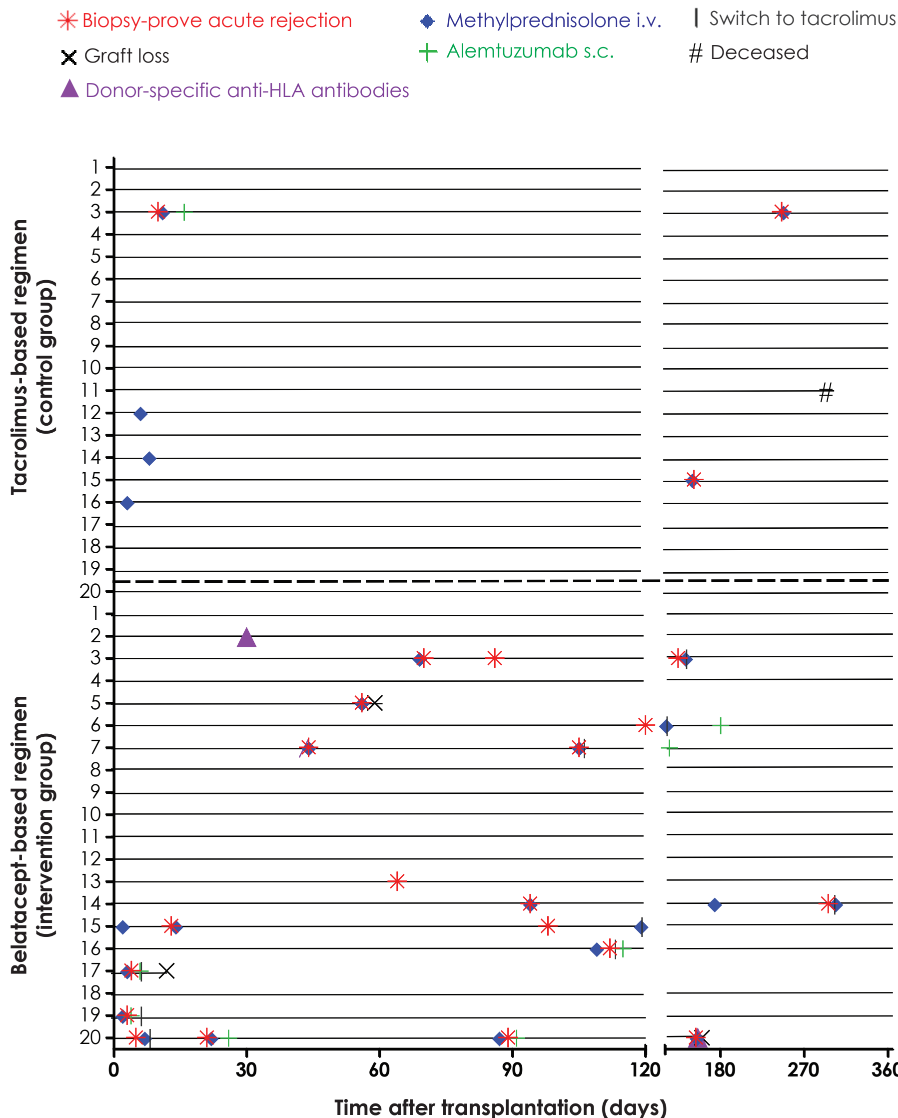



**Figure 3: Clinical outcomes.**

Each line represents the post-transplant course of the 20 individual belatacept- and 20 individual tacrolimus-treated patients (separated by the bold dotted line). Time of BPAR (✳), anti-rejection therapy (methylprednisolone intravenously [◆] or alemtuzumab subcutaneously [+]), switch to tacrolimus (|), development of donor-specific anti-human leukocyte antigen (HLA) antibodies (DSA) (▲), graft loss (✕) and death (#) are shown.In the belatacept group, n = 1 (5%) patient (no. 15) received methylprednisolone for presumed rejection pending the results of a kidney biopsy. Biopsy revealed an alternative diagnosis namely ascending urinary tract infection. In one other case, methylprednisolone was administered for suspected rejection (no. 14). A biopsy was not performed because of a coagulation disorder. In the tacrolimus group, n = 3 (15%) patients received methylprednisolone for presumed rejection pending biopsy results. In all three cases an alternative diagnosis was made: acute tubular necrosis in two patients (no. 12 and 14) and an ascending urinary tract infection in one case (no. 16). One belatacept-treated patient (no. 13) was not treated for rejection, because the diagnosis of vascular rejection (isolated v-lesion) was only made in retrospect after revision of the biopsy.

*Safety*

In total, 205 AEs occurred in the belatacept group (mean 10.3 per patient) and 238 in the tacrolimus group (mean 11.9 per patient); p = 0.41 (SDC, Table 1). Of these, 22 and 35, respectively, were judged to be serious (means per patient 1.1 and 1.8, respectively; p = 0.15), excluding BPAR, graft loss, and death.

eGFR, excluding graft losses, was not different between belatacept-treated and tacrolimus-treated patients 12 months after transplantation (SDC, Table 2): 54 (28–89) and 50 (33–84) mL/min per 1.73m$^2$, respectively; p = 0.57. Median protein/creatinine ratio was 13.2 (5.7–343.8) mg/mmol in the belatacept group and 9.0 (5.3–43.5) mg/mmol in the tacrolimus group; p = 0.44. Additional routine measurements are depicted in SDC, Table 2.

For the on-therapy analysis on month 12; graft function before, during and after BPAR in the belatacept group; the incidence of DSA and non-DSA; and pharmacokinetic drug monitoring, refer to SDC, Results and SDC, Tables 3-5.

**Table 3:** Incidence of rejection according to the treatment group

| | **Belatacept group (n = 20)** | **Tacrolimus group (n = 20)** | **p** |
|---|---|---|---|
| Borderline | 0 (0%) | 0 (0%) | - |
| Type 1 | | | 1.00 |
| • 1A | 0 (0%) | 0 (0%) | |
| • 1B | 1 (5%) | 1 (5%) | |
| Type 2 | | | 0.003 |
| • 2A | 2 (10%) | 1 (5%) | |
| • 2B | 6 (30%) | 0 (0%) | |
| Type 3 | 1 (5%) | 0 (0%) | 1.00 |
| Mixed | 1 (5%) | 0 (0%) | 1.00 |
| Total BPAR | 11 (55%) | 2 (10%) | 0.006 |

The incidence of the first rejection episodes is given. The highest Banff score is depicted if sequential biopsies were performed.

BPAR, Biopsy-proven acute rejection

*Immunological primary end-points (biomarkers)*

Three potential biomarkers for (belatacept-resistant) rejection were measured pre-transplantation, namely CD8$^+$CD28$^-$ T-cells, CD4$^+$CD57$^+$ PD1$^-$ T-cells, and CD8$^+$CD28$^{++}$ EMRA T-cells. There were no significant differences in the numbers or percentages of these cells at baseline between the tacrolimus and belatacept groups (Table 4). The limited number of

patients experiencing BPAR in the tacrolimus group (n = 2) precluded a meaningful statistical comparison between rejectors and non-rejectors in this group. Gating strategies, pre-transplant numbers and percentages of the above-mentioned cell subsets are depicted for future rejectors and non-rejectors in the belatacept group (SDC, Table 6; Figure 4), and no statistically significant differences were observed. Intracellular Granzyme B (GrB) expression was measured in the cell subsets (Figure 4A). Next, we analyzed whether high numbers or proportions of these cell types increased BPAR risk within the first 12 after transplantation by conducting univariable Cox regression analyses (Table 5):

**Table 4:** Absolute numbers and percentages of T cell subsets pre-transplantation

| | **Belatacept group (n = 20)** | **Tacrolimus group (n = 20)** | **p** |
|---|---|---|---|
| $CD8^+$ $CD28^-$ T-cells/uL | 95 (13 – 696) | 135 (26 – 371) | 0.72 |
| $CD28^-$ % of $CD8^+$ T-cells | 26.5 (6.4 – 82.8) | 35.8 (7.4 – 75.3) | 0.95 |
| $CD4^+$ $CD57^+$ $PD1^-$ T-cells/uL | 6 (1 – 126) | 9 (2 – 73) | 0.18 |
| $CD57^+$ $PD1^-$ % of $CD4^+$ T-cells | 0.8 (0.2 – 38.2) | 1.3 (0.2 – 8.1) | 0.95 |
| $CD8^+$ $CD28^{++}$ EMRA T-cells/uL | 5 (1 – 74) | 11 (0 – 118) | 0.84 |
| $CD28^{++}$ % of $CD8^+$ EMRA T-cells | 10.8 (1.8 – 49.3) | 9.0 (2.1 – 35.8) | 0.82 |

Data represent medians (plus ranges).



1) *$CD8^+CD28^-$ T-cells*

$CD8^+CD28^-$ T-cells are mostly effector-memory cytotoxic T-cells that produce large amounts of pro-inflammatory cytokines,(15-17) and are not susceptible to co-stimulation blockade by belatacept. Almost 70% (31–89%) of $CD8^+CD28^-$ T-cells produced GrB. Higher numbers and proportions of pre-transplant $CD8^+CD28^-$ T-cells (irrespective of their intra-cellular GrB expression) did not significantly increase BPAR risk in the first 12 months after transplantation (Hazard Ratio [HR] 1.06; 95%-CI 0.61 to1.83 and HR 1.05; 95%-CI 0.50 to 2.20, respectively; Table 5).

2) *$CD4^+CD57^+PD1^-$ T-cells*

Next, pre-transplant $CD4^+CD57^+PD1^-$ T-cells were compared between rejecting and non-rejecting belatacept-treated patients. These cells were recently described as being cytolytic, $CD28^-$, and to be associated with belatacept-resistant rejection.(18) The proportion of pre-transplant $CD4^+CD57^+PD1^-$ T-cells was low (<2% of the $CD4^+$ T-cell population in most patients). Approximately 24% (1–74%) of these cells were GrB positive. Neither the absolute number nor the proportion of these cell predicted BPAR (HR 0.89; 95%-CI 0.58 to 1.27, and HR 0.90; 95%-CI 0.59 to 1.38, respectively; Table 5).

3) *CD8⁺CD28⁺⁺ EMRA T-cells*

Finally, $CD8^+CD28^{++}$ EMRA T-cells were analyzed as high numbers of these cells predicted belatacept-resistant rejection in primates.(19) It was postulated that these cells rapidly down-regulate their surface CD28 expression after transplantation, making them resistant to co-stimulatory blockade.(19) Circa 3% (0–3%) of these cells expressed intracellular GrB. The absolute numbers or proportions of pre-transplant $CD28^{++}$ cells within the $CD8^+$ EMRA T-cell population did not increase BPAR risk (HR 0.86; 95%-CI 0.58 to 1.27, and HR 1.23; 95%-CI 0.64 to 2.33, respectively; Table 5) Interestingly, from the 5 patients with >35 $CD8^+CD28^{++}$ EMRA T-cells/µL, 4 were rejectors and only 1 was a non-rejector (Figure 4B). In the tacrolimus group the n=2 rejectors had <10 $CD8^+CD28^{++}$ EMRA T-cells/µL pre-transplantation.

**Table 5:** Univariable Cox regression analyses for the risk of biopsy-proven acute rejection

| Independent variables | Hazard Ratio | 95%-CI | | p |
|---|---|---|---|---|
| | | Lower | Upper | |
| Age (years) | 1.002 | 0.964 | 1.042 | 0.91 |
| Gender (female *vs* male) | 1.540 | 0.474 | 5.015 | 0.47 |
| Ethnicity (Non-Caucasian vs Caucasian) | 0.090 | 0.002 | 5.431 | 0.25 |
| Highest PRA (%) | 0.853 | 0.668 | 1.088 | 0.20 |
| HLA total mismatches (4 or more *vs* less than 4) | 1.568 | 0.512 | 4.800 | 0.43 |
| HLA DR mismatches (2 *vs* 1) | 0.623 | 0.138 | 2.810 | 0.54 |
| CMV seropositivity (positive *vs* negative) | 0.453 | 0.148 | 1.389 | 0.17 |
| Treatment group (belatacept *vs* tacrolimus) | 7.206 | 1.592 | 32.614 | 0.01 |
| $CD8^+$ $CD28^-$ T-cells/uL pre-transplantation | 1.061 | 0.614 | 1.832 | 0.83 |
| $CD28^-$ % of $CD8^+$ T-cells pre-transplantation | 1.048 | 0.500 | 2.196 | 0.90 |
| $CD4^+$ $CD57^+$ $PD1^-$ T-cells/uL pre-transplantation | 0.886 | 0.577 | 1.360 | 0.58 |
| $CD57^+$ $PD1^-$ % of $CD4^+$ T-cells pre-transplantation | 0.904 | 0.592 | 1.382 | 0.64 |
| $CD8^+$ $CD28^{++}$ EMRA T-cells/uL pre-transplantation | 0.858 | 0.581 | 1.268 | 0.44 |
| $CD28^{++}$ % of $CD8^+$ EMRA T-cells pre-transplantation | 1.226 | 0.644 | 2.331 | 0.54 |
| CD86 molecules/monocyte | 0.327 | 0.049 | 2.192 | 0.25 |

CI, confidence interval; CMV, cytomegalovirus; HLA, human leukocyte antigen; PRA, panel reactive antibodies

N.B.: To ensure normal distribution, numbers and proportions of the biomarkers and CD86 molecules/monocyte were log transformed before including them in the univariable Cox regression analyses. This means that the hazard ratio represents the relative increase in risk for each log unit increase of the independent variable rather than one unit increase of this variable.

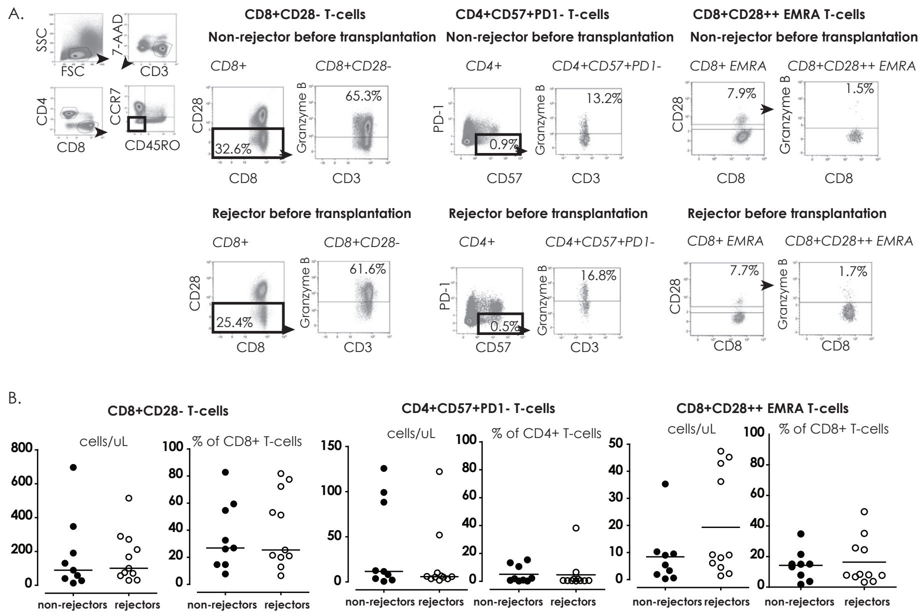



**Figure 4: CD8+CD28-, CD4+CD57+PD1- and CD8+CD28++ EMRA T-cells pre-transplantation.**

CD4+ and CD8+ T-cells were gated from 7-AAD negative CD3+ lymphocytes (based on forward and sideward scatter) and EMRA T-cells were gated as CCR7- and CD45RO- T-cells. Typical examples are given for non-rejectors and rejectors in the belatacept group for CD8+CD28-, CD4+CD57+PD1- and CD8+CD28++ EMRA T-cells and their intracellular Granzyme B expressions (A). The absolute numbers and percentages of CD8+CD28-, CD4+CD57+PD1- and CD8+CD28++ EMRA T-cells are presented for non-rejectors and rejectors (B).



The above-mentioned cell surface biomarkers were also measured in belatacept-treated patients during acute rejection and before additional anti-rejection therapy was given, and were compared with the month 3 samples from patients who remained rejection-free (SDC, Figure 1). No statistically significant differences were observed between rejecting and non-rejecting belatacept-treated patients.

The only significant risk factor for rejection in this study population was the use of a belatacept-based immunosuppressive regimen (HR 7.2; 95%-CI 1.6 to 32.6; p = 0.01) compared to tacrolimus-based therapy (Table 5). Since no other variable significantly influenced acute rejection risk and the sample size was small, no multivariable Cox regression analysis was conducted.

*Pharmacodynamic monitoring of belatacept*

The pharmacodynamic effect of belatacept was monitored by measuring free CD86 molecules on circulating monocytes. CD86 was saturated by belatacept at all time points, in both rejectors as non-rejectors. Moreover, pre-transplantation CD86 molecules/monocyte were not predictive for BPAR (HR 0.33, 95%-CI 0.1-2.2). For details about CD86-expression on monocytes in belatacept- and tacrolimus-treated patients, refer to SDC, Results and SDC, Figure 2.

## Discussion

In this RCT, a belatacept-based and a tacrolimus-based immunosuppressive regimen without lymphocyte-depleting induction therapy were compared head-to-head for the first time in *de novo* kidney transplantation. The results of this trial demonstrate that belatacept is not as potent as tacrolimus in preventing rejection.

In comparison to the 1-year results of the BENEFIT-trial where ciclosporin was used as comparator,(7) we found a more pronounced difference in both BPAR incidence and severity. Ninety-one percent of BPAR in the belatacept group was classified as type II (or higher),(28) while in the BENEFIT-trial this was 69%. The use of lymphocyte-depleting therapy to treat rejection was comparable: circa 50% of BPAR in the BENEFIT-trial *vs.* 55% in this study. The incidence of graft loss caused by BPAR was higher in this study than in the BENEFIT-trial: 3 of 11 *vs.* 2 of 39 rejecting patients, respectively.

This larger difference in rate and severity of BPAR is not explained by dissimilarities between study groups. In the present study 1) there were no transplantations with deceased donors; 2) there were no patients with a PRA >30%; and 3) the proportion of Caucasians was larger. All 3 characteristics are associated with a lower BPAR risk.(29-35) In contrast, the proportion of pre-emptive transplantations was high in our study (55% of included patients), which may have led to the inclusion of patients with a more potent immune system.(36-38) Another explanation for the higher BPAR-rate could be that in this study TDM for MPA was performed, whereas this was not the case in the BENEFIT-trial. It is therefore, theoretically possible that belatacept-treated patients in BENEFIT were exposed to higher MPA concentrations.(1) However, we feel that this is an unlikely explanation as ciclosporin lowers exposure to MPA, whereas tacrolimus does not have such an effect.(39)

Our findings are in line with the higher BPAR rates observed in large retrospective studies and a small cohort study comparing belatacept to tacrolimus.(13, 14, 18) Wen *et al*. conducted a retrospective cohort study using registry data of a time period of 3 years, and compared 1-year clinical outcomes between belatacept- and tacrolimus-treated adult kidney transplant recipients.(39) Although the incidence of BPAR was not as high as in the present trial, Wen *et al*. also observed significantly higher BPAR rates among belatacept-treated patients as compared with tacrolimus-treated patients who would have been eligible for participation in the BENEFIT-study: 15% of patients treated with belatacept and lymphocyte depleting antibody therapy, *versus* 23% of patients treated with belatacept without lymphocyte depleting antibody therapy, *versus* 6% of tacrolimus-treated patients. Nonetheless, it is important to stress that the higher incidence of BPAR in the present study should be interpreted with caution, because the study here included limited numbers of patients, had limited statistical power and may therefore be a chance finding.

In this study, no suitable pre-transplant biomarker was found to predict belatacept-resistant BPAR.(15, 16, 18, 19) The first potential biomarker, pre-transplant $CD8^{+}CD28^{-}$ T-cell number, seemed a logical choice as these highly cytotoxic cells lack surface CD28 and are therefore not susceptible to belatacept.(15-17) Possible explanations for the observation that these cells were not associated with BPAR may be that 1) even though these cells are highly cytotoxic, they lack proliferative capacity,(40) and 2) the CD28-CD80/86 pathway is not the sole mediator of belatacept-resistant rejection. Targeting other co-stimulatory pathways, like

CD40-40L, simultaneously with belatacept, might be more efficient to prevent BPAR.(41, 42) Preliminary data from Cortes-Cerisuelo *et al*. suggest that not the lack of CD28 on these cells before transplantation, but the potential to down-regulate CD28 after donor antigen stimulation is associated with BPAR in belatacept-treated patients.(43)

The second biomarker, pre-transplant $CD4^+CD57^+PD1^-$ T-cell number, was associated to belatacept-resistant rejection in an observational cohort study.(18) These findings were not confirmed here. Apart from differences in study design, the dissimilarities in study populations may explain this discrepancy. [23-26] Our study population 1) was mostly Caucasian; 2) received mostly pre-emptive transplants; and 3) was shorter on dialysis. These factors have, however, not been associated with CD57 expression, and the proportions of $CD4^+CD57^+PD1^-$ T-cells were similar pre-transplantation. Age and CMV status, which influence these proportions(40, 44-48), were also comparable (data not shown).

The final biomarker, $CD8^+CD28^{++}$ EMRA T-cell number, showed potential to predict BPAR under belatacept, even though the group medians did not differ between rejectors and non-rejectors. One of the 9 non-rejectors and 4 of the 11 rejectors had high numbers of these cells pre-transplantation (>35 cells/μL). In-depth analysis of the antigen-specificity of these $CD8^+CD28^{++}$ EMRA T-cells in larger studies seems warranted.(49)

Pharmacodynamic drug monitoring in the form of measuring free, non-belatacept bound CD86 molecules on circulating monocytes was not useful to predict BPAR under belatacept therapy, since free molecules were not higher in rejectors pre-transplantation, and followed the same dynamics in rejectors as in non-rejectors.

Limitations of this study are the small sample size and the resulting increased chance of type II errors. The increased rejection risk among belatacept-treated patients therefore needs to be confirmed in larger RCTs. Ideally, such trials will also include biomarker studies and analyze pre-transplant donor-specific immunity. Also, research on regulatory T-cells would be of interest since blockade of CD80/86 leads to anergic T-cells,(50) which consequently may fail to activate regulatory T-cells via CD28. Studies on antigen-specific biomarkers, such as the IFNγ Elispot assay, would also be useful to study in larger, prospective trials.(51, 52)

In conclusion, this small RCT showed that belatacept-based immunosuppressive therapy results in a significantly higher rejection-rate and severity compared with standard, tacrolimus-based therapy. There were no differences in pre-transplant cellular biomarkers between rejectors and non-rejectors. Belatacept adequately blocked the CD28-CD80/86 co-stimulatory pathway in all patients, making insufficient saturation an unlikely explanation for this higher rejection risk. These results should be regarded as hypothesis-generating and need to be further validated in independent cohorts.



