Aus der Klinik mit Schwerpunkt Nephrologie

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Early detection of the immunophenotyping after conversion to belatacept in comparison to conventional immunosuppressive therapy in renal transplant patients

zur Erlangung des akademischen Grades

Doctor rerum medicinalium (Dr. rer. medic.)

vorgelegt der Medizinischen Fakultät

Charité – Universitätsmedizin Berlin

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Datum der Promotion: 22.09.2017

Gutachter: 1. N.N.

2. N.N.

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ABSTRACT (English)

Beside short term success, long-term results in regard to renal transplant survival are still unsatisfactory for patients and clinicians. The underlying major limits to achieving long-term graft function are determined in late antibody-mediated rejection and side effects of immunosuppression. Conventional immunosuppressive drugs like calcineurin inhibitors (CNI) or mTOR inhibitors (mTORi) may cause toxicity, which limits patient compliance and long-term allograft survival. Belatacept, a specific co-stimulator blocker, offers a new option for patients with declining renal transplant function and CNI- or mTORi-related side effects.

The aim of the present study was to investigate the individual immunophenotype, especially those of T- and B-cell subsets before and after conversion to belatacept from CNI or mTORi in 20 renal transplant patients with matched control patients in a prospective manner over 6 months.

Conversion from CNI to belatacept caused decrease in T- and B- cell subsets, for instance for CD4+ cells, Tregs, CD28-CD57+ on CD4+/CD8+ central memory/naive cells, plasmablasts and memory B cells; whereas Tregs, CD4+ central/effector memory, CD8+ effector memory cells, CD28-CD57+ on CD4+/CD8+ effector cells, CD19+ cells and plasmablasts were reduced after conversion from mTORi. In contrast, an increase of CD4+ naive cells and CD8+ effector memory cells after conversion from CNI and of CD4+/CD8+ naïve cells after conversion from mTORi were observed. After conversion to belatacept, lower expression levels of Tregs, Th17 cells, CD8+ cells, CD4+ central memory cells, CD8+ effector memory cells and CD28-CD57+ on CD4+ naïve cells were found comparable to matched controls. No changes in Th1/Th2 cells were observed after conversion to belatacept. The conversion to belatacept from CNI or mTORi seemed to have a different impact on the immunophenotype of T- and B- cells after renal transplantation.

We also found that effector T (Teff) cells had less proliferation in belatacept conversion from CNI. Tregs suppressed Teff cells' proliferation more in belatacept- than CNI- treated

patients. We found decreasing CD80 expression on CD19+ cells in belatacept conversion from CNI. CD86 was expressed at a higher level in belatacept compared to matched-CNI controls.

Conclusion: these studies indicated that belatacept has a different impact on T- and B-cell subpopulations' phenotyping and functions in renal transplant patients, as compared with pre-conversion and matched- CNI or mTORi patients. The observed changes of the phenotype suggest subsequent immunosuppressive studies with different aspects. Nevertheless, further and extensive studies are required to prove an inhibition of B cell proliferation by belatacept.

ABSTRACT (Deutsch)

Neben kurzfristigem Erfolg sind die Langzeit-Ergebnisse in Bezug auf das Nieren-Transplantat-Überleben noch unbefriedigend für Patienten und Kliniker. Die zugrunde liegenden Limitationen der langfristigen Transplantatfunktion liegen in einer späten Antikörper vermittelte Abstoßung und Nebenwirkungen der Immunsuppression. Die konventionellen Immunsuppressiva wie Calcineurin-Inhibitoren (CNI) oder mTOR-Inhibitoren (mTORi) verursachen Toxizität, dies begrenzt die Patienten-Compliance und das langfristige Transplantat-Überleben. Der spezifische Co-Stimulator-Blocker Belatacept bietet eine neue Option für Patienten mit rückläufiger Transplantatfunktion und CNI- oder mTORi Nebenwirkungen.

Das Ziel dieser prospektiven Studie war es, den individuellen Immunphänotyp zu analysieren, insbesondere von T- und B-Zell-Untergruppen vor und nach der Umstellung auf Belatacept von CNI oder mTORi bei 20 Nierentransplantationspatienten mit angepassten Patientenkontrollen über 6 Monate.

Die Umwandlung von CNI zu Belatacept führte zu einer Abnahme von T- und B-Zell-Untergruppen, zum Beispiel von CD4 + -Zellen, Tregs, CD28-CD57+ auf CD4+/CD8+ Zentralgedaechtnis/naiven Zellen, Plasmablasten und Gedaechtnis B-Zellen; wohingegen Tregs, CD4+ Zentral/Effektor