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## Supplemental Digital Content (SDC)

### *Supplemental Materials and Methods*

*Additional (immunosuppressive) treatment – detailed information*

The additional immunosuppressive therapy was identical in both groups and consisted of basiliximab (Simulect®; Novartis Pharma B.V., Arnhem, the Netherlands) in a dose of 20 mg administered intravenously on day 0 (immediately before kidney transplant reperfusion) and day 4 after transplantation. Patients also received a starting dose of 1000 mg mycophenolate mofetil (MMF; CellCept®; Roche Pharmaceuticals, Woerden, the Netherlands) twice daily aiming for plasma mycophenolic acid (MPA) predose concentrations between 1.5 and 3.0 mg/L. In addition, all patients received prednisolone in a dose of 50 mg twice daily intravenously on days 0–3, followed by 20 mg orally once daily (on days 4–14), after which the dose was tapered to 5 mg at month 3 after transplantation. Patients continued to receive 5 mg of prednisolone for the rest of the first post-transplant year.

All patients received trimethoprim/sulfamethoxazole prophylaxis for Pneumocystis Jirovecii pneumonia for at least 3 months. Patients receiving a kidney from a cytomegalovirus (CMV)-positive donor and patients who were seropositive for CMV received prophylaxis with valganciclovir for a duration of 6 months.

Additional anti-rejection therapy consisted of 3 doses of 1000 mg methylprednisolone intravenously for 3 consecutive days. In case of glucocorticoid-resistant rejection, lymphocyte-depleting therapy with 1 dose of 30 mg of alemtuzumab was administered subcutaneously.(1)

*Primary end points – BPAR scoring methods*

BPAR was scored as part of routine clinical care by a renal pathologist (M.C.C.) according to the Banff '15 classification using 2 µm paraffin sections stained for HE, PAS, Jones and also immunohistochemistry for C4d on 4 µm sections. After the completion of the study, all biopsies were reviewed again in a blinded fashion by two pathologists (M.C.C. and J.v.d.T.) according to the Banff '15 classification.(2) In case of discrepancy, biopsies were reviewed and consensus was reached.

*Safety*

Data on clinical outcomes and (serious) adverse events [(S)AEs] were collected for safety and included patient- and graft survival, estimated GFR (eGFR), proteinuria, and development of donor-specific anti-HLA antibodies (DSA). DSA were retrospectively measured in patient sera one day before transplantation, and 1, 6 and 12 months after transplantation. In addition, we monitored delayed graft function, malignancies, (opportunistic) infections, post-transplant diabetes mellitus (PTDM), neurologic events, and acute tacrolimus-induced nephrotoxicity. PTDM was defined as the need for glucose-lowering medical therapy that persisted after month 3 post-transplantation in a patient not needing such treatment pre-transplantation. Acute tacrolimus nephrotoxicity was defined as any ≥15% increase of serum creatinine with a return to baseline after tacrolimus dose reduction and after exclusion of other causes of renal transplant function deterioration.

Routine laboratory investigations included blood glucose, glycated hemoglobin (HbA1c), thrombocytes, leucocytes, hemoglobin (Hb), mean corpuscular volume (MCV), low-density lipoproteins (LDL), high-density lipoproteins (HDL) and triglycerides.Blood pressure and body weight were measured at every visit to the outpatient clinic.

*Laboratory Studies – detailed information*

Blood samples were collected on days 0 (pre-transplant), 4, 30, 90, and months 6 and 12. Serum was collected on days 0, 15, 30, and months 6 and 12. Blood and sera were also collected during clinically suspected rejection, before additional anti-rejection therapy was given. In addition, blood and urine samples were collected on a routine basis as part of routine clinical care. Proportions of CD8+CD28-, CD4+CD57+PD1- and CD8+CD28++ EMRA T-cells were determined pre-transplantion (1 day before transplantation) and post-transplant (3 months after transplantation or during rejection) on thawed isolated peripheral blood mononuclear cells.

*Absolute numbers of cells in blood*

The Becton & Dickinson (BD Biosciences, San José, CA) multi-test 6-color®, CD14 FITC (Serotec, Kidlington, United Kingdom) and TruCount Tubes® were used to measure absolute numbers of CD3+ T-cells, CD4+ T-helper cells, CD8+ cytotoxic T-cells, and CD14+ monocytes. Absolute numbers were measured in 50 µL blood in the presence of 0.5 mL BD Pharm Lyse. All proportions of subsets measured in PBMCs (see below) were calculated back to these absolute numbers.

*Flow cytometry of cytotoxic T-cells in peripheral blood mononuclear cells (PBMCs)*

Using the Ficoll density method, PBMCs were isolated and stored at -190°C before further characterization. T-cells were identified by CD3 (AF700, BD), CD4 (V450, BD) and CD8a (APC-eF780, eBioscience). The immuno-regulatory receptor PD-1 (PE, BioLegend), the cytotoxic marker CD57 (FITC, BD), and the co-stimulatory molecule CD28 (APC, BD) were determined on CD4+ and CD8+ T-cells. EMRA CD8+ T-cells were defined by CD8+ CCR7-CD45 RO-, using CCR7 (PE, BD) and CD45RO (PE-Cy7, BD). Intracellular expression of GrB (PE-CF594, BD) was also assessed.

*Saturation of CD86 on monocytes and B-cells in blood*

The surface expression of free CD86 on CD14+ monocytes was assessed using the Lyse-Wash method according to the manufacturer's instruction. Cells were surface-stained in 100 µL blood and erythrocytes were subsequently lysed in 2 mL BD FACS Lysing solution®, and washed away before measurement. Monoclonal antibodies used were the leukocyte marker CD45 PerCP (BD); CD19 PE-Cy7 (BioLegend); CD14 FITC (Serotec); and the for belatacept competitive binder of the co-stimulatory molecules of the CD28-pathway, CD86 PE (clone HA5.2B7 Beckman Coulter, Brea, CA).(3) Numbers of CD86 molecules per monocyte were calculated by using QuantiBrite beads according to manufacturer's manual (BD).

*Detection of serum DSA*

Using the Luminex single antigen bead assay (Thermo Fisher Scientific, Waltham, MA) as previously described,(4) the development of DSA was determined by measuring the presence of DSA against HLA class I and II before and at different set time points after transplantation in serum. The MFI cut-off for positivity was 1000.

*Panel reactive antibodies*

Sera were tested for HLA-antibody specificities by standard National Institutes of Health

(NIH) complement-dependent microlymphocytotoxicity test (LCT) using a panel of 54 donors yielding a measurement of the PRA (Panel Reactive Antibody). If samples tested positive using a Human Linker for Activation of T cell ELISA (LAT) or a Complement-Dependent Cytotoxicity Crossmatch(CDC), HLA antibodies were specified with Luminex single antigen test (LABScreen SA, One Lambda Inc., Canoga Park, CA, USA).

*Statistical analyses – additional information*

Patient, graft and biopsy-proven acute rejection (BPAR)-free survival were defined as 1) time from transplantation to mortality, 2) time from transplantation to transplant nephrectomy, re-initiation of dialysis or (pre-emptive) re-transplantation, and 3) time from transplantation to the diagnosis of BPAR, respectively, or as the end of the 12-month follow-up period, whichever came earlier.

In addition to intention-to-treat analyses, on-therapy analyses were conducted and included evaluable patients who were still on their assigned regimen 12 months after transplantation.

Categorical variables (+ reference groups) in the univariable Cox regression analyses included treatment arm (belatacept vs. tacrolimus), gender (female vs. male), ethnicity (non-Caucasian vs. Caucasian), HLA mismatches (4 or more vs. less than 4), HLA-DR mismatches (2 vs. 1), highest PRA, and CMV serostatus (positive vs. negative).

## *Supplemental Results*



*On-therapy analysis*

The on-therapy analysis at month 12 revealed that eGFR and protein/creatinine ratios were similar between non-rejecting tacrolimus and belatacept-treated patients: median eGFR 57 (45-89) and 58 (37-84) mL/min per 1.73m2, respectively (SDC, Table 3). Graft-loss censored median eGFR in belatacept-treated patients that suffered from rejection (n = 7) was 36 (28-76) mL/min per 1.73m2 at month 12, which was lower than in the non-rejecting belatacept group, p = 0.001.

*Graft function in time in belatacept-treated rejectors*

The graft function before, during and after BPAR (after additional anti-rejection therapy) is displayed in SDC, Table 4, for the belatacept-group. Before and after BPAR the highest eGFR is depicted for each patient. It should be noted 6 patients had a decrease in eGFR after BPAR was diagnosed (including 3 graft losses), 2 patients had a similar eGFR after treatment for BPAR, and 3 patients had an improved eGFR.

*Donor-specific and non-donor-specific anti-HLA antibodies (DSA and non-DSA)*

None of the patients had DSA pre-transplantation. During the first post-transplant year, 2 patients developed DSA, both in the belatacept group (Figure 3 and SDC, Table 5). One month after transplantation, patient no. 2 in the belatacept group developed DSA against HLA-DQ2 (Median Fluorescence Intensity [MFI] 3787; most likely C1q-negative(5), but these disappeared hereafter without additional therapy and no AR occurred. Patient no. 20 in the belatacept group developed DSA during her fourth rejection episode (right before losing her graft), which were

also detectable in the cross match-dependent cytotoxicity test, against HLA-A1 (MFI 18000), B8 (MFI 22700), DR3 (MFI 11000), DR52 (MFI 5500) and DQ2 (MFI 16500) (SDC, Table 5). At this time she had already been switched to a tacrolimus-based regimen and had been treated with methylprednisolone and alemtuzumab (Figure 3).

Two and three patients, in the belatacept and tacrolimus group, respectively, had non-donor specific anti-HLA antibodies (non-DSA) pre-transplantation. In both the belatacept and the tacrolimus group two patients developed non-DSA after transplantation (SDC, Table 5).

*Pharmacokinetic drug monitoring*

SDC, Table 2 depicts belatacept doses, tacrolimus doses and pre-dose concentrations (C0), MMF doses and mycophenolic acid (MPA) C0, and prednisolone doses. MPA C0 were not different between the belatacept and tacrolimus groups after 12 months: 2.30 (0.99–3.54) and 1.83 (0.57–3.67) mg/mL, respectively; p = 0.25. Also prednisolone doses were similar between the belatacept and tacrolimus group in month 12; p = 0.59.

*Pharmacodynamic drug monitoring*

The number of belatacept-free CD86 molecules on monocytes was calculated by measuring the MFI of bound anti-CD86-PE antibodies. These antibodies bind to CD86 molecules to the same epitope but with lower affinity than belatacept, which allows for measurement of free CD86 molecules.(3) A typical example is depicted for the MFIs of CD86-PE on monocytes for a patient treated with belatacept and a patient treated with tacrolimus (SDC, Figure 2A). As evidenced by a linear mixed model, belatacept significantly decreased free CD86 molecules on monocytes at different time points after transplantation compared to tacrolimus (SDC, Figure 2B). Free CD86 molecules/monocyte were 5.9-fold (95%-CI 5.4 to 7.7-fold) higher on day 4 and 5.3-fold (95%-CI 4.0 to 7.0-fold) higher 1 month after transplantation in tacrolimus-treated patients compared to belatacept-treated patients, p <0.0001. Hereafter the difference in free CD86 molecules/monocyte between the belatacept- and tacrolimus-treated patients reduced, because almost half of the belatacept-treated patients had been converted to tacrolimus-based therapy. In these patients (n = 8), free CD86 expression returned to baseline 3–5 months after conversion (SDC, Figure 2C). Pre-transplant values for (future) rejectors and non-rejectors in the belatacept group were significantly different: 753 (428 – 928) free CD86 molecules/monocyte versus 882 (528 – 1528) cells/monocyte, respectively, p = 0.04 (SDC, Figure 2D). However, the pre-transplant values showed a great overlap between rejectors and non-rejectors, and the numbers of pre-transplant CD86 molecules on monocytes were not associated with acute rejection risk (Table 5). No significant differences between (future) rejectors and non-rejectors were observed in post-transplant dynamics of free CD86 molecules/monocyte (SDC, Figure 2E).

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## *Supplemental Tables*

**SDC, Table 1** Adverse events, intention-to-treat analysis* (*Table continues on next pages*)

| | Belatacept group (n = 20) | Tacrolimus group (n = 20) | p |
|---|---|---|---|
| Blood or lymphatic system | 0.75 (0.97) | 1.00 (0.92) | 0.22 |
| • Leucopenia | 7 | 7 | |
| • Anemia | 6 | 10 | |
| • Thrombocytopenia | 1 | 1 | |
| • Other | 1 | 2 | |
| Bleeding and thrombotic events | 0.30 (0.57) | 0.40 (0.60) | 0.52 |
| • Major bleeding | 0 | 2 | |
| • Minor bleeding | 4 | 2 | |
| • Thrombosis | 2 | 4 | |
| Cancer | 0 | 0 | - |
| Cardiovascular | 0.95 (0.83) | 1.20 (0.83) | 0.33 |
| • Acute coronary syndrome / myocardial ischemia | 1 | 1 | |
| • Cardiac decompensation / volume overload | 2 | 3 | |
| • Hypertension | 12 | 17 | |
| • Other | 4 | 3 | |
| Gastrointestinal | 0.65 (0.67) | 0.60 (1.00) | 0.40 |
| • Diarrhea | 2 | 4 | |
| • Other | 11 | 8 | |
| Infection | 2.25 (1.86) | 1.90 (1.83) | 0.46 |
| • Opportunistic infection | 0.45 (0.69) | 1.90 (1.83) | 0.57 |
| ▪ BKV | 2 | 1 | |
| ▪ CMV | 1 | 2 | |
| ▪ EBV | 1 | 0 | |
| ▪ HSV | 0 | 1 | |
| ▪ VZV | 0 | 0 | |

| | | | |
|---|---|---|---|
| ▪ Fungal | 5 | 2 | |
| • Other infection | 1.80 (1.70) | 1.60 (1.64) | 0.61 |
| ▪ Urinary tract infection | 20 | 14 | |
| ▪ Upper respiratory tract infection | 8 | 4 | |
| ▪ Pneumonia | 2 | 0 | |
| ▪ Gastrointestinal infection | 1 | 2 | |
| ▪ Other | 5 | 12 | |
| Locomotor system disorder | 0.25 (0.55) | 0.20 (0.52) | 0.70 |
| Metabolism or nutrition | 1.75 (1.16) | 2.00 (1.56) | 0.84 |
| • Post-transplant diabetes mellitus | 1 | 7 | |
| • Hypo- / hyperglycemic dysregulation | 4 | 9 | |
| • Calcium disorder (hypo- / hypercalcemia) | 6 | 3 | |
| • Potassium disorder (hypo- / hyperkalemia) | 6 | 9 | |
| • Hypophosphatemia | 6 | 6 | |
| • Dyslipidemia | 8 | 4 | |
| • Liver enzyme abnormality | 3 | 1 | |
| • Other | 1 | 1 | |
| Nervous system | 0.50 (1.00) | 0.65 (0.88) | 0.36 |
| • CVA/TIA | 1 | 0 | |
| • Tremor | 2 | 8 | |
| • Headache | 1 | 1 | |
| • Other | 6 | 4 | |
| Skin-related disorders | 0.15 (0.37) | 0.30 (0.47) | 0.26 |
| Surgical or procedural complication | 0.10 (0.31) | 0.20 (0.52) | |
| • Acute tubular necrosis | 1 | 2 | |
| • Delayed graft function | 1 | 1 | |
| • Renal infarction | 0 | 1 | |
| • Other | 0 | 0 | |
| Tacrolimus-induced nephrotoxicity | 0.05 (0.22) | 0.40 (0.60) | - |
| Urological complication | 0.55 (0.76) | 0.60 (0.88) | 0.96 |
| • Hydronephrosis | 1 | 4 | |
| • Urinary leakage | 2 | 1 | |
| • Other | 8 | 7 | |

| | | | |
|---|---|---|---|
| Wound-related problem | 0.15 (0.37) | 0.25 (0.44) | 0.44 |
| • Wound infection | 2 | 3 | |
| • Other | 1 | 2 | |
| Other | 1.80 (1.44) | 2.20 (2.04) | 0.63 |
| Total | 10.25 (4.18) | 11.90 (5.43) | 0.41 |

*(<Table on previous pages)*

* Mean number of adverse events (+ standard deviation) per patient are depicted for both treatment groups for the different categories of adverse events. Numbers of adverse events per subcategory are depicted per treatment group.

BKV, BK virus; CMV, cytomegalovirus; CVA, cerebrovascular accident; EBV, Epstein-Barr virus; HSV, herpes simplex virus; N/A, not applicable; VZV, varicella zoster virus; TIA, transient ischemic attack.

**SDC, Table 2:** Clinical outcomes, intention-to-treat analysis* (*Table continues on next page*)

| | Belatacept group (n = 20) | | | | | | Tacrolimus group (n = 20) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | n | M3 | n | M6 | n | M12 | n | M3 | n | M6 | n | M12 | p† |
| Blood pressure | | | | | | | | | | | | | |
| • Systolic / diastolic | 18 | 137 (98 - 167) / 83 (40 - 94) | 17 | 138 (93 - 181) / 80 (50 - 109) | 17 | 147 (106 - 165) / 81 (50 - 85) | 20 | 144 (108 – 178) / 85 (59 – 98) | 20 | 138 (96 – 184) / 84 (55 – 95) | 19 | 145 (110 – 170) / 85 (45 – 97) | 0.64 / 0.42 |
| Kidney function | | | | | | | | | | | | | |
| • Creatinine (µmol/L) | 18 | 127 (73 – 276) | 17 | 114 (74 – 219) | 17 | 128 (71 – 207) | 20 | 122 (64 – 242) | 20 | 126 (61 – 179) | 19 | 126 (79 – 179) | 0.80 |
| • eGFR (mL/min) | 18 | 52 (18 – 72) | 17 | 62 (26 – 88) | 17 | 54 (28 – 89) | 20 | 50 (23 – 80) | 20 | 53 (33 – 85) | 19 | 50 (33 – 84) | 0.57 |
| • Protein/Creatinine ratio | 18 | 19.3 (5.2 – 443.2) | 17 | 18.2 (5.8 – 87.7) | 17 | 13.2 (5.7 – 343.8) | 20 | 15.3 (7.3 – 115.0) | 20 | 12.1 (4.2 – 209.6) | 19 | 9.0 (5.3 – 43.5) | 0.44 |
| Glucose metabolism | | | | | | | | | | | | | |
| • Glucose (mmol/L) | 18 | 5.6 (4.7 – 9.4) | 17 | 5.5 (2.9 – 13.7) | 17 | 5.6 (2.9 – 13.7) | 20 | 6.2 (3.7 – 10.7) | 20 | 6.6 (4.7 – 13.5) | 19 | 6.1 (4.3 – 26.7) | 0.06 |
| • HbA1c (mmol/mol) | 6 | 36 (29 – 74) | 3 | 37 (33 – 50) | 5 | 41 (33 – 49) | 6 | 48 (37 – 67) | 5 | 42 (30 – 73) | 10 | 46 (33 – 75) | 0.31 |
| Lipids | | | | | | | | | | | | | |
| • Cholesterol total (mmol/L) | 18 | 4.6 (2.9 – 7.5) | 16 | 4.7 (3.0 – 6.9) | 16 | 4.7 (3.4 – 7.2) | 20 | 4.5 (2.9 – 6.5) | 20 | 4.5 (3.2 – 5.9) | 19 | 4.7 (3.1 – 6.9) | 0.55 |
| • Triglycerides (mmol/L) | 18 | 2.1 (1.1 – 4.1) | 16 | 1.9 (1.1 – 4.0) | 16 | 2.2 (1.2 – 3.2) | 20 | 2.0 (0.8 – 5.3) | 20 | 1.8 (0.7 – 4.2) | 19 | 1.6 (0.9 – 5.9) | 0.13 |
| • HDL-cholesterol (mmol/L) | 18 | 1.1 (0.7 – 3.0) | 16 | 1.2 (0.9 – 3.1) | 16 | 1.2 (0.8 – 3.5) | 20 | 1.3 (0.8 – 2.7) | 20 | 1.2 (0.6 – 2.8) | 19 | 1.4 (0.8 – 3.4) | 0.66 |
| • LDL-cholesterol (mmol/L) | 18 | 3.0 (1.2 – 5.3) | 16 | 2.8 (1.0 – 4.9) | 16 | 2.8 (1.3 – 5.3) | 20 | 2.4 (1.2 – 4.4) | 20 | 2.6 (1.2 – 4.3) | 19 | 2.7 (1.2 – 4.3) | 0.30 |
| Hematology | | | | | | | | | | | | | |
| • Hemoglobin (mmol/L) | 18 | 7.2 (5.0 – 9.5) | 17 | 7.6 (6.3 – 9.6) | 17 | 8.2 (7.0 – 9.9) | 20 | 7.5 (6.5 – 9.4) | 20 | 7.7 (6.2 – 10.5) | 19 | 8.4 (6.5 – 10.5) | 0.85 |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| • MCV (fL) | 18 | 96 (89 – 100) | 17 | 93 (88 – 98) | 17 | 92 (83 – 97) | 20 | 94 (69 – 106) | 20 | 90 (68 – 102) | 19 | 88 (72 – 108) | 0.20 |
| • Thrombocytes (×10^9/L) | 17 | 222 (162 – 401) | 17 | 232 (119 – 477) | 17 | 214 (138 – 394) | 20 | 231 (148 – 495) | 20 | 235 (131 – 457) | 19 | 245 (163 – 380) | 0.21 |
| • Leucocytes (×10^9/L) | 18 | 6.3 (1.0 – 15.5) | 17 | 6.9 (1.9 – 11.1) | 17 | 6.4 (2.2 – 17.4) | 20 | 5.9 (1.3 – 11.8) | 20 | 7.4 (1.7 – 14.2) | 19 | 8.4 (4.0 – 12.0) | 0.12 |
| Pharmacokinetics | | | | | | | | | | | | | |
| • Belatacept dose (mg) | 16 | 800 (575 – 938) | 11 | 400 (300 – 45) | 10 | 381 (300 – 450) | - | N/A | - | N/A | - | N/A | N/A |
| • Tacrolimus dose (mg) | 22 | 10.0 (10.0 – 10.0) | 6 | 5.5 (3.5 – 10.0) | 7 | 5.0 (3.0 – 8.0) | 20 | 4.0 (2.0 – 8.0) | 20 | 4.0 (2.0 – 6.0) | 19 | 4.0 (2.5 – 7.0) | 0.19 |
| • Tacrolimus concentration (ug/L) | 22 | 2.2 (1.5 – 5.5) | 6 | 5.8 (4.2 – 8.3) | 7 | 7.2 (4.5 – 8.6) | 20 | 7.0 (4.1 – 10.7) | 20 | 6.3 (2.6 – 9.9) | 19 | 6.8 (4.4 – 13.3) | 0.53 |
| • Mycophenolate mofetil dose (mg) | 18 | 1000 (500 – 2000) | 17 | 1000 (500 – 2000) | 17 | 1000 (500 – 2000) | 20 | 1000 (500 – 2000) | 19 | 1000 (0 – 2000) | 18 | 1000 (0 – 2000) | 0.47 |
| • Mycophenolate acid concentration (mg/mL) | 17 | 3.04 (0.52- 10.00) | 16 | 2.45 (0.98 – 5.21) | 17 | 2.30 (0.99 – 3.54) | 20 | 2.53 (1.03 – 10.00) | 19 | 1.69 (0.96 – 4.24) | 18 | 1.83 (0.57 – 3.67) | 0.25 |
| • Prednisone dose (mg) | 18 | 5.0 (5.0 – 10.0) | 17 | 5.0 (5.0 – 10.0) | 17 | 5.0 (5.0 – 10.0) | 20 | 5.0 (5.0 – 10.0) | 20 | 5.0 (5.0 – 10.0) | 19 | 5.0 (2.5 – 10.0) | 0.59 |

* Censored for graft loss and death; † Comparison between patients from the belatacept group and the tacrolimus group 12 months after transplantation

Target tacrolimus C0 of 5 – 10 ng/mL were achieved in 75%, 85% and 95% of patients in the tacrolimus group 3, 6 and 12 months after transplantation, respectively. Target MPA C0 of 1.5 – 3.0 mg/mL were achieved in 45%, 40% and 40% of patients in the tacrolimus group respectively 3, 6 and 12 months after transplantation, and in 30%, 40% and 60% of patients in the belatacept group respectively 3, 6 and 12 months after transplantation.

Data present medians (plus ranges).

BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL, high density lipoproteins; LDL, low density lipoproteins; M3, 3 months after transplantation; M6, 6 months after transplantation, M12, 12 months after transplantation; MCV, mean corpuscular volume

**SDC, Table 3:** Graft function 12 months after transplantation

| | **Belatacept non-rejectors (n=9)** | **Belatacept rejectors, censored for graft loss (n=8)** | **Belatacept rejectors, including graft loss (n=11)** | **Tacrolimus (n=19)** | **Tacrolimus non-rejectors (n=17)** |
|---|---|---|---|---|---|
| Creatinine (μmol/L) | 106 (71-143) | 163 (93-207) | - | 126 (79-179) | 119 (79-178) |
| eGFR (mL/min) | 57 (45-89) | 36 (28-76) | 34 (0-76) | 50 (33-84) | 58 (37-84) |
| Protein/ Creatinine ratio | 11.4 (7.9-25.0) | 12.2 (5.7-343.8) | - | 9.0 (5.3-43.5) | 9.0 (5.3-43.5) |

< *Table on previous page*

Data are medians (plus ranges). Graft function was compared between 1) the belatacept-treatedrejectors and belatacept-treated non-rejectors and 2) the tacrolimus-treated and belatacept-treated non-rejectors, using the Mann-Whitney U test. Creatinine concentrationat month 12 was significantly higher and eGFR at month 12 was consequently significantly lower in belatacept-treated rejectors than in belatacept-treated non-rejectors, both p=0.001. These parameters did not differ between non-rejecting belatacept-treated and tacrolimus-treated patients at month 12.

In the group of "Belatacept rejectors, including graft loss" the 3 patients that lost their grafts were set to an eGFR of zero on month 12. Creatinine and Protein/Creatinine ratio were not calculated for this group, since these could not be determined for the 3 patients after graft loss.

eGFR, estimated glomerular filtration rate

**SDC, Table 4:** Response to anti-rejection therapy in belatacept-treated rejectors

| | Creatinine | | | eGFR | | |
|---|---|---|---|---|---|---|
| No. (Patient) | Best before | BPAR | Best after | Best before | BPAR | Best after |
| 3 | 84 | 132 | 93 | 59 | 35 | 52 |
| 5 | 89 | 698 | N/A | 56 | 5 | 0 |
| 6 | 155 | 211 | 136 | 39 | 27 | 45 |
| 7 | 148 | 188 | 164 | 45 | 34 | 40 |
| 13 | 89 | 107 | 93 | 80 | 65 | 76 |
| 14 | 109 | 148 | 110 | 72 | 50 | 71 |
| 15 | 227 | 279 | 145 | 25 | 19 | 41 |
| 16 | 106 | 210 | 152 | 62 | 28 | 41 |
| 17 | 305 | 807 | N/A | 14 | 5 | 0 |
| 19 | 325 | 367 | 161 | 18 | 16 | 41 |
| 20 | 162 | 175 | N/A | 33 | 30 | 0 |

Patient numbers are the same depicted as in Figure 3. For detailed clinical course per patient, please refer to this figure. Creatinine and estimated glomerular filtration rates (eGFR) are given for the 10 belatacept-treated rejectors before, during and after rejection (when applicable, before second rejection episodes). Both before and after rejection the highest measuredeGFRs are depicted. Patients no. 6, 16 and 19 were switched to tacrolimus (almost) immediately after rejection occurred. Patients no. 5 lost her graft immediately after rejection, and patients no. 17 and 20 were switched to tacrolimus, but still lost their grafts thereafter (eGFRs after rejection were set to zero). Patients no.7, 14 and 15 were switched to tacrolimus after a second episode of acute rejection. Patient no. 13 was diagnosed with BPAR after revision of the biopsy, and was therefore not treated with additional anti-rejection therapy. This patient had an isolated v-lesion which may explain the excellent outcome despite no treatment. Finally, patient no. 3 was switched after his third rejection episode.

N/A, not applicable

**SDC, Table 5:** Anti-HLA antibodies in serum

| | | Belatacept group (n = 20) | Tacrolimus group (n = 20) | p |
|---|---|---|---|---|
| Donor-specific | *Pre-existent* | - | - | - |
| | *De novo* | 2 (10%) *[Patients no. 2 and 20]* | - | 0.49 |
| Non donor-specific | *Pre-existent* | 2 (10%) *[Patients no. 2 and 12]* | 3 (15%) *[Patients no. 3, 11 and 12]* | 1.00 |
| | *De novo* | 2 (10%) *[Patients no. 7 and 20]* | 2 (10%) *[Patients no. 6 and 20]* | 1.00 |

Patient numbers are the same as in Figure 3. None of the patients had donor-specific anti-human leukocyte antigen (HLA) antibodies (DSA) pre-transplantation. During the first post-transplant year, 2 patients developed DSA, both in the belatacept group. Patient no. 2 in the belatacept group developed DSA against HLA-DQ2 (MFI 3787) one month after transplantation, but these disappeared hereafter without additional therapy and no acute rejection occurred. Patient no. 20 in the belatacept group had DSA, which were also detectable in the cross match-dependent cytotoxicity test, against HLA-A1 (MFI 18,000), -B8 (MFI 22700), -DR3 (MFI 11000), -DR52 (MFI 5500) and -DQ2 (MFI 16500) during her fourth rejection episode right before losing her graft. At this time she was already switched to a tacrolimus-based regimen and had been treated with multiple methylprednisolone and alemtuzumab gifts (Figure 3).

Two and three patients, in the belatacept and tacrolimus group, respectively, had non donor-specific anti-HLA antibodies (non-DSA) pre-transplantation. Patient no. 2 in the belatacept group had non-DSA against DR1 that remained present after transplantation, without clinical consequences. Patient no. 12 in the belatacept group had non-DSA against HLA-Dp11 which disappeared after transplantation. No rejection occurred. Patients no. 3, 11 and 12 in the tacrolimus group had non-DSA pre-transplantation against HLA-B76, -DP1, and -DP; -DR4; and -B15; respectively. Only patient no. 3 suffered from an acute rejection Banff type 2B. No serum was available from this patient at the time of rejection, but in sera from month 1 to 12 no non-DSA were detected. Also in the other 3 patients pre-existent anti-HLA antibodies disappeared after transplantation.

Two patients in the belatacept group (no. 7 and no. 20) developed non-DSA. Patient no. 7 in the belatacept group developed non-DSA against HLA-DQ3 (measured on day 30) before he was diagnosed with an acute Banff type 2B rejection 44 days after transplantation. These non-DSA were also positive during rejection. After treatment with methylprednisolone they were no longer detectable and remained so throughout follow-up. Patient no. 7 in the belatacept group developed non-DSA against HLA-A24, -A68, and -DQ3 simultaneously with DSA. Two patients in the tacrolimus group (no. 6 and no. 20) developed non-DSA, without clinical consequences in the first year after transplantation against HLA-DP14 and HLA-A24, respectively.

**SDC, Table 6:** Baseline characteristics of (future) rejectors and non-rejectors in the belatacept group (Table continues on next page)

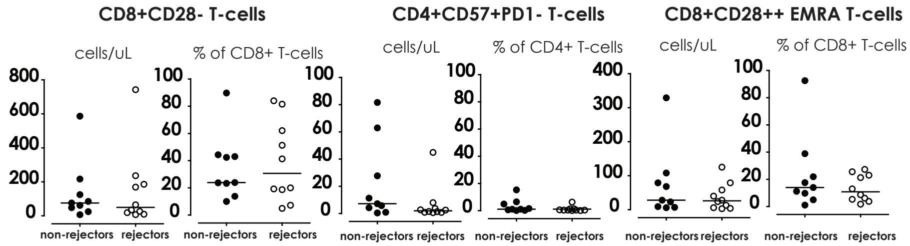
| | Belatacept (n = 20) | | p |
|---|---|---|---|
| | rejectors (n = 11) | non-rejectors (n = 9) | |
| Age at transplantation (years) | 47 (25-76) | 60 (40-74) | 0.41 |
| Male / female | 7 (64%) / 4 (36%) | 7 (78%) / 2 (22%) | 0.49 |
| Ethnicity | | | 0.07 |
| • Caucasian | 11 (100%) | 6 (67%) | |
| • African | - | 2 (22%) | |

| | | | |
|---|---|---|---|
| • Asian | - | 1 (11%) | |
| • Hispanic | 83.3 (63.5 - 111.4) | 76.0 (56.6 - 98.6) | 0.26 |
| Body weight (kg) | 1.0 (± 0.6) | 1.1 (± 0.8) | 0.84 |
| HLA A mismatch (mean ± SD) | 1.4 (± 0.5) | 1.2 (± 0.4) | 0.63 |
| HLA B mismatch (mean ± SD) | 1.2 (± 0.4) | 1.0 (± 0.5) | 1.00 |
| HLA DR mismatch (mean ± SD) | 0 (0 - 4) | 0 (0 - 5) | 0.55 |
| Current PRA (%) | 4 (0 - 6) | 4 (0 - 5) | 0.37 |
| Peak PRA (%) | | | 0.37 |
| CMV status at transplantation | 1 (9%) | 2 (22%) | |
| • Donor + / Recipient - | 2 (18%) | 2 (22%) | |
| • Donor + / Recipient + | 6 (55%) | 1 (11%) | |
| • Donor - / Recipient - | 2 (18%) | 4 (44%) | |
| • Donor - / Recipient + | 60 (43 - 69) | 53 (24 – 71) | 0.33 |
| Donor age at transplantation (years) | 4 (36%) / 7 (64%) | 2 (22%) / 7 (78%) | 0.64 |
| Related / unrelated donor | | | 0.90 |
| Cause of end-stage renal disease | 1 (9%) | 2 (22%) | |
| • Diabetes mellitus | - | 2 (22%) | |
| • Hypertension | - | 1 (11%) | |
| • IgA nephropathy | 2 (18%) | 1 (11%) | |
| • Polycystic kidney disease | 2 (18%) | 1 (11%) | |
| • Obstructive nephropathy | 3 (27%) | 2 (22%) | |
| • Unknown | 3 (27%) | 0 (0%) | |
| • Other | | | 0.37 |
| Renal replacement therapy | 7 (64%) | 3 (33%) | |
| • None (pre-emptive) | 3 (27%) | 4 (44%) | |
| • Hemodialysis | 1 (9%) | 2 (22%) | |
| • Peritoneal dialysis | 560 (147-2633) | 425 (123-2782) | 1.00 |
| Time on dialysis therapy (days) | | | 1.00 |
| Number of kidney transplantation | 10 (91%) | 9 (100%) | |
| • First | 1 (9%) | - | |
| • Second | 47 (25-76) | 60 (40-74) | 0.41 |

Continuous variables are presented as medians (plus ranges) and categorical variables as numbers (plus percentages), unless otherwise specified

BPAR, biopsy-proven acute rejection; CMV, cytomegalovirus; HLA, human leukocyte antigen; PRA, panel reactive antibodies; SD, standard deviation.

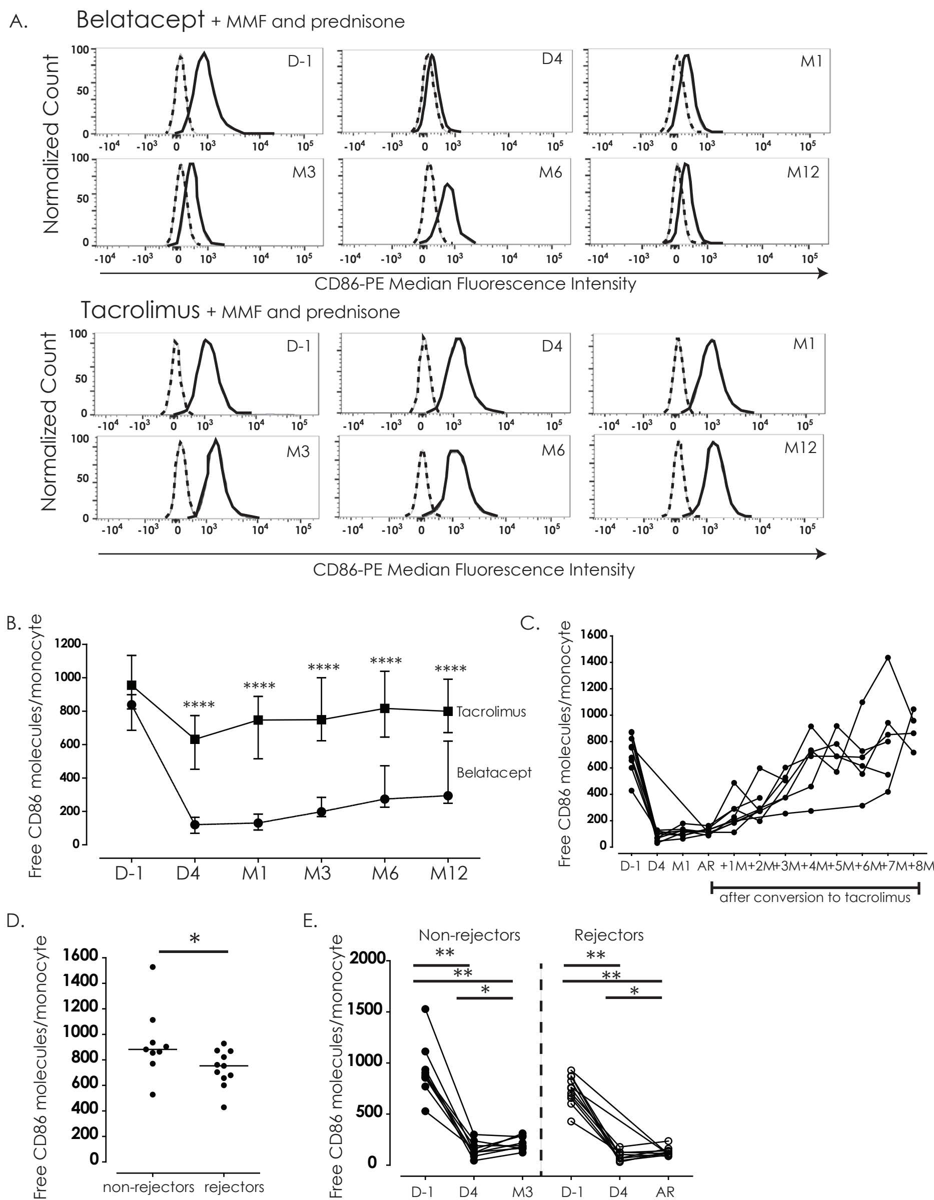
## *Supplemental Figures*




**SDC, Figure 1. CD8+CD28-, CD4+CD57+PD1- and CD8+CD28++ EMRA T-cells during rejection or 3 months after transplantation.**

CD4+ and CD8+ T-cells were gated from 7-AAD negative CD3+ lymphocytes (based on forward and sideward scatter) and EMRA T-cells were gated as CCR7- and CD45RO- T-cells (See Figure 4). The absolute numbers and percentages of CD8+CD28-, CD4+CD57+PD1- and CD8+CD28++ EMRA T-cells are presented for non-rejectors 3 months after transplantation and for rejectors during acute rejection before additional anti-rejection therapy was given.

N.B.: From 1 rejector no materials were obtained during rejection, because biopsy-proven acute rejection was diagnosed in retrospect after revision by a second pathologist.




**SDC, Figure 2. Pharmacodynamic drug monitoring of belatacept.**
The median fluorescence intensity (MFI) of CD86 was assessed on circulating monocytes in belatacept and tacrolimus-treated patients using a competitive monoclonal antibody (clone HA5.2B7, solid line) with an IgG control (dotted line) (A). Free CD86 molecules per monocyte were calculated from MFIs (medians + interquartile ranges) and compared between the belatacept (triangles) and tacrolimus (squares) group on different time points in an intention-to-treat analysis using a linear mixed model (B). Free CD86 molecules/ monocyte in tacrolimus-treated patients compared to belatacept-treated patients were 5.9-fold (95% CI 4.5 to 7.7-fold) higher on day 4; 5.3-fold (95% CI 4.0 to 7.0-fold) higher on month 1; 3.7-fold (95% CI 2.8 to 4.8-fold) higher on month 3; 2.6-fold (95% CI 2.0 to 3.4-fold) higher on month 6; and 2.1-fold (95% CI 1.6 to 2.8-fold) on month 12. Free CD86 molecules/monocytes were measured in n = 8 patients which were converted to a tacrolimus-based therapy after acute belatacept-resistant rejection (C). Numbers of free CD86 molecules/ monocytes pre-transplantation were compared between non-rejectors

> *Continuation of Figure Legend*

(n = 9) and rejectors (n = 11) in the belatacept group (D), as well as CD86 molecules/monocyte on day 4 and month 3 or during rejection after transplantation (E). AR, acute rejection; D-1, one day pre-transplantation; D4, four days after transplantation; M1, one month after transplantation; M3, three months after transplantation; M6, six months after transplantation; M12, twelve months after transplantation; MMF, mycophenolate mofetil

* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001

N.B.: In (D) black lines represent the medians; the upper and lower border of the boxes represent the 25th and 75th percentiles; the error lines represent 10th and 90th percentiles.

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# Chapter 6

## An acute cellular rejection with detrimental outcome occurring under belatacept-based immunosuppressive therapy: an immunological analysis

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## Abstract

### Background

Belatacept has been associated with an increased acute rejection rate after kidney transplantation. This case report sheds light on the possible immunological mechanisms underlying this phenomenon by analyzing the immunological mechanisms in patient serum, peripheral blood mononuclear cells, rejected kidney tissue, and graft infiltrating cells.

### Methods

A 61-year old female treated with belatacept, who received her first kidney transplant from her husband was admitted with an acute, vascular rejection 56 days post-transplantation which necessitated a transplantectomy. Histology and immunohistochemistry were performed on biopsy and explant tissue. CD86-expression on peripheral monocytes was assessed. Using Ficoll density methods, peripheral blood and graft infiltrating lymphocytes were isolated and phenotyped.

### Results

The explant showed a vascular rejection (Banff ACR grade III) and a perivascular infiltrate mostly consisting of T-cells. No evidence for antibody-mediated rejection was found. In contrast to the peripheral blood monocytes, CD86 was still expressed by part of the mononuclear cells in the explant. Isolated graft cells were mostly CCR7-CD45RO+ effector-memory $CD4^+$ and $CD8^+$ T-cells (60-70%). CD28-positive as CD28-negative T-cells were present in the explant, showing a great IFNγ production capacity and expressing granzyme B.

### Conclusions

We postulate that this glucocorticoid-resistant cellular rejection occurring under belatacept was predominantly mediated by cytotoxic memory T-cells, which are less susceptible to co-stimulatory blockade by belatacept, or resulted from incomplete CD80/86 blockade at the tissue level.

## Introduction

Belatacept is the first Food and Drug Administration-approved inhibitor of the CD28-CD80/86 co-stimulatory pathway used for the prevention of kidney allograft rejection.(1) Belatacept treatment results in a significantly better renal function compared with cyclosporin-based therapy (2, 3), but acute rejection occurs more frequently in belatacept-treated patients, and these rejections are also more severe according to Banff criteria.(4) However, 1-year graft survival was comparable between belatacept- and cyclosporin-treated patients despite the increased rejection risk.(4) The higher acute rejection incidence may be explained by differences in the susceptibility of immune cells to the inhibiting effects of belatacept. Cytotoxic T-memory lymphocytes do not express CD28 and are not dependent on this molecule for their activation.(5, 6) However, little data exists on the immunological processes in peripheral blood and at the tissue level, during rejection under belatacept-based treatment.(7, 8)

Here, a case is described of early, glucocorticoid-resistant, acute cellular rejection in a belatacept-treated patient, which led to acute kidney allograft loss. Lymphocytes and sera isolated from blood and lymphocytes from the explanted allograft were studied to gain more insight into the immunological mechanisms responsible for this severe rejection.

## Case presentation

A 61-year-old female with end-stage renal disease caused by recurrent urinary tract infections, received her first, blood group-compatible, preemptive, living-unrelated donor (her husband), kidney transplant on December 17, 2013. The transplant was 1-2-2 mismatched (for HLA A2, B18, B60, DR4 and DR8, respectively). The pretransplant complement-dependent cytotoxicity (CDC) cross-match was negative. Current and historical panel reactive antibodies (PRA) were 4%. She had 2 children with her husband, and never received blood transfusions. She was treated with belatacept according to the Less-Intensive regimen (3) as part of a clinical trial (NTR4242, Dutch Trial Register, www.trialregister.nl), and with basiliximab, mycophenolate mofetil (MMF) and prednisolone.

The initial post-operative course was uneventful, and she was discharged with a serum creatinine of 97 µmol/mL. Fifty-six days after transplantation, she was admitted with fever, malaise, headache and cough. Apart from mild hypertension (153/87 mmHg), tachycardia (118 bpm), and graft tenderness, the physical examination was unremarkable.

Laboratory examination revealed renal insufficiency with a rise of her serum creatinine from 100 µmol/L (measured on post-operative day 53) to 698 µmol/L at the time of presentation (post-operative day 56). C-reactive protein and the leucocyte count were elevated (277 mg/L and 15.4 x $10^9$ cells/L). Lactate dehydrogenase was 691 IU/mL. The urinary sample contained leucocytes (4+), albumin (2+), and erythrocytes (1+), but was negative for nitrite. The mycophenolic acid plasma concentration 3 days before admission was 2.16 mg/L. Abdominal ultrasound showed a normal-sized and perfused kidney allograft without hydronephrosis.

Because of presumed urinary or upper respiratory tract infection, she was treated with broad-spectrum antibiotics. A renal biopsy was obtained, after which she received intravenous methylprednisolone 1 gram daily for three consecutive days, which is the first line therapy for acute rejection at our center. This did not result in an improvement of her renal function.

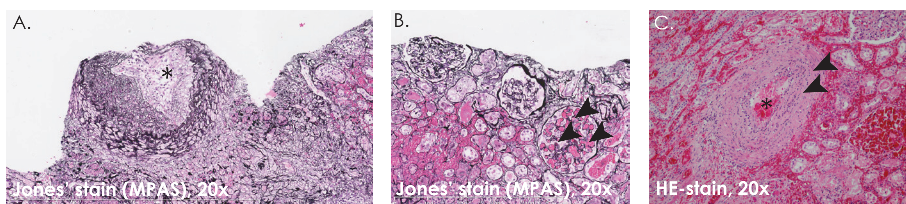


**Figure 1. Histology of the renal graft under belatacept treatment.**

(A) Severe endarteritis with subtotal occlusion of the artery is depicted (Jones 20x). (B) Diffuse cortical necrosis and hemorrhage and glomerular congestion is shown (Jones 20x). (C) Endothelialitis, fibrinoid necrosis and transmural infiltration are depicted (HE, 20x).

N.B.: A and B, The histology is shown for the renal graft biopsy prior to transplantectomy. C, The histology is shown for the explant.

C-reactive protein, leucocyte count, and LDH rose to a maximum of 306 mg/L, 42 x $10^9$/L, and 954 IU/mL, respectively. Additional testing revealed an Epstein-Barr virus (EBV) reactivation with low-grade viremia (280 copies/mL) prior to the start of pulse-glucocorticoid therapy. Renal scintigraphy demonstrated absent perfusion of the allograft. Therefore, no T-cell depleting therapy was given and a transplantectomy was immediately performed on post-operative day 59.

## Investigation of the kidney biopsy and explant

The renal allograft biopsy demonstrated an acute vascular rejection (Banff ACR grade II, i1 t1 v2 g3 ptc3 C4d0 ti3) with large vessel thrombosis and diffuse cortical necrosis (Figure 1A and 1B). Three days later, the explant showed a severe vascular rejection (Banff ACR grade III, i1 t1 v3 g3 ptc3 C4d0 ti3) with endothelialitis, fibrinoid necrosis and transmural infiltration (Figure 1C). Histopathology and additional immunohistochemistry showed no signs of antibody-mediated rejection. (Supplementary Figure 1) No subendothelial deposits nor CD138+ plasma cells were present in the biopsy and explant. Light microscopy also did not show large amounts of granulocytes or macrophages expressing Fc-receptors (not shown). The presence of glomerulitis and peritubular capillaritis could fit the vascular antibody-mediated rejection (AMR/V+) pattern, as well as the vascular T-cell mediated rejection (TCMR/V+) pattern as described by Lefaucheur et al.(9) because these 2 overlap. The high grade of interstitial inflammation pleads against AMR. Moreover, transplant glomerulopathy was absent. Furthermore C4d, IgM and IgG were negative. (Supplementary Figure 1)

## Donor-specific anti-HLA antibodies (DSA)

The pretransplant and posttransplant sera were screened for the presence of HLA

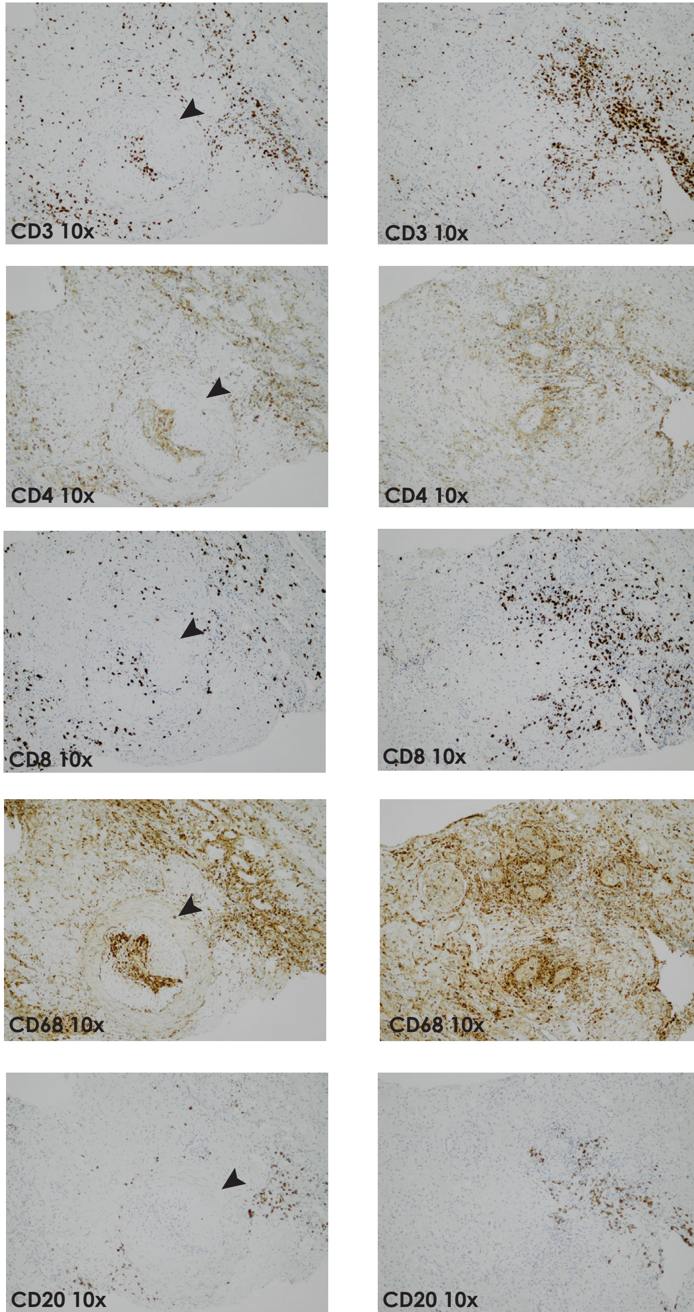


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**Figure 2. Immunohistochemistry of the kidney biopsy.**

Immunohistochemistry demonstrated severe endothelialitis and tubulitis. Left panel indicates infiltrate in the vascular wall (arrow) with endothelitis. Right panel indicates tubulointerstitial infiltration. CD3+ T cells were located perivascular and endovascular, as were CD4+ and CD8+ cells. CD68+ macrophages were diffusely present in the explant. A small amount of CD20+ B cells were also located perivascular.

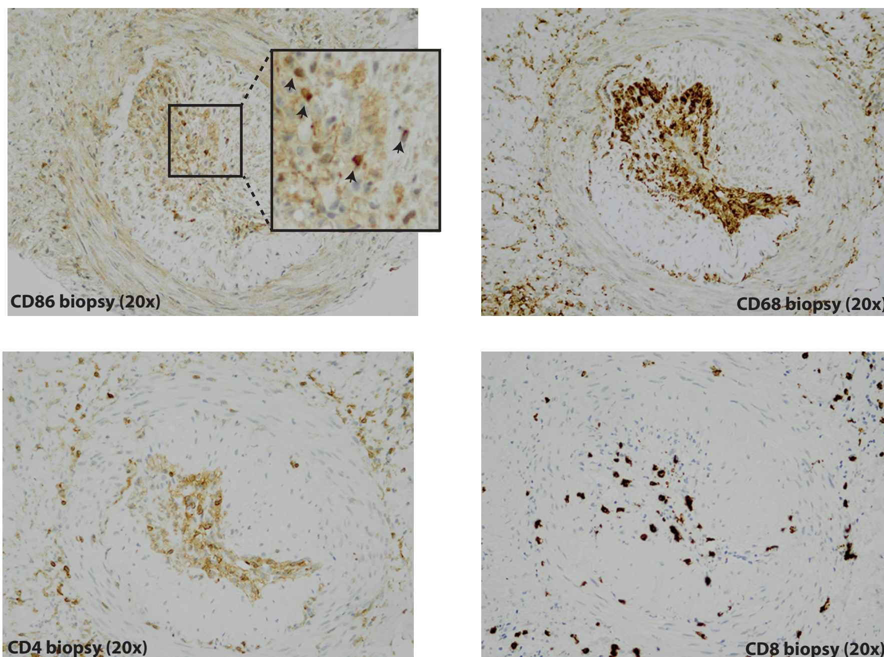


**Figure 3. CD86 was still expressed on part of the CD4+ mononuclear cells in the kidney biopsy.**

Surface staining for CD86 was assessed (arrows) including CD68, CD4 and CD8 stainings on sequential slides. The renal biopsy during rejection in the belatacept-treated patient showed small amounts of CD86+ cells (15-20 CD86-positive cells per 100 mononuclear cells). Original magnification 20x. The location and morphology of these CD86+ cells suggests mostly CD4+ monocytes expressed CD86.

antibodies by CDC, and these sera were negative. Additional screening using the Luminex assay by single-antigen-LABScreen beads (OneLambda, Canoga Park, CA, USA) was performed, which revealed DSA against only HLA-DR4 at the time of rejection (median fluorescence intenstity [MFI] 600). In retrospect, DSA against HLA-DR4 could also be detected before transplantation (MFI 722). However, the MFI cut-off for clinically relevant DSA in our facility (Leiden University Medical Center, which also harbors The Eurotransplant Reference Laboratory) is 1000 or greater and hence, these signals were considered negative. In addition, these DSA were negative in the C1q-binding antibody assay (C1qScreen, One Lambda). No *de novo* DSA were formed after transplantation as analyzed by CDC and Luminex. During the work-up for a second transplantation, after all immunosuppression was withdrawn and her transplant had been

removed, the titer of the DSA against HLA-DR4 increased with an MFI of 2600 (144 days after transplantectomy), but remained C1q-negative. No DSA against HLA-A, -B, -Cw, -DP or -DQ were found before transplantation or during rejection.

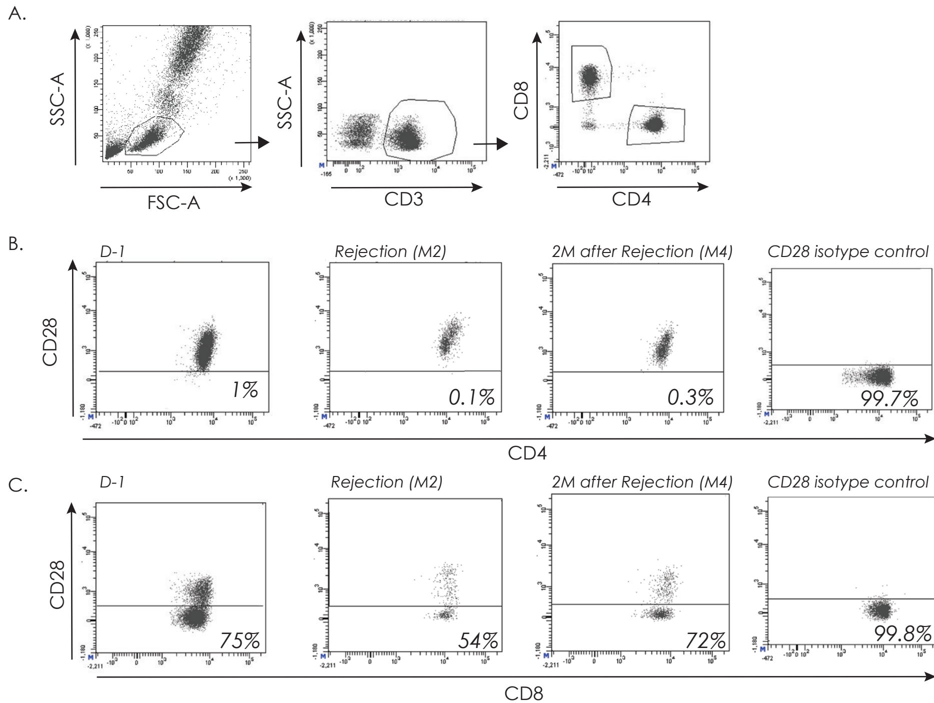


**Figure 4. The CD28^NULL CD4+ and CD28^NULL CD8+ T-cells decreased in the blood during rejection.**

(A) The gating strategy for CD4+ and CD8+ is depicted. First cells were gated by size and granularity in the forward and sideward scatter. CD4+ and CD8+ were gated from CD3+ cells. (B-C) In both T-cell subsets CD28-positive and CD28-negative cell percentages were determined before transplantation, during rejection and 2 months after rejection (after removal of the graft).

D-1 = 1 day before transplantation, M2 = 2 months after transplantation, M4= 4 months after transplantation.

## Immunohistochemistry of the kidney biopsy and explant

A severe endothelialitis was observed in the renal cortex (Figure 2). CD3+ T-lymphocytes were present perivascular and endovascular, and composed both CD4+ and CD8+ cells. The CD4+ expression could also be explained by the numerous CD68+ infiltrating macrophages. Also, a small amount of perivascular CD20+ B-lymphocytes was observed. No CD138+ plasma cells nor IgM or IgG were detectable (Supplementary Figure 1). The immunohistochemistry of the explant showed a comparable picture (Supplementary figure 2).

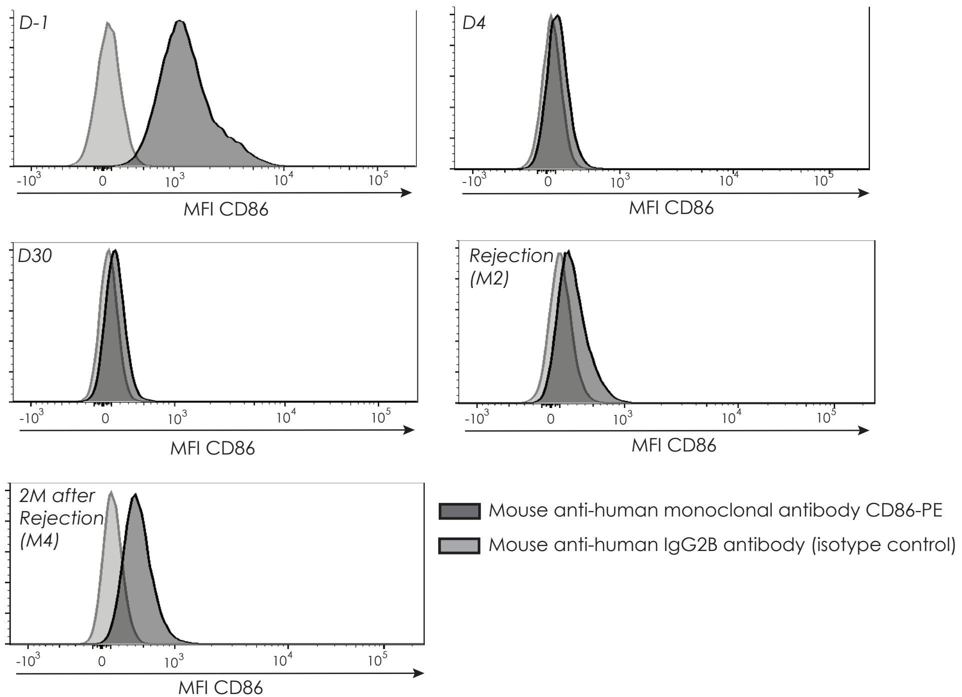


**Figure 5. The CD86 molecules on CD14+ monocytes were completely blocked by belatacept after transplantation, including the moment of rejection.**

The Median Fluorescence Intensity (MFI) is given for CD86-PE (dark grey) and the negative isotype control (IgG2B, light grey). D-1 = 1 day before transplantation, D4 = 4 days after transplantation, D30 = 30 days after transplantation, M2 = 2 months after transplantation, M4= 4 months after transplantation.

The expression of CD86 in the biopsy and explant was measured to assess whether this co-stimulatory molecule was fully blocked by belatacept (Figure 3 and Supplementary Figure 3). In both specimens, small numbers of CD86+ cells were found in the infiltrate. These cells were mononuclear and were CD86+ in both cytoplasm and on the surface, and were most likely CD4+ monocytes based on their morphology. For comparison, a type II vascular T-cell mediated rejection biopsy under tacrolimus was stained for CD86 and CD68 (Supplementary Figure 3C). A high CD86 expression was observed. The location and morphology of these CD86+ cells suggested these cells were mostly CD68+ monocytes and macrophages.

Fluorescence *in situ* hybridization of the perivascular infiltrate confirmed that >95% of the infiltrating cells was from recipient origin (double X-chromosome positive, Supplementary figure 4). No EBV encoded small RNAs (EBERs) were present in the transplant (not shown).

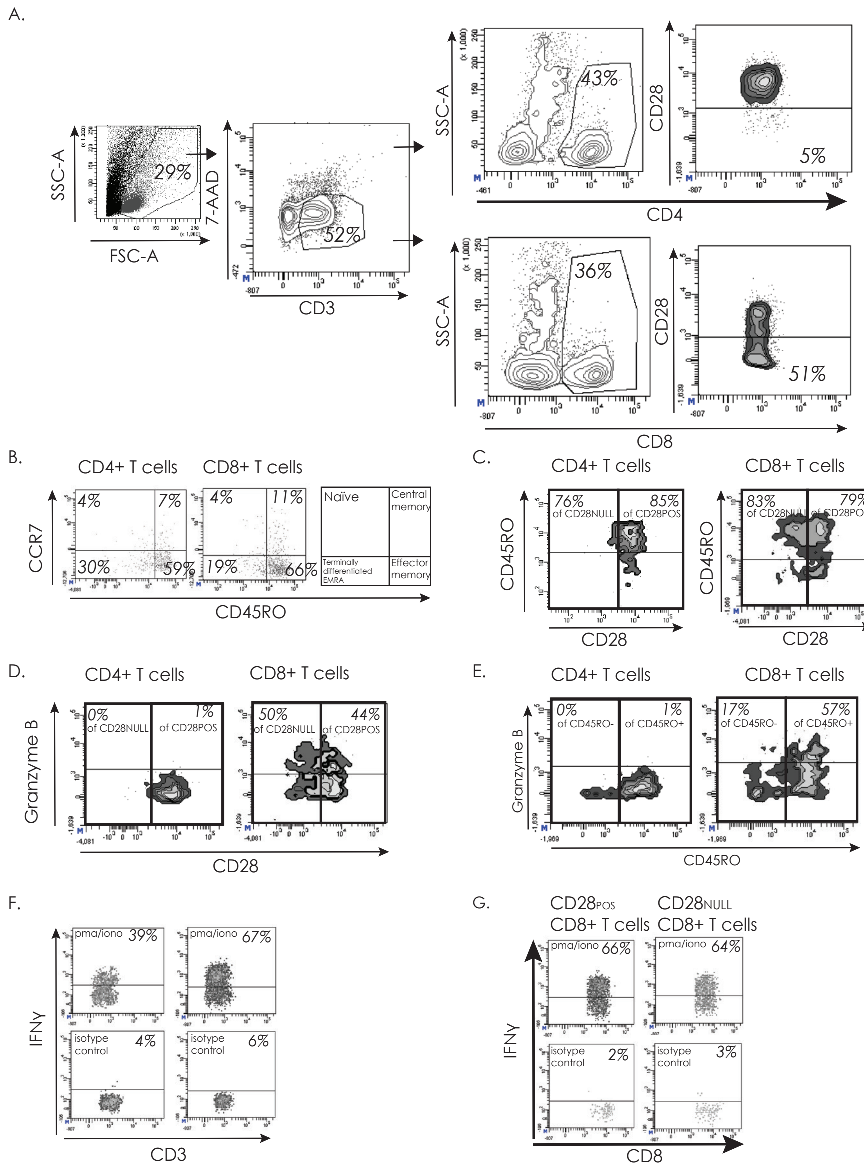


**Figure 6. The rejected renal graft contained CD28$^{NULL}$ T-cells, granzyme B producing CD8+ T-cells and both CD4+ and CD8+ T-cells with great IFNγ-production capacity.**

(A) The gating strategy for CD4+ and CD8+ is depicted. First, graft-infiltrating lymphocytes were gated by size and granularity in the forward and sideward scatter. CD4+ and CD8+ were gated from viable (i.e. 7-AAD negative) CD3+ cells. In both T-cell subsets CD28-positive and CD28-negative were determined. In addition, the memory phenotype was determined, with the use of CCR7 and CD45RO, in the total CD4+ and CD8+ T-cell population (B) as well as in CD28POS and CD28NULL T-cells (C). The intracellular granzyme B expression was determined in CD4+ and CD8+ T-cells in CD28NULL and CD28POS T-cells (D) and in CD45RO+ and

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CD45RO- T-cells (E). The intracellular IFNγ-production capacity was determined in CD4+ and CD8+ T-cells upon 4 hours of stimulation with PMA/ionomycin (F). The intracellular IFNγ-production is also specified for CD28$^{POS}$ and CD28$^{NULL}$ CD8+ T-cells (G).

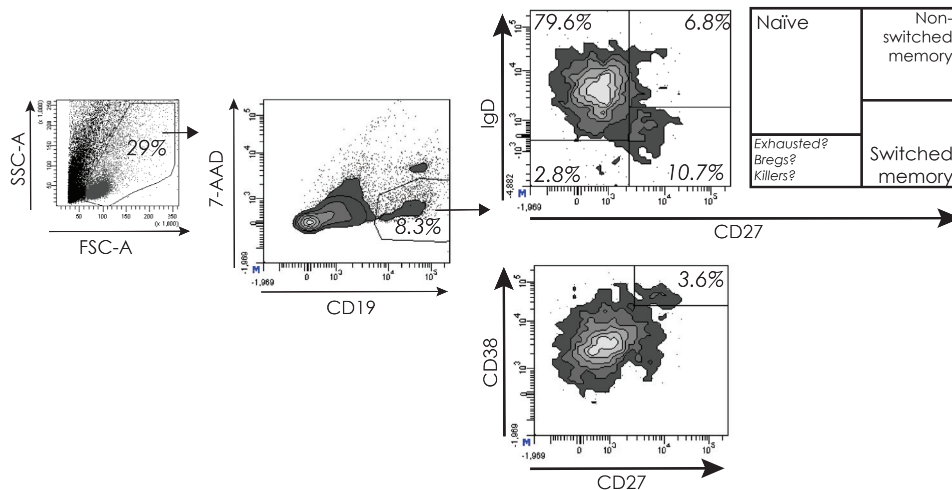
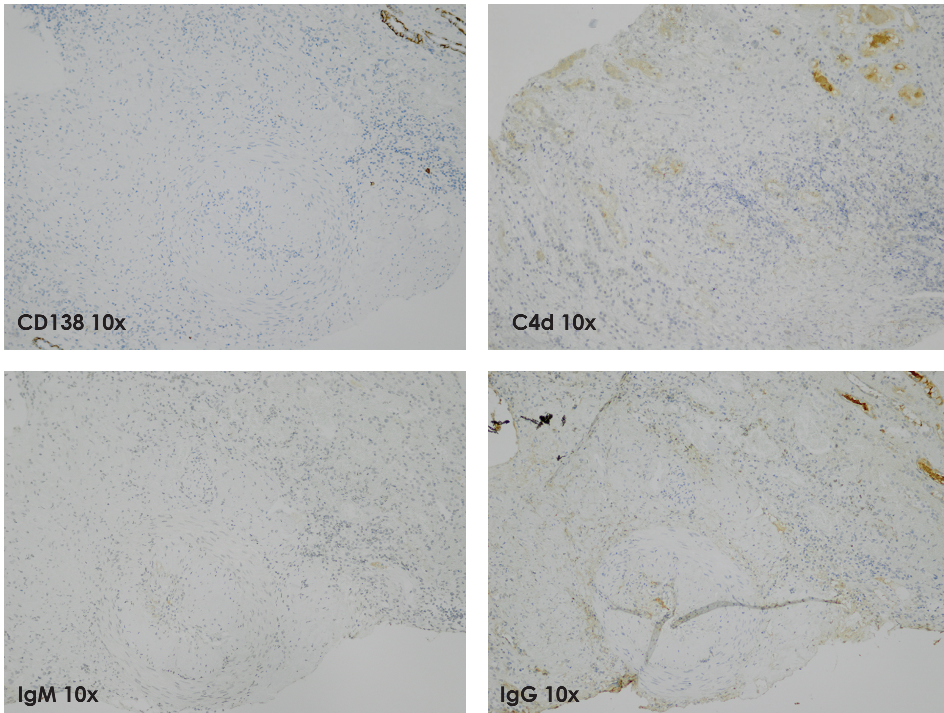



**Figure 7. A small proportion of the graft infiltrating cells were B cells. The gating strategy for CD19+ cells is depicted.** First cells were gated by size and granules content in the forward and sideward scatter. Memory phenotype was determined within viable (i.e. 7-AAD negative) CD19+ cells, using IgD and CD27. The percentage of CD27+CD38HIGH plasmablasts was also determined (within viable CD19+ B-cells).

## Immunophenotyping of peripheral blood lymphocytes

CD28 expression on T-cells was assessed before transplantation, during rejection and 2 months after transplantectomy when immunosuppressive drugs had been stopped. (Figure 4) Seventy-five percent of the CD8+ T-cells (280 cells/μL), and 1% of the CD4+ T-cells (12 cells/μL), did not express CD28 before transplantation. Interestingly, during rejection, a decrease in CD4+CD28-negative (CD28$^{NULL}$) (from 1% to 0.1%) and CD8+CD28$^{NULL}$ (from 75% to 54%) was observed (0.6 cells/μL and 57 cells/μL, for CD4 and CD8, respectively). After rejection, the proportions of these cells almost returned to baseline levels (CD4+CD28$^{NULL}$: 0.3% [3 cells/μL] and CD8CD28$^{NULL}$ 72% [161 cells/μL]).

To assess whether belatacept blocked the co-stimulatory pathway in blood, the expression of CD86-molecules on monocytes and B-cells was measured using a whole-blood assay (Figure 5). Before transplantation, CD86 was measured on all monocytes, but it was fully blocked after the first dose (day 4) and before the 4$^{th}$ belatacept dose (day 30), and during rejection. Two months after discontinuation of belatacept, CD86 expression approached pretransplantation levels. CD86 was not expressed on peripheral B-cells before nor after transplantation (not shown).

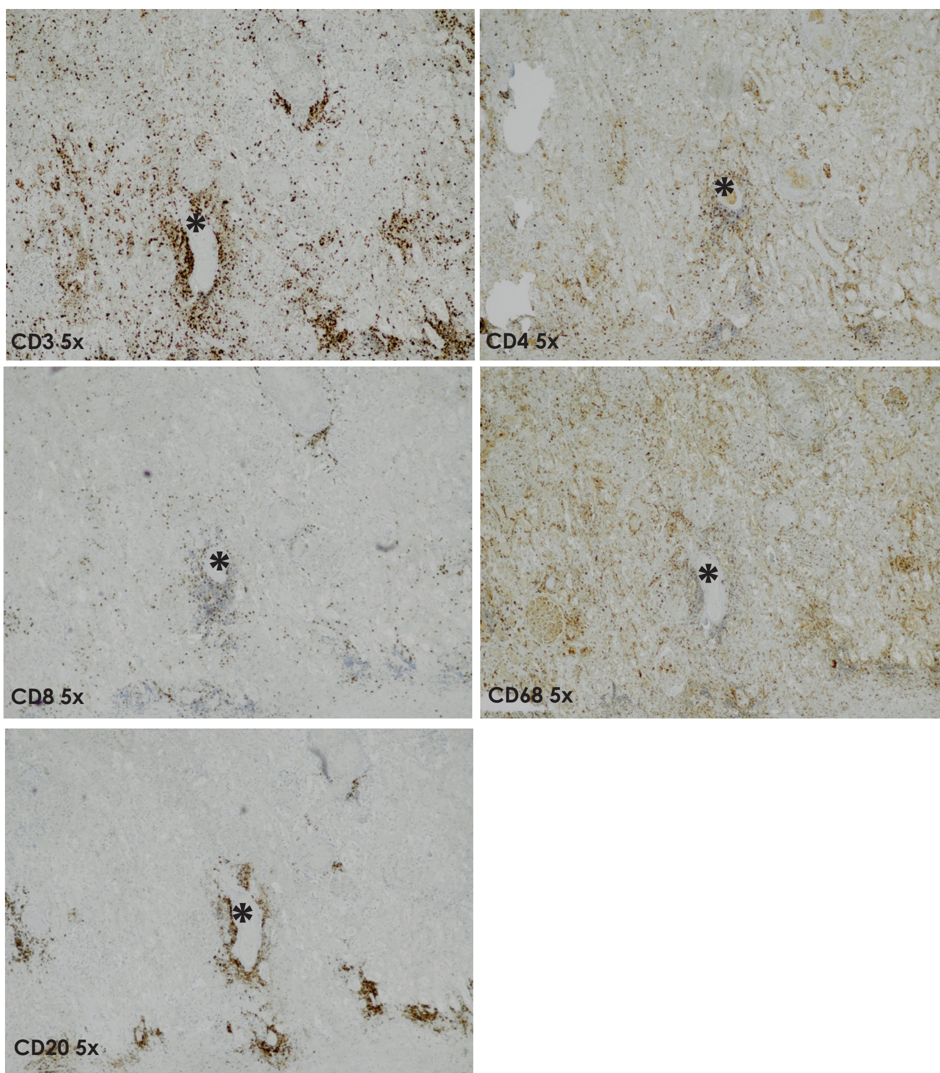



**Supplementary figure 1. No signs of humoral reactivity during rejection under belatacept.**

No CD138+ plasma cells, C4d, IgM or IgG could be detected in the kidney biopsy.

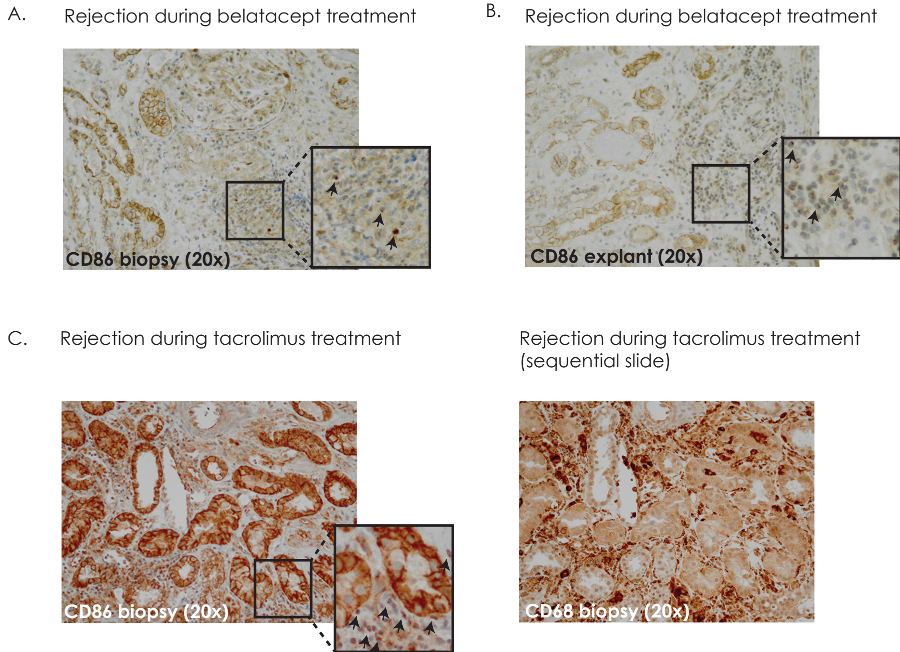


The phenotype of CD4+FoxP3+ T-cells was compared to the phenotype of a stable belatacept-treated patient at month 3 after transplantation and to the phenotype of a tacrolimus-treated patient during an acute rejection episode 9 days after transplantation. (Supplementary Figure 5) No differences were found in intracellular FoxP3 and surface CTLA-4 expression between these two patients and the case described here. Also the expression of intracellular Helios was similar between the 3 patients.




**Supplementary figure 2. Immunohistochemistry of the explant.**

CD3+ T cells were located perivascular (arterisk [*]), as were CD4+ and CD8+ cells. CD68+ macrophages were diffusely present in the explant. A small amount of CD20+ B cells were also located perivascular.



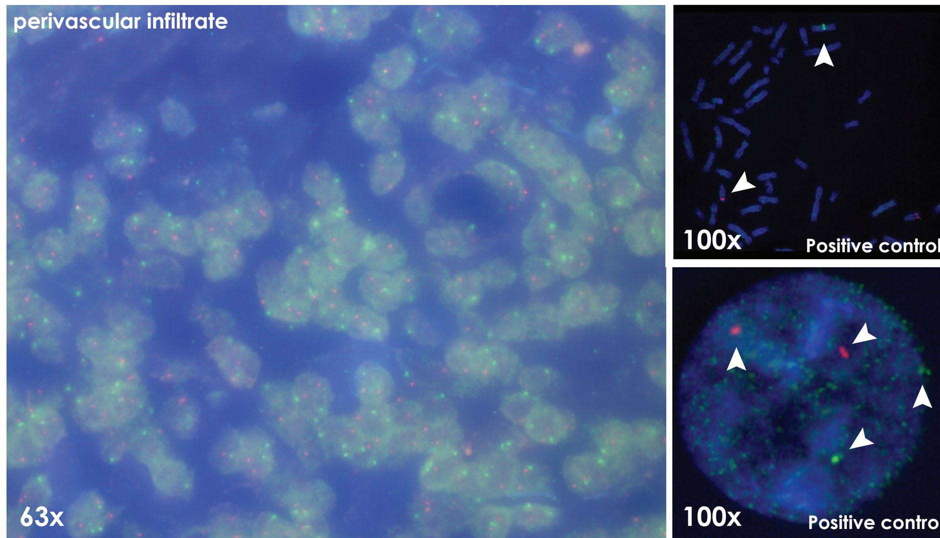

**Supplementary Figure 3. CD86 was expressed in the kidney biopsy (before anti-rejection therapy) and in the explant (after anti-rejection therapy), but this expression was lower than in a rejection under tacrolimus.**

A similar expression of CD86 (15-20 CD86+ cells per 100 mononuclear cells) was shown in the biopsy (A) and in the explant (B). As a control a type 2B (C4d0) rejection under a tacrolimus-based regimen is shown (C) with CD68 staining in a sequential staining (70-80 CD86+ cells per 100 mononuclear cells). The location and morphology of these CD86+ cells suggests mostly CD68+ monocytes and macrophages expressed CD86.



## Isolated graft lymphocytes

The CD4:CD8 ratio of the isolated graft lymphocytes was ~1:1 (43% CD4+ and 36% CD8+, Figure 6A). The proportion of $CD28^{NULL}$ within CD4+ T cells was 5% (compared to 0.1% in peripheral blood) and the proportion of $CD28^{NULL}$ within CD8+ T cells was 51% (compared to 54% in peripheral blood; Figure 6A). The majority of the isolated CD4+ and CD8+ T-lymphocytes were effector memory T-cells (59% and 66%) and terminally-differentiated EMRA cells (30% and 19%; Figure 6B). The majority of both CD28-positive ($CD28^{POS}$) as $CD28^{NULL}$ T-cells was CD45RO+ (Figure 6C). Intracellular granzyme B was present in CD8+ T-lymphocytes, but not in CD4+ T-lymphocytes (Figure 6D-E). Of the $CD8+CD28^{POS}$ T-lymphocytes, 47% expressed granzyme B, and of the $CD8+CD28^{NULL}$ T-lymphocytes, 50% was positive for granzyme B (Figure 6D). About 60% of CD45RO+ memory CD8+ T-cells and 17% of the CD45RO- CD8+ T-cells expressed granzyme B (Figure 6E). The IFNγ-production capacity was high in CD4+ and CD8+ T-cells: 39% and 67%, respectively (Figure 6F). Both $CD28^{POS}$ and $CD28^{NULL}$ CD8+ T-cells had a high IFNγ-production capacity (66% and 64%, respectively; Figure 6G).



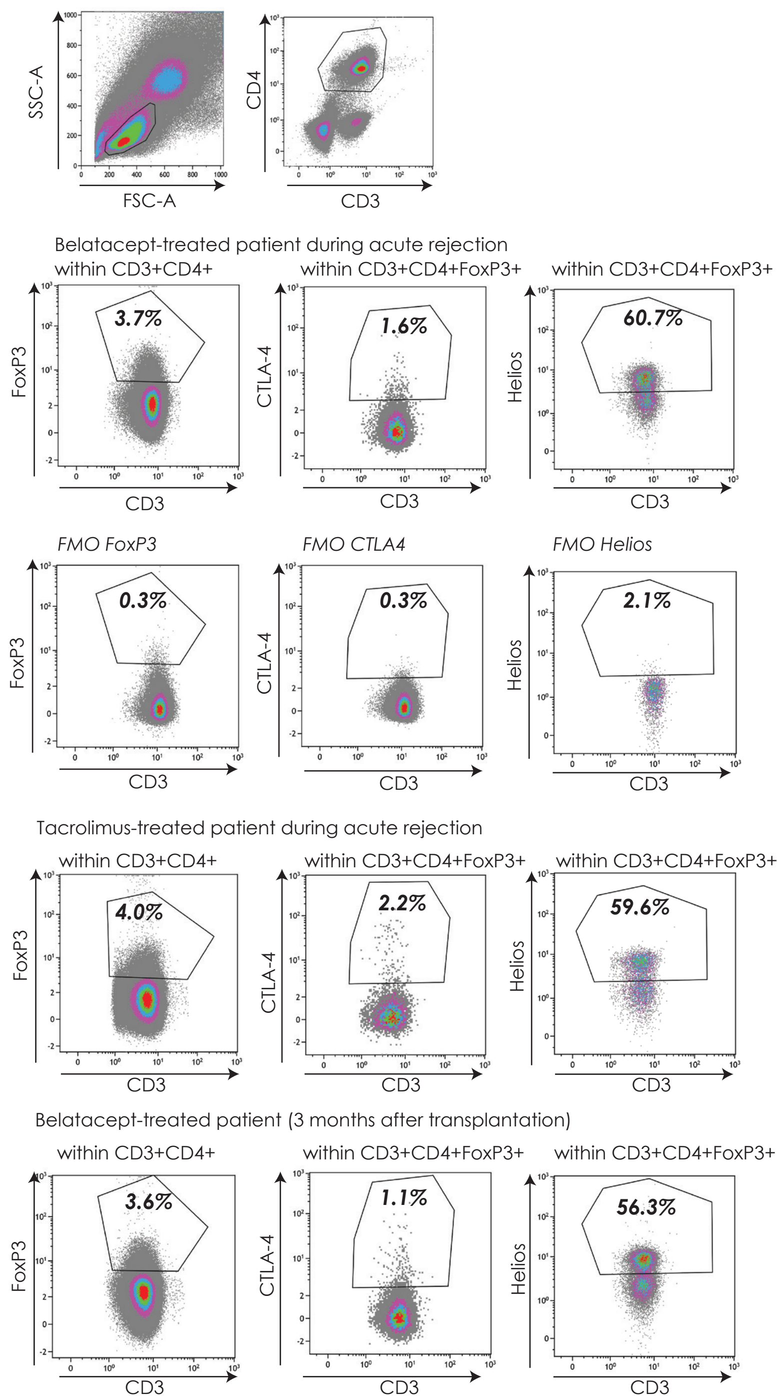

**Supplementary figure 4. Fluorescence In Situ Hybridization (FISH) of the perivascular infiltrate.**

Probes: X-chromosomes (green) and control chromosomes 15 (red). Every cell which contains a double X signal is from female origin and therefore from the recipient. Female chromosomes and female lymphocyte on the right served as a positive control.

A small proportion of the isolated lymphocytes consisted of B-cells (8.3%), which were mostly naïve CD27-IgD+ (79.6%) and switched memory CD27+IgD- (10.7%) B-cells. (Figure 7) About 3.6% of the B-cells were CD27+CD38$^{HIGH}$ plasmablasts.

## Discussion

This is the first immunological characterization, both in peripheral blood and in allograft tissue, of a severe, glucocorticoid-resistant, acute T-cell-mediated, kidney transplant rejection occurring under belatacept-based immunosuppression. We believe there was little evidence to support an antibody-mediated mechanism of this rejection. Although this possibility cannot be ruled out, histological examination was compatible with a T-cell mediated rejection.(9) The DSA were negative before transplantation and during rejection, and non-complement binding.

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**Supplementary Figure 5. Similar phenotype of FoxP3+ CD4+ T-cells in acute rejection under belatacept treatment, acute rejection under tacrolimus treatment and a stable/non-rejecting belatacept-treated patient.**

Lymphocytes were gated by size and granularity in the forward and sideward scatter. CD3+CD4+ T-cells expressing intracellular FoxP3 were considered as regulatory T cells (Tregs).

Although serum DSA could have been negative due to deposition in the renal graft (10), the absence of C4d, IgM and IgG from the tissue pleads against this (Supplementary Figure 1).

The occurrence of this rejection during belatacept-based immunosuppression therapy may have several explanations: (i) The co-stimulatory CD28-CD80/86 pathway was not sufficiently inhibited at the tissue level (*i.e.* in lymph nodes and/or the kidney); (ii) memory T-cells, which are independent of the CD28/CD80-86 pathway for their activation, mediated the rejection; (iii) the rejection was initiated through other co-stimulatory pathways; and (iv) belatacept inhibited the negative regulators of alloreactive T-cells, *i.e.* Tregs.

With regard to the first explanation, the CD86-occupancy on CD14+ monocytes was measured prospectively in whole blood. Monocyte CD86-occupancy correlates with the inhibition by belatacept of *in vitro* effector T-cell function and with serum belatacept concentrations.(8) In the present case, CD86 on peripheral blood monocytes was completely blocked both before and during rejection. However, in the kidney allograft, CD86 expression was not completely blocked as evidenced by the presence of CD86+ mononuclear cells. This difference between allograft tissue and peripheral blood might be explained by the small volume of distribution of belatacept, which implicates lower tissue than plasma concentrations.(11) The high CD86-expression in a rejection-biopsy of a tacrolimus-treated patient (Supplementary Figure 3) indicated that this anti-CD86 monoclonal antibody was also competitive with belatacept, as was the monoclonal antibody used in the whole blood flowcytometric assay (Figure 5). (12)

Regarding the second possibility, the large number of $CD28^{NULL}$ T-cells in peripheral blood before transplantation is a reflection of the presence of memory T-lymphocytes. As a consequence of her 2 pregnancies, part of these effector-memory T-cells may have been directed against HLA antigens of her husband.(13) The decrease of these blood $CD28^{NULL}$ T-lymphocytes during rejection and the presence of $CD28^{NULL}$ T-lymphocytes within isolated lymphocytes from the rejected graft suggest these cells may have migrated from the periphery into the allograft. Alternatively, the decrease of these cells was the result of immunosuppression. However, $CD28^{NULL}$ effector-memory T-cells are less susceptible to immunosuppressants (14, 15), so this is a less likely explanation. In fact, the documented low susceptibility of memory T-cells to the effects of glucocorticoids is in line with the clinical course of this case.(16, 17)

A significant proportion of the effector T-cell memory population probably was directed against antigens other than HLA. Memory against EBV could be an explanation since the patient had a sub-clinical EBV reactivation at the time of rejection.(18) EBV-specific memory T-cells can be cross-reactive to HLA-B*44:02 (19) but the donor did not express this HLA-antigen. The mild EBV reactivation therefore probably did not cause this rejection. Nevertheless, EBV-specific memory T-cells could have a yet unknown cross-reactivity with other HLA molecules. (20)

Third, this rejection could be explained by memory T-cells which were activated through other co-stimulatory pathways, like OX40-OX40L and ICOS-ICOSL.(21) This remains speculative as these pathways have been studied mostly in animals. Finally, impaired immunoregulation may have played a role as CD28 is also an important mediator of Treg homeostasis.(22, 23) Blockade of CD80/86 leads to anergic T-cells (24), which consequently may fail to activate Tregs via CD28. This could explain the lower expression of FOXP3 on mRNA level in renal tissue in belatacept-treated patients compared to the levels in cyclosporin-treated patients.(7) In contrast, in another study, FOXP3+ protein concentrations in T-cells were higher in belatacept-treated patients during rejection than in cyclosporin-treated patients.(8) In our patient the Treg phenotype was similar to a non-rejecting belatacept-treated patient and a tacrolimus-treated patient undergoing rejection (Supplementary Figure 5). The effect of belatacept on Treg function requires more research. The comparable expression of FoxP3, CTLA4, and Helios do not point to impaired Treg function by belatacept. Finally, CD80/86 signaling might also be important for suppression of Th17 cells via CD28 and CTLA4.(25)

In conclusion, this case sheds light on the immunologic mechanisms possibly underlying the higher rejection risk associated with belatacept-based immunosuppression. This glucocorticoid-resistant cellular rejection occurring under belatacept was predominantly mediated by cytotoxic memory T-cells, which are less susceptible to co-stimulatory blockade by belatacept, and/or resulted from incomplete CD80/86 blockade at the tissue level.

## Materials and methods

*Study design*

The patient described here participated in an ongoing, randomized-controlled trial in kidney transplant patients and was treated with belatacept (approved by the Medical Ethical Committee of the Erasmus MC, University Medical Centre Rotterdam; MEC-2012-42, EUDRACT CT # 2012-003169-16). In this trial, a total of 40 patients were included, of which 20 patients were randomized for treatment with belatacept and 20 patients for treatment with tacrolimus (the control group). Both groups were treated with basiliximab (Simulect®, Novartis, Basel, Switzerland) on the day of transplantation and on day 4. During the first three post-operative days methylprednisolone was administered intravenously in a dosage of 100 mg/day. Subsequently prednisolone was given orally in a dose of 20 mg and tapered to 5 mg/day at month 3. Mycophenolate mofetil was given in a starting dose of 2000 mg/day divided in two doses, and then adjusted to pre-dose levels (1.5 – 3.0 mg/L). The tacrolimus-treated patients received tacrolimus from the day of transplantation twice a day with a starting dose of 0.2 mg/kg/day. Thereafter, tacrolimus was adjusted to predose concentrations: 12-15 ng/mL (week 1-2), 8-12 ng/mL (week 3-4), and 5-10 ng/mL (from week 5 onwards). Belatacept-treated patients received 10 mg/kg/day intravenously on the day of transplantation, and on days 4, 15, 30, 60, and 90 after transplantation. From month 4 onwards, patients received monthly infusions of 5 mg/kg belatacept according to the less-intensive regimen.(4) The primary aim of this trial is to monitor and compare the alloreactive T-cell response during tacrolimus and belatacept therapy.

*Single bead Luminex assay*

DSA including C1q-binding capacity were determined in thawed sera before transplantation, 1 month after transplantation and during rejection. Twenty microliter of serum was incubated for 30 minutes with 2 µl Single Antigen beads mix from LABScreen Single Antigen class I and class II kits (One Lambda®, Canoga Park, CA). After protocol washing procedures, serum samples were incubated with 1 µl goat anti-human IgG-PE per well (One Lambda®). Microbeads were analyzed with a Luminex LabscanTM 100 analyzer using the Luminex 100IS software and analyzed using the HLA Fusion 3.0 software. All samples fulfilled the quality criteria for reactivity of the control beads.

*Flow cytometric phenotyping of peripheral blood lymphocytes*

Fresh heparin whole-blood was collected from the patient one day before transplantation, on day 4 and month 1 after transplantation, during rejection (month 2 after transplantation) and 2 months after rejection (month 4 after transplantation). The following monoclonal antibodies were added to 100 µL of whole blood for 15 minutes on room temperature to determine the CD28-negative ($CD28^{NULL}$) and CD28-positive ($CD28^{POS}$) T-cells: CD3 Brilliant Violet 510 (Biolegend, San Diego, CA), CD4 Brilliant Violet 421 (Biolegend), CD8 APC-Cy7 (Biolegend), and CD28 PerCP-Cy5 (Becton, Dickinson & company [BD], Franklin Lakes, NJ). Number of monocytes were determined using CD14 FITC (Serotec, Oxford, United Kingdom), CD45 PerCP (BD) and TruCount tubes® (BD). Erythrocytes were lysed using FACS Lysing Solution® (BD).

The CD86-expression on CD14+ monocytes was determined using CD86 PE (Beckman Coulter, Brea, CA), which is competitive with belatacept for CD86, but binds with lower affinity, *i.e.* cannot replace belatacept from the CD86-receptor.(12) In this manner only free, non-belatacept bound CD86 is measured. The whole-blood was incubated with the monoclonal antibodies for 30 minutes on ice, and thereafter erythrocytes were lysed using FACS Lysing Solution® (BD FACS, Franklin Lakes, NJ). Mouse IgG2B (Beckman Coulter) was used as an isotype control for the CD86-antibody.

Intracellular Forkhead box P3 (FoxP3) was stained using an anti-Human Foxp3 staining set (eBioscience, San Diego, CA). In short, thawed PBMCs were surface stained, using the following monoclonal antibodies: CD3 BV510 (BioLegend), CD4 BV421 (BioLegend), CTLA4 PeCy7 (BioLegend). Subsequently, the PBMCs were fixated and permeabilized and intracellularly stained for Helios (PerCP-Cy5.5, BioLegend) and FoxP3 (APC, eBioscience). Regulatory T cells were defined as CD3+CD4+FoxP3+ T cells.

*Immunohistochemistry of the kidney biopsy*

The renal graft biopsy and the explant were used for immunohistochemistry. The renal tissue was paraffin-embedded, formalin-fixed and cut into four-micrometer sections. Immunohistochemistry was performed by routine diagnostics on the Benchmark Ultra Stainer (Ventana, Basel, Switzerland), using the following moAbs: CD3 (1:150 dilution, DAKO, Denmark)

was used to detect pan-T cells; CD4 (undiluted, Ventana, AZ) for T-helper cells; CD8 (1:50, DAKO) for cytotoxic T-cells; and CD20 (1:400 dilution, DAKO) for B-cells. Also CD68 (1:1600, DAKO) for macrophages, CD138 (undiluted, Cell Marque, Rocklin, CA) for plasma cells, and CD80 (undiluted, R&D, Minneapolis, MN) and CD86 (undiluted, Abcam, Cambridge, UK) for the co-stimulatory molecules on antigen-presenting cells. Incubation with antibodies was done for 30 minutes and anti-rabbit or anti-mouse amplifiers were used.

Positive controls were used for immunoglobulins (lymph nodes), C4d (humoral rejection tissue) and CD138 (a mixture of tissue samples consisting of lymph node, bowel and pancreas). Positivity was required in these samples to exclude technical errors when patient material samples were negative.

*Flow cytometric phenotyping of explant lymphocytes*

A section of the kidney explant was embedded in collagenase at 37°C to detach the cells from the tissue. Thereafter, lymphocytes were isolated using the Ficoll density method. Tryptan-blue dye revealed that these cells were viable (> 90%). Explant lymphocytes were incubated with the following monoclonal antibodies for 30 minutes on room temperature to stain the cells for CD3 Brilliant Violet 510 (BioLegend, San Diego, CA), CD4 Brilliant Violet 421 (BioLegend), CD8 APC-Cy7 (BioLegend), CD28 PerCP-Cy5 (BD, Franklin Lakes, NJ) or CD28 APC (BD), CCR7 PE (BD Pharm), and CD45RO Pacific Blue (BioLegend). 7-AAD PerCP (BD Biosciences, San José, CA) was added as viability marker. In addition, the isolated explant lymphocytes were stained for intracellular granzyme-B (PE, Sanquin, Amsterdam, the Netherlands) after permeabilization with PERM II® 1:10 (BD FACS permeabilization solution, San Jose, CA). The IFNγ-production capacity was assessed by stimulating isolated graft lymphocytes for 4 hours with PMA 0.05 µg/ml and Ionomycine 1 µg/ml (Sigma-Aldrich, St. Louis, MO) at 37°C in the presence of Golgiplug 10 µL/mL (BD). IFNγ-FITC from BD Pharmingen (San Jose, CA) was used.

*Fluorescence in-situ hybridization (FISH)*

To establish the infiltrating cells were of patient (double X-chromosomes) and not of donor origin (single X-chromosome), a FISH was performed as described in previous studies.(26, 27) In short, the presence of two X-chromosomes per cell was determined using an X-probe (Xq13, RP11-1083G9). As a positive control a probe for chromosome 15 was used simultaneously (15q25, RP11-121E15). The paraffin-embedded, formalin-fixed graft tissue was first incubated with pepsine before the probes were added.

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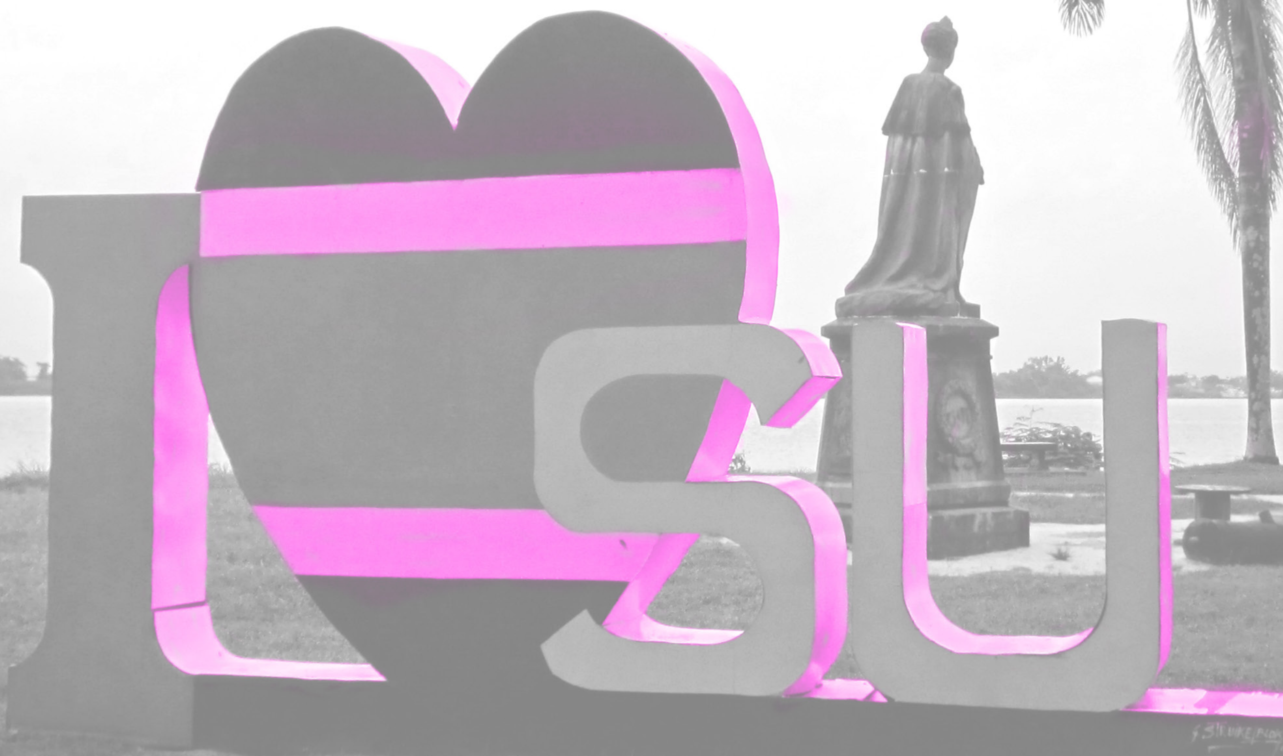


# Chapter 7

## Summary, discussion and conclusions

## Summary


The introduction of the currently used protocols in patient care and calcineurin inhibitor (CNI)-based immunosuppressive regimens improved outcomes in kidney transplantation.(1-7) Further progress in long-term patient and graft survival could be realized by the development and implementation of immunosuppressive drugs, which when compared to CNIs 1) are not inferior in preventing acute allograft rejection; 2) exert less severe adverse events, e.g. infections, malignancies, nephrotoxicity and cardiovascular events; and 3) are more efficient in preventing chronic graft destruction and dysfunction by inhibiting the formation of donor-specific anti-human leukocyte antigen (HLA) antibodies (DSA). Belatacept, the CD28-CD80/86 pathway inhibitor,(8) might meet the second and third criteria, but violates the first criterion by increasing acute rejection risk.(9-11)

In this dissertation, we aimed to learn more about the immune mechanisms involved in alloreactivity in patients treated with belatacept or tacrolimus after kidney transplantation. In particular, we sought to explain the higher acute rejection rate in belatacept-treated patients by studying cytotoxic cell populations which are potentially less susceptible to co-stimulatory inhibition, and to explain the lower incidence of DSA in previous trials by studying the effects of belatacept on Tfh-B cell interaction. Additionally, we tried to characterize belatacept-resistant rejection clinically and immunologically, and to find a biomarker to distinguish between patients who will reject under belatacept-treatment and those who will not.

In **Chapter 2**, we investigated if Tfh cells still mediated humoral reactivity shortly after kidney transplantation under the currently most prescribed immunosuppressive regimen. We found Tfh cells obtained from tacrolimus-treated patients can still mediate the differentiation of B cells into immunoglobulin-producing plasmablasts shortly after transplantation. Moreover, the numbers of these Tfh cells were positively correlated to pre-existent DSA, and Tfh cells co-localized in follicular-like structures with B cells and immunoglobulins in kidney biopsies of T-cell-mediated acute rejection. This implies Tfh-B cell interaction, which could lead to DSA formation, is not sufficiently inhibited by the tacrolimus-based immunosuppressive regimen. Interleukin (IL)-21 receptor blockade *in vitro* demonstrated efficient inhibition of Tfh-driven plasmablast formation and immunoglobulin production. This emphasizes the importance of IL-21 in Tfh-B cell interaction and the potential of immunosuppressive compounds interfering in the IL-21 pathway.

In **Chapter 3**, we studied the effects of belatacept and tacrolimus on Tfh-B cell interaction to explain the lower incidence of DSA observed in CNI-treated patients compared to belatacept-treated patients.(10) In PBMCs obtained from kidney transplant patients, surprisingly, no superior inhibition by belatacept was observed in Tfh cell generation, IL-21 production or plasmablast formation when compared to tacrolimus. Even though both drugs reduced IgM production, only tacrolimus could prevent plasmablast formation. We hypothesized redundant co-stimulatory pathways, e.g. the CD40-CD40L pathway, could circumvent co-stimulation blockade by belatacept. Moreover, upregulation of CD86 on activated B cells was not completely inhibited by belatacept. The attenuated suppression by belatacept might have the advantage IL-10+ transitional B cells, which are potentially immune regulatory, were not affected, but this was not confirmed in a multivariable regression analysis. The overestimation of the inhibitory effects by belatacept on Tfh-B cell interaction could be because belatacept has always been studied in combination with other immunosuppressive drugs, which could be accountable for the successful suppression.

In **Chapter 4,** we investigated the effect of belatacept on CD28-positive T cells, which in theory should be indirectly affected by costimulatory blockade. CD28-positive T cells were, however, capable of proliferating, differentiating and producing the cytokine IFNγ in the presence of belatacept. Furthermore, CD28-positive T cells could down regulate their surface CD28 after donor antigen stimulation and thus transform into CD28-negative T cells which are not susceptible to belatacept.

In **Chapter 5**, we tried to identify a biomarker for belatacept-resistant rejection for the first time in a randomized controlled trial in *de novo* kidney transplant patients ($n$ = 40) comparing a belatacept-based to a tacrolimus-based regimen. Acute rejection rate was higher in the belatacept group than anticipated (50% *vs.* 10% in the tacrolimus group) and these rejections were more severe, leading to 3 graft losses in the first months after transplantation. We postulated that this acute rejection rate was higher than in previous trials, because mycophenolic acid concentrations might have differed between the study populations and because the proportion of pre-emptive transplantations was higher in our study. Potential biomarkers were tested and compared pre-transplantation between future rejectors and non-rejectors: high proportions and absolute numbers of CD28-negative $CD8^+$ T cells, CD57-positive PD1-negative $CD4^+$ T cells, and CD28-high positive end-stage terminally differentiated ($CD28^{++}$ EMRA) $CD8^+$ T cells were not predictive for belatacept-resistant rejection. $CD28^{++}$ EMRA $CD8^+$ T cells showed the most potential as biomarker, but these findings need to be validated in a larger prospective study. CD86 receptor occupancy by belatacept on circulating monocytes proved to be a feasible method of pharmacodynamic therapeutic drug monitoring, but could also not distinguish rejectors from non-rejectors.

In **Chapter 6**, one illustrative case of a belatacept- and steroid-resistant rejection leading to acute graft loss was described and immunologically analyzed in detail. The characterization of isolated graft-infiltrating lymphocytes, and the immunohistochemistry of graft biopsy and tissue revealed a severe vascular T-cell mediated rejection consisting of CD28-positive and CD28-negative effector-memory T cells that expressed Granzyme B and produced IFNγ. No signs of antibody-mediated rejection were found. ~15% of $CD4^+$ mononuclear cells in the graft, morphologically classified as monocytes, still expressed CD86, which suggests an incomplete blockade of the CD28-CD80/86 pathway by belatacept on the tissue level.

In Figure 1, a summary is depicted of the different postulated mechanisms of the immune system to circumvent the effects of belatacept.

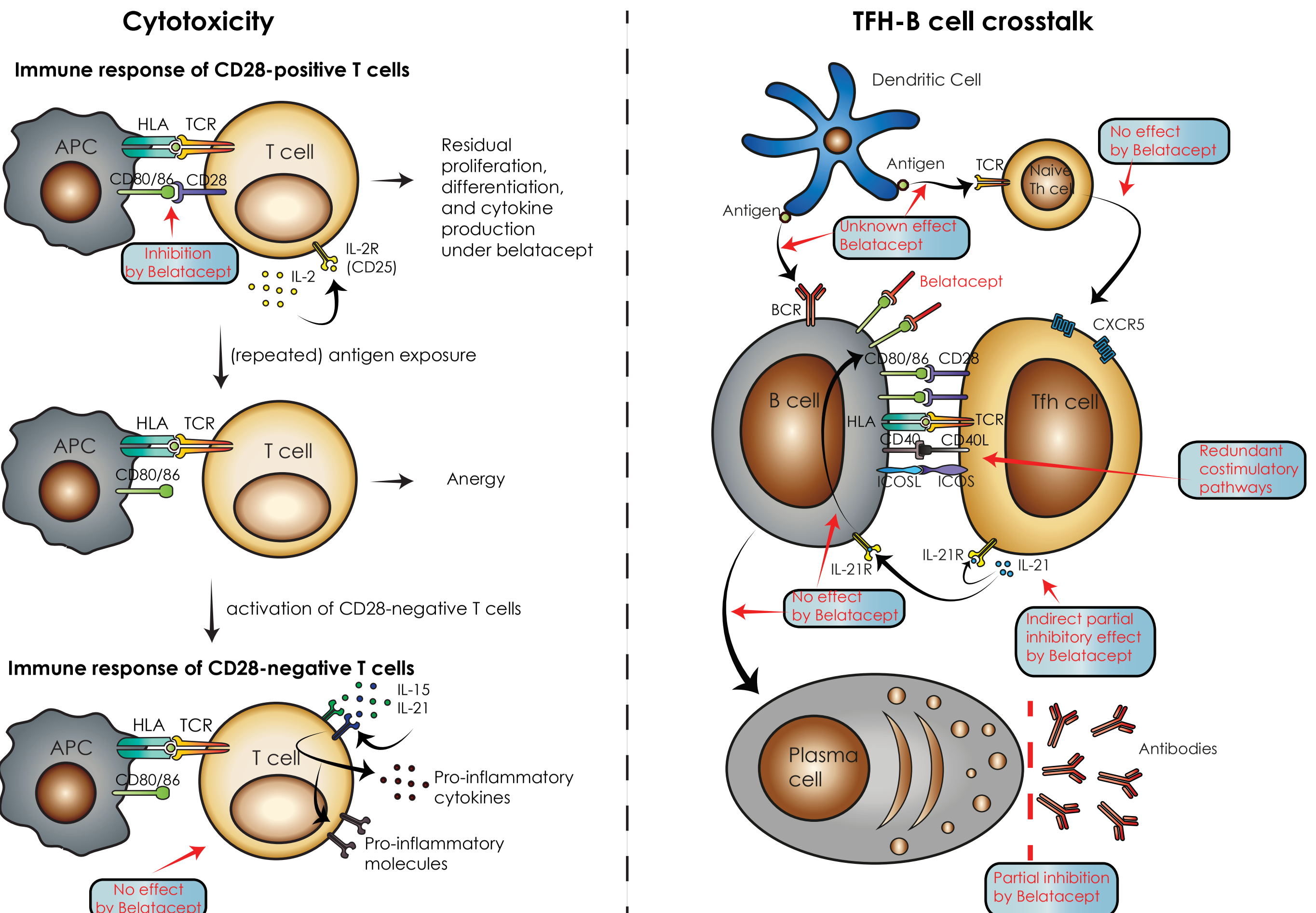


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**Figure 1. Hypothesized mechanisms of the immune system to circumvent co-stimulatory blockade by belatacept.**

Both cytotoxic T cells as follicular T cells (Tfh) and B cells could be less or not susceptible to belatacept. CD28-positive T cells down-regulate surface CD28 upon repeated antigen exposure, e.g. donor antigen, and become anergic when only their T-cell receptor is stimulated without co-stimulatory signals. CD28-negative T cells can, however, be activated again by external stimulating factors like the cytokines interleukin (IL)-15 or IL-21.(1-3) This leads to the upregulation and production of pro-inflammatory molecules and cytokines by CD28-negative T cells, which makes them dangerous to the graft, but not susceptible to belatacept. Moreover, a part of the T cells that remain CD28-positive can still proliferate, differentiate and produce pro-inflammatory cytokines under belatacept.

Tfh and B cells are activated by the same (donor)antigen. The formation of Tfh cells from T helper (Th) cells is not affected by belatacept, but the activation of Tfh cells and the consequent IL-21 production are partially inhibited. Tfh and B cells can still stimulate each other in the presence of belatacept by redundant CD86 on B cells and redundant co-stimulatory pathways, e.g. CD40-CD40L and ICOS-ICOSL. Consequently, Tfh-driven plasmablast formation is not prevented by belatacept. The IgM production is partially inhibited by blockade of the CD28-CD80/86 pathway. The inhibitory effects of belatacept on the interaction between dendritic cells (DCs) and Tfh or B cells is unknown.

References: (1) Traitanon et al. AJT 2014; 14: 1277–1289; (2) Alonso-Arias et al. Aging Cell 2011; 10, pp844–852; (3) Mou et al. AJT 2014; 14: 2460–2466

## Discussion - Clinical implications

The following recommendations can be made based on this dissertation and other studies: 1) belatacept should not be prescribed as first-line immunosuppressive therapy in *de novo* kidney transplantation, but should be reserved to special patient populations and/or occasions; 2) the identification of a biomarker for belatacept-resistant rejection is important for the implementation of belatacept in kidney transplantation; and 3) despite the introduction of belatacept, the search for more efficient immunosuppressive drugs against the development of DSA, by targeting Tfh-B cell interaction, should be continued.

The first recommendation, stating belatacept should not be used as first-line immunosuppressant, arises from the higher than expected acute rejection rate we observed in kidney transplant patients with a relatively low-immunological risk profile, mostly receiving their first graft from a living donor (**Chapter 5**). Probably since not only aggressive CD28-negative effector-memory T cells can escape blockade by belatacept,(12-14) but also a substantial part of their indirect targets, CD28-positive T cells (Figure 1; **Chapter 4 and 6)**. The severity of rejections under belatacept resulted in the increased necessity of using lymphocyte-depleting anti-rejection therapy, *i.e.*, alemtuzumab, followed by discontinuing belatacept-treatment and conversion to tacrolimus (**Chapter 5**). As most of these rejections occurred within the first months after transplantation, starting with a tacrolimus-based regimen the first months after transplantation and switching to a belatacept-based regimen hereafter, could be an elegant solution to prevent early acute rejections, and benefit from the long-term effects of a CNI-free regimen.(11) In the only reported randomized controlled belatacept-conversion trial (and its extension study) tacrolimus and cyclosporine A were switched to belatacept 6 months after kidney transplantation, which still resulted in an increased acute rejection rate, but equal patient and graft survival compared to patients that remained on tacrolimus.(15, 16) Reasons

for conversion from CNIs to belatacept in an observational study and various case reports were CNI nephrotoxicity,(17-19) tacrolimus-induced microangiopathy (TMA),(20, 21) and CNI intolerance(22, 23) All studies that reported renal function showed an increase of glomerular filtration rate after conversion to belatacept.(15, 16, 19, 20, 22, 23) In some studies belatacept could contribute in resolving TMA.(20, 21, 23) In addition, *in theory* certain patient populations could still benefit from belatacept more than they will suffer from the down sides of belatacept-resistant rejection, such as insulin-resistant diabetes patients and non-compliant patients.(11, 24) The question remains if stable belatacept-treated patients could have also profited from a regimen containing low doses of tacrolimus or no tacrolimus at all, but only glucocorticoid and mycophenolate mofetil therapy.

The second recommendation, that a biomarker for belatacept-resistant rejection is imperative for the implementation of the co-stimulatory inhibitor, results from the importance to distinguish patients who can benefit from a CNI-free belatacept-based regimen and patients who will only be harmed by irreversible graft injury caused by rejection or the detrimental effects of lymphocyte-depleting anti-rejection therapy. No definite immunological biomarker has been found yet, but the absolute numbers of pre-transplantation $CD28^{++}$ EMRA $CD8^{+}$ T cells warrant further investigation in a larger randomized controlled trial than conducted here. Our study did not have enough power to reliably compare characteristics between rejectors and non-rejectors. Nonetheless, belatacept is a milder immunosuppressant compared to tacrolimus. Thus by taking into account generally accepted risk factors for acute rejection,(25) e.g. higher donor age and younger recipient age, and complications caused or abrogated by CNIs like mentioned earlier, certain patients can be selected for belatacept-based treatment.

Finally, the third recommendation, that the search for a drug that effectively abrogates Tfh-B cell interaction should continue, comes from our *in vitro* studies that did not show a superior inhibition of this interaction by belatacept compared to tacrolimus. This was not in line with observations made in the BENEFIT-trial(10) and could be explained by an overestimation of the inhibitory effects by belatacept, because 1) it was combined with mycophenolate mofetil and prednisone; 2) mycophenolic acid concentrations could have been higher than usual; and 3) the comparator was the less potent CNI ciclosporin A.(10, 26, 27) The attenuated effects of belatacept on Tfh-B cell interaction possibly result from inadequate saturation of CD86 molecules on B cells and redundancy of other costimulatory pathways and cell types (Figure 1; **Chapter 3**). Furthermore, in the trial presented in **Chapter 5** we found that two patients in the belatacept group developed DSA the first year after transplantation *vs.* none in the tacrolimus group. Belatacept- (and also tacrolimus-) based regimens could be improved by the addition of other compounds interfering with Tfh-B cell interaction. The interleukin (IL)-21-receptor antagonist is a promising drug to prevent DSA formation, because the cytokine IL-21 is important for Tfh-cell-driven differentiation into immunoglobulin-producing plasma cells (**Chapter 2**). A study to assess the safety and tolerability of an IL-21-receptor antagonist ATR-107 was, however, terminated in 2011 due to the development of anti-drug antibodies in more than 75% of healthy volunteers, resulting in rapid clearance and low bioavailability of the drug.(28) Recently, a phase-I and –II trial have been completed about the efficacy and safety of other IL-21-receptor antagonists (NNC0114-0005 and -0006) in patients with rheumatoid arthritis and Crohn's disease, respectively (NCT01208506 and NCT01751152). However, so far, the results of these trials are not published and no trials have been conducted in kidney transplant patients. Another compound, targeting the CD40-CD40L pathway named CFZ533, could also be an useful addition to a belatacept-based regimen.(8, 29, 30) Currently, a phase-II study by Novartis® is recruiting *de novo* kidney transplant

patients to assess the safety and efficacy of CFZ533.(31) A third compound, tocilizumab, used for the treatment of rheumatoid arthritis, interferes with Tfh-B cell interaction by blocking the IL-6 receptor,(32, 33) and proved to be safe in a small group of highly sensitized kidney transplant patients.(34) Currently, kidney transplant patients are recruited in a phase-II trial to investigate the efficacy and safety of tocilizumab for treatment of inflammation in the graft.(35)

For now, belatacept does not meet the requirements to be the new corner stone in immunosuppression after kidney transplantation, because short-term outcomes are inferior to tacrolimus. However, belatacept-treatment could be beneficial and increase quality of life for a selected group of patients. The search for an immunosuppressive drug or combination of immunosuppressants, possibly including belatacept, to further improve kidney transplant patients' outcomes continues.

## Conclusions

- Follicular T helper cells mediate important humoral alloreactivity shortly after kidney transplantation under the currently used tacrolimus-based immunosuppressive regimen.
- Co-stimulation blockade by belatacept *in vitro* is less efficient than calcineurin inhibition by tacrolimus in preventing donor antigen-driven plasmablast formation resulting from Tfh-B cell crosstalk.
- Not only CD28-negative, but also CD28-positive, mostly effector-memory T cells can escape co-stimulatory blockade by down regulating their surface CD28 after antigen exposure.
- Belatacept-based therapy resulted in significantly higher and more severe acute rejection compared to tacrolimus-based therapy.
- Cytotoxic $CD4^+$ and $CD8^+$ T cells, *i.e.*, $CD4^+CD57^+PD1^-$, $CD8^+CD28^-$ and $CD8^+CD28^{++}$ EMRA T cells, are no suitable immunological biomarkers for belatacept-resistant rejection.
- Measuring free CD86 molecules on circulating monocytes is a feasible method for pharmacodynamic therapeutic drug monitoring of belatacept, but does not distinguish between (future) rejectors and non-rejectors.
- Belatacept-resistant rejection is a classic T-cell-mediated process including $CD28^+$ and $CD28^-$ effector-memory T cells, monocytes and macrophages.
- CD86 was not fully blocked by belatacept on $CD4^+$ mononuclear cells during rejection in a kidney graft.

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# Chapter 8

## Dutch summary (Nederlandse samenvatting)

## Samenvatting

De nieren zijn belangrijke organen in het menselijk lichaam en hebben de volgende functies:

- Het vormen van urine om schadelijke stoffen of stoffen waar we teveel van hebben uit het bloed te filtreren
- Het tegenhouden van belangrijke eiwitten en het actief terughalen van belangrijke stoffen uit de urine die we nodig hebben in het lichaam
- De balans van zouten en water onderhouden in het lichaam
- Het reguleren van de bloeddruk
- Het maken van hormonen om de aanmaak van rode bloedcellen te stimuleren
- Het omzetten van inactief vitamine D naar actief vitamine D

Elke minuut worden er door onze nieren 120 milliliter (mL) aan urine gevormd (dus meer dan 7 liter per uur), waarvan een groot deel door de nieren wordt heropgenomen naar de bloedbaan. Nieren kunnen beschadigd raken door hoge bloeddruk, suikerziekte, medicijnen en auto-immuunziektes (ziektes waarbij het immuunsysteem het eigen lichaam aanvalt, zoals lupus). Er wordt gesproken van chronische nierziekte wanneer de nieren nog minder dan 60 mL urine per minuut kunnen vormen. Wanneer de nieren zo slecht zijn dat er minder dan 10 mL urine per minuut wordt geproduceerd, kan de patiënt niet overleven zonder medische hulp. Een manier om nierpatiënten te helpen is door meerdere malen per week het bloed te filtreren door een machine in het ziekenhuis (hemodialyse) of door het bloed te zuiveren door spoelingen van de buikholte te verrichten thuis of in het ziekenhuis (peritoneaal dialyse). Deze oplossingen zijn echter niet perfect, omdat ze niet volledig de functie van een nier kunnen vervangen. Een betere oplossing is een niertransplantatie. Dit is realiseerbaar, doordat het mogelijk is om met één nier te leven in plaats van twee zonder hier nadelen aan te ondervinden.

Niertransplantatiepatiënten hebben een betere kwaliteit van leven en een betere levensverwachting dan dialysepatiënten.(1-3) Deze kwaliteit en levensverwachting zijn echter nog niet gelijk aan die van gezonde personen, omdat transplantatiepatiënten levenslang medicijnen moeten slikken om te voorkomen dat hun donornier wordt afgestoten en deze kapot gaat. Een afstoting betekent dat de cellen van het immuunsysteem van de patiënt de donornier ingaan en aanvallen. Dit kan worden vastgesteld door de combinatie van achteruitgang van nierfunctie en het binnendringen van immuuncellen in de donornier. Dit laatste wordt vastgesteld met een nierbiopt. Een acute afstoting, in het eerste jaar na transplantatie, bestaat vaak uit T cellen die de donornier binnendringen. Een chronische afstoting, die meer geleidelijk en jaren na transplantatie plaatsvindt, wordt veroorzaakt door gespecialiseerde B cellen, plasmacellen, die antistoffen vormen tegen de donornier. Meestal zijn acute afstotingen te behandelen door extra afweerremmende medicijnen, zoals prednison, te geven waardoor het transplantaat behouden blijft. Voor chronische afstotingen bestaat nog geen bewezen effectieve behandeling.

De meest gebruikte afweerremmende medicijnen die patiënten dagelijks moeten innemen zijn “calcineurine-remmers”, die celgroei en activatie van immuuncellen remmen,

in combinatie met de afweerremmers mycofenolzuur en prednison. Hierdoor treedt er veel minder vaak een afstoting op dan als er geen afweerremmers zouden worden gebruikt. Calcineurine-remmers kunnen T cellen effectief remmen, maar zijn niet goed in het voorkomen van antistofproductie door de plasmacellen van de patiënt.(4, 5) Hierdoor kan de nier jaren na transplantatie nog vernietigd worden door een chronische afstoting. Bovendien hebben calcineurine-remmers bijwerkingen als infecties en kanker, en zijn ze slecht voor hart en vaten. (6-9) Ten slotte beschadigen calcineurine-remmers de donornier.(10)

Er valt dus nog veel te verbeteren in de behandeling van niertransplantatiepatiënten. Het ideale afweerremmend middel voor deze patiënten zou de volgende drie eigenschappen bezitten:

- voorkomen dat de patiënt zijn donornier door een afstoting kwijtraakt;
- minder bijwerkingen hebben dan calcineurine-remmers;
- beter voorkomen dat er antistoffen worden gemaakt tegen de donornier, zodat deze ook op langere termijn niet beschadigd raakt.

Belatacept is nieuw afweerremmend geneesmiddel dat in 2012 geregistreerd werd voor niertransplantatiepatiënten en valt in de groep "co-stimulatieremmers". Het remt, net als calcineurine-remmers, de immuuncellen van de patiënt, maar via blokkade van co-stimulatiemoleculen. Het bindt op het molecuul CD86 op het oppervlak van de antigeen-presenterende cellen (monocyten, macrofagen, dendritische cellen en B cellen), en voorkomt zo het co-stimulatie signaal dat door binding aan CD28 op T cellen, naast de binding van antigen aan de T-cel receptor, nodig is voor de activatie van T cellen.(11) Dit middel geeft minder bijwerkingen dan calcineurine-remmers en is mogelijk beter in het voorkomen van antistoffen die worden gemaakt tegen de donornier.(12-14) Het nadeel van dit geneesmiddel is dat het minder krachtig is dan calcineurine-remmers, waardoor er meer acute afstotingen plaatsvinden de eerste maanden na transplantatie.(12)

In dit proefschrift willen we de immuunreacties die plaatsvinden onder behandeling met belatacept of tacrolimus beter leren begrijpen. In het bijzonder, proberen we een verklaring te geven voor het hogere aantal afstotingen in patiënten die behandeld worden met belatacept.(12) Hiervoor hebben we verschillende subgroepen van immuuncellen bestudeerd die mogelijk minder gevoelig zijn voor behandeling met belatacept en daardoor een afstoting kunnen veroorzaken, met name "cytotoxische T cellen". Dit zijn cellen die agressief zijn tegen de donornier en schadelijke stoffen maken, zoals cytokines of proteasen. Verder hebben we op celniveau bestudeerd hoe belatacept ervoor zorgt dat er minder antistoffen worden gemaakt tegen de donornier. Hierbij hebben we in het bijzonder T helper cellen en B cellen bestudeerd, omdat het samenspel van deze twee soorten immuuncellen zorgt voor de vorming van antistoffen. We hebben een uitgebreid onderzoek gedaan naar het immuunsysteem en de donornier van een patiënt die haar nier is kwijtgeraakt door een acute afstoting, terwijl ze werd behandeld met belatacept. Ten slotte, hebben we gezocht naar verschillen in het immuunsysteem tussen patiënten die wel en patiënten die geen acute afstoting hebben gehad onder behandeling met belatacept.

In **Hoofdstuk 2**, hebben we onderzocht of "folliculaire T helper cellen" (T helper cellen

die belangrijk zijn voor het stimuleren van B cellen) kort na niertransplantatie nog steeds B cellen en antistofproductie kunnen activeren, terwijl de patiënten behandeld worden met een calcineurine-remmer genaamd tacrolimus. We hebben gevonden dat folliculaire T helper cellen van deze patiënten in de kweek inderdaad B cellen kunnen stimuleren om plasmacellen te worden die antistoffen kunnen maken tegen de donor. Wanneer patiënten voor transplantatie meer antistoffen hadden die schadelijk kunnen zijn voor de donornier, hadden ze ook een hoger aantal folliculaire T helper cellen in hun bloed na transplantatie. In biopten van patiënten met een afstoting werden deze T cellen gezien op dezelfde plaats in de donornier als B cellen en antistoffen. Al deze resultaten wijzen er op dat de calcineurine-remmer tacrolimus het samenspel tussen folliculaire T helper en B cellen niet goed kan remmen, waardoor antistoffen tegen de donornier kunnen ontstaan. Wanneer we in een kweekbakje een antistof tegen de interleukine 21 receptor toevoegden, werd de B cel activatie en de vorming van antistoffen geremd, omdat interleukine 21 belangrijk is voor het samenspel tussen T helper en B cellen. Dit zou kunnen betekenen dat deze anti-interleukine 21 receptor-blokker ook in patiënten de vorming van antistoffen kan remmen.

In **Hoofdstuk 3**, hebben we getest of belatacept inderdaad beter de samenwerking tussen T en B cellen remt dan tacrolimus. We bestudeerden in de kweek terwijl er belatacept of tacrolimus was toegevoegd of 1) immuuncellen van niertranplantatiepatiënten folliculaire T cellen konden vormen, 2) er interleukine 21 werd gemaakt en 3) er vorming was van plasmacellen. Tegen de verwachting in werd dit niet beter geremd door belatacept. Beide middelen konden de vorming van antistoffen verminderen, maar alleen tacrolimus kon de vorming van plasmacellen voorkomen. Een verklaring zou kunnen zijn dat belatacept alleen CD86 afdekt en de T en B cellen nog geactiveerd kunnen raken via andere moleculen. Een andere verklaring is dat de B cellen zoveel CD86 moleculen op hun oppervlak hebben na activatie, dat ze niet allemaal geblokkeerd kunnen worden door belatacept. Er was ook een voordeel aan het feit dat belatacept minder goed remt dan tacrolimus: namelijk dat speciale B cellen die het immuunsysteem onderdrukken en mogelijk de kans op afstoting verminderen ("transitionele B cellen" die interleukine 10 maken) ook niet werden geremd door belatacept, maar wel door tacrolimus. Wanneer we echter in onze analyse corrigeerden voor andere factoren, zagen we deze beschermende werking voor transitionele B cellen door belatacept niet meer. De reden dat we niet hetzelfde hebben gevonden als eerdere studies die de remming van belatacept op T en B cellen bestuderen, kan zijn doordat wij voor het eerst het geïsoleerde effect van belatacept testen. De andere studies keken naar het effect van belatacept in combinatie met andere middelen.

In **Hoofdstuk 4**, hebben we bestudeerd wat het effect van belatacept is op T cellen met CD28 moleculen op hun oppervlakte. Aangezien belatacept het activerende signaal naar CD28 blokkeert door te binden aan CD86 moleculen van antigeen-presenterende cellen, moet belatacept er indirect voor zorgen dat deze CD28-positieve T cellen niet geactiveerd kunnen worden. Een deel van deze cellen kon na stimulatie met donormateriaal, ondanks de aanwezigheid van belatacept in de kweek, nog steeds delen, veranderen in gespecialiseerde geheugen cellen en het schadelijke cytokine Interferon-gamma produceren. Bovendien verdween bij een deel van de cellen de CD28 moleculen van het oppervlak door de stimulatie met donormateriaal. Hierdoor veranderden ze in CD28-negatieve T cellen die via andere manieren dan CD86 van antigeen-presenterende cellen kunnen worden geactiveerd en dus niet meer gevoelig zijn voor de indirecte remming door belatacept.

In **Hoofstuk 5**, hebben we gezocht naar een biomarker om te voorspellen welke patiënten een acute afstoting zouden krijgen onder behandeling met belatacept en welke patiënten niet. Hierbij werden 40 patiënten *at random* toegewezen aan een behandeling met belatacept of tacrolimus (in combinatie met twee standaard gebruikte afweerremmende medicijnen in niertransplantatie: mycofenolaat mofetil en prednison). De helft van de 20 patiënten in de belatacept groep had een acute afstoting, terwijl maar 2 van de 20 patiënten in de tacrolimus groep een afstoting had. De afstotingen in de belatacept groep waren ook ernstiger, waardoor er 3 nieren verloren zijn gegaan in de eerste paar maanden na transplantatie. Het hogere aantal acute afstotingen dan in eerdere onderzoeken kan komen doordat de spiegels van de actieve vorm van mycofenolaat mofetil (mycofenolzuur) mogelijk verschilden tussen de studiepopulaties en doordat het aantal transplantaties bij patiënten die nog niet dialyseren in ons centrum hoger is (zij hebben een sterker immuunsysteem dan dialysepatiënten en dus meer kans op een afstoting). We hebben voor transplantatie de volgende cytotoxische T cellen getest als biomarkers: CD28-negatieve $CD8^+$ T cellen, CD57-positieve PD1-negatieve $CD4^+$ T cellen en CD28-hoogpositieve zeer-gedifferentieerde geheugen $CD8^+$ T cellen ($CD8^+CD28^{++}$ EMRA). Geen van deze biomarkers kon worden gebruikt om te voorspellen welke patiënt een afstoting zou ontwikkelen onder belatacept behandeling. De $CD8^+CD28^{++}$ EMRA T cellen hadden de meeste potentie om een goede biomarker te zijn, maar deze resultaten moeten worden getest in een groter onderzoek. Het vrije aantal CD86 moleculen op monocyten kon gebruikt worden om de werking van belatacept te monitoren, maar niet om onderscheid te maken tussen patiënten die wel of geen afstoting zouden krijgen met belatacept behandeling.

In **Hoofdstuk 6** is een casus beschreven over een patiënt die haar nier kwijtraakte door afstoting onder belatacept behandeling. Deze afstoting kon niet worden geremd met hoge doses prednison. We bestudeerden de immuuncellen die de donornier hadden aangevallen en vernietigd, en hebben ook naar het weefsel van de donornier zelf gekeken. Hieruit konden we concluderen dat dit een ernstige afstoting was, waarbij de bloedvaten naar de nier dicht zaten door de immuunreactie. Deze reactie bestond uit zowel CD28-positieve als CD28-negatieve T cellen die schadelijke cytokines en proteasen produceerden, genaamd Interferon-gamma en Granzyme B. Er werden geen antistoffen van de patiënt gevonden in de bloedbaan of donornier. In de kapotte donornier werden er monocyten gezien die nog steeds vrije CD86 moleculen hadden op hun oppervlak, ondanks de patiënt met belatacept was behandeld.

## Beschouwing – Gevolgen voor niertransplantatiepatiënten

Op basis van dit proefschrift en andere onderzoeken kunnen de volgende aanbevelingen gedaan worden:

1) Belatacept is niet de eerste keus voor afweerremmende medicatie bij niertransplantatie, maar moet worden gebruikt in een selecte groep patiënten of situaties;

2) Het is belangrijk om een biomarker te vinden om te kunnen voorspellen welke patiënt wel en welke niet zal afstoten onder belatacept behandeling, voordat dit middel vaker gebruikt kan worden;

3) De zoektocht moet worden voortgezet naar een ander middel dan belatacept dat de vorming van antistoffen remt door het samenspel tussen folliculaire T helper en B cellen te verstoren.

De eerste aanbeveling, dat belatacept niet de eerste keus is als afweerremmend middel, is gebaseerd op het feit dat we veel meer acute afstotingen observeerden in niertransplantatiepatiënten die werden behandeld met belatacept. Ondanks deze patiënten van tevoren een laag ingeschat risico hadden op een afstoting en dat de meesten voor de eerste keer een nier hadden ontvangen, en deze van een levende donor was. Het hoge aantal afstotingen komt waarschijnlijk doordat niet alleen de agressieve CD28-negatieve geheugen T cellen kunnen ontsnappen aan belatacept,(15-17) maar ook een groot deel van hun indirecte doelwitten, CD28-positieve T cellen (**Hoofdstuk 4** en **6**). De afstotingen waren zodanig ernstig dat er aanvullende afweerremmende medicatie gegeven moest worden dat het hele immuunsysteem van de patiënt onderdrukt (alemtuzumab). Bovendien konden deze patiënten niet doorgaan met belatacept, en hebben ze tacrolimus voorgeschreven gekregen (**Hoofdstuk 5**). Omdat de meeste afstotingen in de eerste maanden na transplantatie plaatsvonden, de periode waarvan bekend is dat de kans op afstoting hoger is, zou een elegante oplossing kunnen zijn om de eerste maanden te behandelen met tacrolimus en daarna over te gaan op belatacept. Dit zou de vroege afstotingen voorkomen, maar als voordeel hebben dat er geen lange termijn schade aan de nier optreedt door tacrolimus.(14) In het enige beschreven gerandomiseerde onderzoek waarbij patiënten met calcineurine-remmers werden omgezet naar belatacept 6 maanden na transplantatie, was er een stijging in het aantal acute afstotingen, maar gelijke overleving van de donornier en de patiënt vergeleken met patiënten die nog steeds calcineurine-remmers gebruikten.(18, 19) Redenen om patiënten over te zetten van calcineurine-remmers naar belatacept in verschillende studies zijn nierschade,(20-22) bloedstolsels in de kleine niervaten (trombotische micro-angiopathie) (23, 24) of overgevoeligheid veroorzaakt door calcineurine-remmers.(25, 26) In de studies waar de nierfunctie werd beoordeeld, werd gezien dat deze patiënten allemaal een betere functie van hun donornier hadden nadat ze waren overgezet op belatacept.(18, 19, 22, 23, 25, 26) In sommige studies waren de bloedstolsels in de niervaten zelfs opgelost nadat belatacept gestart en tacrolimus gestopt werd.(23, 24, 26) Verder zouden *in theorie* bepaalde patiënten meer voor- dan nadelen kunnen halen uit behandeling met belatacept, zoals patiënten met suikerziekte of patiënten die hun medicatie niet trouw slikken (belatacept wordt maandelijks via een infuus in het ziekenhuis gegeven).(14, 27) De vraag blijft echter of patiënten die geen afstoting hebben onder belatacept-behandeling het ook goed zouden doen met lagere doseringen tacrolimus of helemaal geen tacrolimus, maar alleen

mycofenolaat mofetil en prednison.

De tweede aanbeveling, dat de zoektocht naar een biomarker voor afstoting onder belatacept behandeling moet worden voortgezet, resulteert uit het feit dat het belangrijk is om patiënten te kunnen onderscheiden die voordeel hebben aan een behandeling zonder calcineurine-remmers en patiënten die alleen maar schade ondervinden aan ernstige acute afstotingen bij belatacept behandeling of bijwerkingen van extra anti-afstotingstherapie (alemtuzumab). Er is nog geen biomarker gevonden, maar het aantal $CD8^{+}CD28^{++}$ EMRA T cellen voor transplantatie moet verder worden onderzocht als biomarker in een groter onderzoek dan wij hier hebben verricht. Onze studie was niet groot genoeg om betrouwbaar patiënten die wel of niet afstoten met belatacept te vergelijken. Desalniettemin is belatacept een zwakker afweerremmend medicijn dan tacrolimus en zou daarom alleen gegeven moeten worden aan patiënten met een lager ingeschat risico op afstoting,(28) zoals oudere patiënten of patiënten die een nier ontvangen van een levende jonge donor. Hierbij moeten ook patiënten worden geselecteerd die duidelijke nadelen ondervinden van calcineurine-remmers, zoals eerder genoemd.

Ten slotte, de derde aanbeveling, dat de zoektocht moet worden voortgezet naar een ander middel dan belatacept dat het samenspel tussen folliculaire T helper cellen en B cellen verstoort, komt doordat wij hebben gezien dat belatacept dit samenspel eigenlijk niet beter remt dan tacrolimus in de kweek. Dit komt niet overeen met wat in een Amerikaanse studie (de BENEFIT-trial) gevonden is(13) en kan worden verklaard doordat de remmende werking van belatacept in die studie overschat is, omdat 1) het gecombineerd werd met 2 andere afweerremmende middelen (mycofenolaat mofetil en prednison); 2) de concentraties van de werkzame stof van mycofenolaat mofetil (mycofenolzuur) hoger geweest konden zijn; en 3) belatacept werd vergeleken met een oudere en zwakkere calcineurine-remmer dan tacrolimus, namelijk ciclosporine A.(13, 29, 30) De verminderde werking van belatacept op het samenspel tussen folliculaire T helper cellen en B cellen komt waarschijnlijk doordat het niet alle CD86 moleculen kan afdekken op geactiveerde B cellen en doordat de activatie van de immuuncellen via andere moleculen kan plaatsvinden (**Hoofdstuk 3**). Bovendien hadden twee patiënten behandeld met belatacept in onze studie in **Hoofdstuk 5** antistoffen tegen de donor gemaakt, terwijl geen patiënten behandeld met tacrolimus deze antistoffen ontwikkelden. Behandeling met belatacept (of tacrolimus) zou kunnen worden verbeterd door andere middelen toe te voegen die het samenspel tussen folliculaire T helper en B cellen remmen. De blokker van de interleukine-21-receptor is een veelbelovend middel om antistofvorming te voorkomen, omdat de cytokine interleukine-21 belangrijk is voor de samenwerking tussen folliculaire T helper en B cellen die leidt tot antistof-producerende plasmacellen (**Hoofdstuk 2**). Een onderzoek naar de veiligheid van zo een interleukine-21 blokker genaamd ATR-107 is echter vroegtijdig gestaakt, omdat meer dan driekwart van de gezonde vrijwilligers antistoffen maakten tegen het geneesmiddel zelf, waardoor deze werd afgebroken en niet meer werkzaam kon zijn. (31) Onlangs zijn er twee onderzoeken verricht, waarvan nog geen resultaten zijn gepubliceerd, naar de veiligheid en effectiviteit van andere interleukine-21 blokkers (NNC0114-0005 en -0006) in patiënten met een auto-immuunziekte (trial nummers NCT01208506 en NCT01751152). Maar deze afweerremmers zijn nog niet onderzocht in niertransplantatiepatiënten. Een ander afweerremmend middel genaamd CFZ533, dat het CD40 molecuul bindt dat ook belangrijk is voor T en B cel samenspel, zou een goede toevoeging kunnen zijn aan behandeling met belatacept.(11, 32, 33) Er wordt momenteel een studie verricht door Novartis® waarbij de veiligheid en effectiviteit van dit middel wordt getest op patiënten die voor het eerst een

donornier krijgen.(34) Een derde middel genaamd tocilizumab, dat van oorsprong gebruikt wordt in reumapatiënten, blokkeert de interleukine-6 receptor, die ook belangrijk is voor de samenwerking tussen folliculaire T helper en B cellen.(35, 36) Dit middel bleek veilig te zijn in een kleine groep nierpatiënten die al voor transplantatie veel antistoffen hadden.(37) Er is een studie gaande om de effectiviteit en veiligheid van dit middel te testen in niertransplantatiepatiënten met een immuunreactie in hun donornier.(38)

Vooralsnog voldoet belatacept niet aan de eisen om de nieuwe hoeksteen van de afweerremmende behandeling te worden na niertransplantatie, omdat de korte termijn uitkomsten van belatacept minder goed zijn dan die van tacrolimus. Behandeling met belatacept kan echter voordelen hebben en de kwaliteit van leven verbeteren voor een selecte groep aan patiënten. De zoektocht naar een afweerremmend middel of een combinatie van afweerremmende middelen, mogelijk inclusief belatacept, om de uitkomsten in niertransplantatiepatiënten te verbeteren, duurt voort.

## Conclusies:

- Folliculaire T helper cellen reguleren belangrijke B cel processen al kort na niertransplantatie ondanks patiënten worden behandeld met tacrolimus.
- Het samenspel tussen folliculaire T helper en B cellen wordt minder goed geremd door belatacept dan door tacrolimus.
- Niet alleen CD28-negatieve, maar ook CD28-positieve geheugen T cellen kunnen ontsnappen aan belatacept, doordat hun oppervlakte CD28 verdwijnt na blootstelling aan antigenen.
- Behandeling met belatacept resulteert in meer en ernstigere afstotingen dan behandeling met tacrolimus.
- Cytotoxische $CD4^{+}$ en $CD8^{+}$ T cellen, namelijk $CD4^{+}CD57^{+}PD1^{-}$, $CD8^{+}CD28^{-}$ en $CD8^{+}CD28^{++}$ EMRA T cellen, zijn geen geschikte biomarkers om afstoting onder belatacept behandeling te voorspellen.
- Het meten van vrije CD86 moleculen op monocyten in de bloedbaan is een goede methode om het werkingsmechanisme van belatacept te bestuderen, maar niet om onderscheid te maken tussen patiënten die wel of niet afstoten.
- Bij afstoting onder belatacept zijn zowel CD28-positieve als CD28-negatieve geheugen T cellen betrokken die de donornier aanvallen samen met monocyten en macrofagen.
- CD86 was, ondanks behandeling met belatacept, niet volledig geblokkeerd op monocyten in de afgestoten donornier.

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# Appendices

## Curriculum Vitae Auctoris

Gretchen Norine de Graav was born on September 19th 1986 in Rotterdam, the Netherlands, after her parents migrated from Paramaribo, Suriname in 1985. From 1998 to 2004 she attended the vwo at Farel College in Ridderkerk, the Netherlands. Hereafter, she started her Medicine study at the Erasmus University Rotterdam, and obtained her medical degree January 2011. For over a year she worked as a resident at the Department of Internal Medicine at the Maasstad Hospital, Rotterdam. In May 2012 she started her PhD project at the Transplantation Laboratory of the Internal Medicine Department, Division of Nephrology and Transplantation, at the Erasmus MC, under supervision of prof. dr. Carla Baan, prof. dr. Willem Weimar and dr. Dennis Hesselink. This research is presented in this thesis. In January 2017 Gretchen started her training in Internal Medicine at Maasstad Hospital, Rotterdam, under supervision of dr. M. van den Dorpel. She lives with Rens Kraaijeveld whom she met at the Transplantation Laboratory.

# List of publications


1. Baan CC, **de Graav GN**, Boer K. T Follicular Helper Cells in Transplantation: The Target to Attenuate Antibody-Mediated Allogeneic Responses? *Current Transplantation Reports* 2014; 1:166-172

2. **de Graav GN**, Dieterich M, Hesselink DA, Boer K, Clahsen-van Groningen MC, Kraaijeveld R, Litjens NHR, Bouamar R, Vanderlocht J, Tilanus M, Houba I, Boonstra A, Roelen DL, Claas FHJ, Betjes MGH, Weimar W, Baan CC. Follicular T helper cells and humoral reactivity in kidney transplant patients. *Clinical and Experimental Immunology* 2014; 180:329-340

3. Spaan M, Kreefft K, **de Graav GN**, Brouwer WP, de Knegt RJ, ten Kate FJW, Baan CC, Vanwolleghem T, Janssen HLA, Boonstra A. CD4+CXCR5+ T cells in chronic HCV infection produce less IL-21, yet are efficient at supporting B cell responses. *Journal of Hepatology* 2015; 62:303-310

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5. **de Graav GN**, Hesselink DA, Dieterich M, Kraaijeveld R, Douben H, de Klein A, Roelen DL, Weimar W, Roodnat JI, Clahsen-van Groningen MC, Baan CC. An Acute Cellular Rejection With Detrimenal Outcome Occuring Under Belatacept-Based Immunosuppresive Therapy: An Immunological Analysis. *Transplantation* 2016; 100: 1111-1119.

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8. **de Graav GN,** Baan CC, Clahsen-van Groningen MC, Kraaijeveld R, Dieterich M, Verschoor W, von Thusen JH, Roelen DL, Cadogan M, van de Wetering J, van Rosmalen J, Weimar W, Hesselink DA. Belatacept Causes A Higher Incidence Of Acute Rejection Compared with Tacrolimus after De Novo Kidney Transplantation: A Randomized Controlled Trial. *Transplantation 2017;* under review after major revision

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## PhD Portfolio

| | |
|---|---|
| **Name PhD Student** | Gretchen Norine de Graav |
| **Erasmus MC department** | Internal Medicine, section Nephrology and Transplantation |
| **Research school** | Postgraduate School Molecular Medicine |
| **PhD period** | 2012 – 2017 |
| **Promotors** | Prof.dr. C.C. Baan |
| | Prof.dr. W.Weimar |
| **Copromotor** | Dr. D.A. Hesselink |

**Courses and workshops**

2012 Basic Course about the Rules and Organization in Clinical Trials (BROK cursus)*

2012 NIHES Statistics Course*

2012 Practical Radiation Protection Course level 5B*

2013 Immunology Course*

2013 Biomedical English Writing and Communication*

2013 Adobe Photoshop & Illustrator Course*

2014 Research Integrity Course*

2014 Hesperis Course Transplantation

2015 Master Class in Kidney Transplantation I Sanofi

2015 Winter school Dutch Kidney Foundation

2016 Refreshment Course of BROK*

2016 Adobe InDesign Course*

2016 Master Class in Kidney Transplantation II Sanofi

* Erasmus MC, Rotterdam

**Participation and presentations at conferences**

| | | |
|---|---|---|
| 2012 | American Society of Nephrology (ASN) Congress, San Diego, USA | Oral + Poster |
| 2012 | Annual meeting Dutch Transplant Society (NTV Bootcongres), Maastricht, the Netherlands | Participation |
| 2013 | Dutch ASN Congress, Utrecht, the Netherlands | Oral |
| 2013 | American Transplant Congress (ATC), Seattle, USA | Poster |
| 2013 | European Society of Organ Transplantation (ESOT) congress, Vienna, Austria | Oral |
| 2013 | ESOT Basic Science Meeting and TTS Basic Science Symposium, Paris, France | Oral |
| 2014 | Science Days, Antwerp, Belgium | Poster |
| 2014 | Annual meeting Dutch Transplant Society (NTV Bootcongres), Leiden, the Netherlands | Oral |
| 2014 | World Transplant Congress (WTC), San Francisco, USA | Poster |
| 2014 | ESOT and AST Joint Meeting, Madrid, Spain | Participation |
| 2014 | Dutch Society for Immunology Congress, Kaatsheuvel, the Netherlands | Poster |
| 2015 | Science Days, Antwerp, Belgium | Poster |
| 2015 | Molecular Medicine Day, Rotterdam, the Netherlands | Poster |
| 2015 | Joint British and Dutch Transplant Societies Congress, Bournemouth, United Kingdom | Poster |
| 2015 | ESOT congress, Brussels, Belgium | Oral + Poster |
| 2015 | International Congress of Therapeutic Drug Monitoring & Clinical Toxicology, Rotterdam, the Netherlands | Oral |
| 2016 | Science Days, Antwerp, Belgium | Poster |
| 2016 | Molecular Medicine Day, Rotterdam, the Netherlands | Participation |
| 2016 | Annual meeting Dutch Transplant Society (NTV Bootcongres), Groningen, the Netherlands | Oral |
| 2016 | ATC, Boston, USA | Oral |
| 2016 | The Transplantation Society (TTS) congress, Hong Kong, China | Oral |
| 2017 | Annual meeting Dutch Transplant Society (NTV Bootcongres), Zeist, the Netherlands | Poster |
| 2017 | ATC, Chicago, USA | Oral |

**Travel grants and awards**

2013 Young Investigator Award Dutch ASN

2014 Travel grant Vereniging Trustfond Erasmus Universiteit Rotterdam

2014 Best Abstract Award Bootcongres

2016 Young Investigator Award ATC

2016 Mentor-mentee Award TTS

**Teaching activities**

2014-2016 Counseling PhD students and technicians in setting up study protocols

2016 Counseling and lecturing second year Medical students in writing a systematic review

**Memberships**

2012 – present Nederlandse Transplantatie Vereniging

2012 – present The Transplantation Society

2012 – present The European Society of Organ Transplantation

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Ling, thank you for always being your happy self and making me laugh with your sharp remarks. I realy envy your ability to eat a lot of chocolate without getting fat. You shared some difficult periods in your life with me and I want to thank you for your trust in me.

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Kitty, je was als een verfrissende wervelwind toen je bij ons begon. Heerlijk enthousiast en een tikkeltje chaotisch, maar al meteen veel inzicht (#flawless). Vanaf de eerste dag konden we het al goed vinden en ons hart luchten bij onze onofficiële bela-meetings bij de Doppio. Bedankt dat je niet gek werd van al mijn Hello Kitty spullen aan de muur en de fijne tijd samen in ons kantoor.

Lin, you are a very sweet and enthusiastic lady. Good luck in the cold and rainy Netherlands!

Ruud, ik leerde je pas echt kennen toen we tegenover elkaar zaten in het Na-gebouw. Bedankt voor de onvergetelijke tijd in de Efteling en je oog voor detail. Het gaat je goed!

Nynke, met jou voelde het alsof we er een nieuw zusje bij hadden in de bela-groep. Samen sloegen we ons door de pittige maandagochtend meetings heen en waren we elkaars steun en toeverlaat tijdens cursussen en congressen. In het vliegtuig vormden we een gezellig koppeltje (de één met reisziekte, de ander met vliegangst). Ook jij bedankt voor de onofficiële bela-meetings.

Samantha, geheel tegen mijn eerste verwachtingen in, kon ik het erg goed met je vinden en ben je nu officieus lid van mijn “Asian Posse”. Ondanks je me regelmatig pestte met mijn smetvrees, zorgde je voor persoonlijke hygiëne in de groep. Bedankt hiervoor en uiteraard voor alle gezelligheid op en buiten het werk.

Franka, we delen onze grote passies “wijn drinken” en “TV series binge watchen”. Gelukkig zijn we ondanks de “Wine-throwing-gate” en de “What’s-behind-you?-You’re-youth!-escapade” wel vrienden geworden. Bedankt voor het geven van lachstuipen en het beschikbaar stellen van je haar voor extensions.

Fleur, met je befaamde knot en voetbaltalenten tegenover mijn liefde voor Hello Kitty en alles roze, konden we bijna niet meer verschillen. Toch heb ik veel met je gelachen wanneer je op een droge manier de meest hilarische verhalen vertelde. Bedankt voor het tolereren van mijn meisjesdingetjes.

Jesus, you, me and your beard had lots of laughs. Keep being your funny self during the rest of your career!

Anusha, we kennen elkaar nog niet lang, alhoewel we al minimaal 6 keer aan elkaar zijn voorgesteld. Veel succes op het Transplantatielab!

Anja, Anne, Marieke, Martijn en Ramin, mijn oudere AIO-broers en zussen. We zaten samen in een benauwd hok en jullie hebben ervoor gezorgd dat ik niet verzoop in de PhD-stress de eerste periode van mijn traject. Het gaat jullie allen goed!

Ik wil ook de post-docs bedanken voor hun kennis, hulp en gezelligheid op de werkvloer. Het zou niet hetzelfde zijn geweest zonder jullie input.

Dr. Wu, I respect the way you adapted to the Dutch culture and that you were always in for a party. Sometimes you even entertained us with Chinese music in the office. Thank you for our talks about different cultures and good luck with the rest of your career.

Marcel.la, although you are back home now, I still think about you. With your down-to-earth attitude, your sporty figure and yoga fitness, you’re an example to us all. Good luck with your family and career in Barça.

Martin, ik heb nog nooit iemand gezien met zoveel kalmte en geduld als jij. Bedankt voor het compenseren van alle drukte op het lab.

Nicolle, als jij iets niet weet, weet niemand het. Heel veel dank dat ik altijd naar binnen kon lopen om advies te vragen over protocollen, antilichamen en FACS-plaatjes.

Ana, thank you for your Southern-Europe mentality that looks a lot like the Surinamese attitude. I always felt really comfortable with you and I applaud your ways to interact with the people around you. I hope I will see you again at the Feria Córdoba the coming years.

Karin, jij doet je nooit anders voor dan je bent en zegt gewoon waar het op staat. Als Rotterdammert stel ik dat zeer op prijs. Op gebied van onderzoek (te moeilijke kleuringen op

biopten) kon ik op je hulp rekenen. Dank hiervoor.

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Alle analisten wil ik ook bedanken voor het opvoeden van mij tot iemand die je alleen durft achter te laten op een lab.

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Joke, het opperhoofd van de kweek. We hadden soms onze mini-uitbarstingen, maar we konden het toch goed vinden met elkaar. Je hebt me leren ficollen, terwijl we modetips aan elkaar uitwisselden. Ik kreeg soms zelfs mooie kleding of schoenen van je. Ik hoop dat ik er later half zo modieus uit zie als jij. Geniet van je vrije tijd!

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Derek, de nieuwe stille kracht op het transplantatielab. Succes!

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# Abbreviations

| | |
|---|---|
| (S)AE | (Severe) Adverse event |
| 7-AAD | 7-aminoactinomycin |
| ABMR | Antibody-mediated rejection |
| AF647 | Alexa Fluor 647 |
| AMR | Antibody-mediated rejection |
| APC | Antigen-presenting cell |
| APC | Allophycocyanin |
| APC-Cy7 | Allophycocyanin-Cyanine7 |
| AR | Acute rejection |
| Bcl-6 | B-cell lymphoma 6 |
| Bela | Belatacept |
| BENEFIT (study/trial) | Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial |
| BKV | BK virus |
| BMI | Body mass index |
| BPAR | Biopsy-proven acute rejection |
| BSc | Bachelor of Science |
| BV | Briliant Violet |
| $C_0$ | Pre-dose concentration |
| C4d | Complement factor 4d |
| CD | Cluster of differentiation |
| CDC | Complement-dependent cytotoxicity |
| CFSE | Carboxyfluorescein succinimidyl ester |
| CMV | Cytomegalovirus |
| CNI | Calcineurin inhibitor |
| CsA | Cyclosporin A |
| CTLA-4 | Cytotoxic T-lymphocyte-associated protein 4 |
| CVA | Cerebrovascular accident |
| CXCR5 | C-X-C chemokine receptor 5 |
| D-1 | One day before transplantation |
| DC | Dendritic cell |
| DSA | Donor-specific anti-human leucoyte antigen antibodies |
| Dx | x days after transplantation |
| EBV | Epstein-Barr virus |
| eGFR | Estimated glomerular filtration rate |

| | |
|---|---|
| ELISA | Enzyme-linked immunosorbent assay |
| ESRD | End-stage renal disease |
| FACS | Fluorescence-activated cell sorting |
| FITC | Fluorescein isothiocyanate |
| FMO | Fluorescence-minus-one |
| FoxP3 | Forkhead box P3 |
| FSC-A | Forward scatter area |
| GrB | Granzyme B |
| Gy | Gray |
| HDL | High density lipoproteins |
| HIGH | high-positive |
| HIV | Human immunodeficiency virus |
| HLA | Human leukocyte antigen |
| HR | Hazard ratio |
| HSV | Herpes simplex virus |
| $IC_{50}$ | Half maximal (50%) inhibitory concentration |
| ICOS | Inducible T cell co-stimulator |
| ICOSL | Inducible T cell co-stimulator ligand |
| IFN | Interferon |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IL-21-R | Interleukin-21 receptor |
| KT | Kidney transplantation |
| LDL | Low density lipoproteins |
| MCV | Mean corpuscular volume |
| MD | Medical Doctor |
| MFI | Median fluorescense intensity |
| MGUS | Monoclonal gammopathy of unknown significance |
| MLR | Mixed lymphocyte reaction |
| MMF | Mycophenolate mofetil |
| MPA | Mycophenolic acid |
| mTor | Mammalian target of rapamycin |
| Mx | x months after transplantation |
| N/A | Not applicable |
| NR | Non-rejector |
| NULL | Negative |
| PA | Pathology |

| | |
|---|---|
| PBMCs | Peripheral blood mononuclear cells |
| PD-1 | Programmed death 1 |
| PD-L1 | Programmed death ligand 1 |
| PE | Phycoerythrin |
| PE-Cy7 | Phycoerythrin-Cyanine7 |
| PerCP | Peridinin chlorophyll |
| PerCP-Cy5 | Peridinin chlorophyll-Cyanine 5.5 |
| PhD | Philosophical Doctor |
| PKH | Paul Karl Horan dye |
| PMA | Phorbol 12-myrisate 13-acetate |
| $^{POS}$ | Positive |
| PRA | Panel reactive antibodies |
| PTDM | Post-transplant diabetes mellitus |
| PTLD | Post-transplant lymphoproliferative disease |
| R | Rejector |
| RCT | Randomized controlled trial |
| SD | Standard deviation |
| SDC | Supplemental Digital Content |
| SE | Standard error |
| SEB | Superantigen Staphylococcus aureus antigen B |
| SPSS | Statistical Package for the Social Sciences |
| SSC-A | Sideward scatter area |
| Tac | Tacrolimus |
| $T_{CM}$ | Central-memory T cells |
| TCMR | T-cell mediated rejection |
| TDM | Therapeutic drug monitoring |
| $T_{EM}$ | Effector-memory T cells |
| $T_{EMRA}$ | End-stage terminally differentiated effector-memory T cells expressing CD45RA |
| Tfh cell | Follicular T helper cell |
| TIA | Transient ischemic attack |
| $T_N$ | Naïve T cells |
| TNF | Tumor necrosis factor |
| Treg | Regulatory T cell |
| VZV | Varicella zoster virus |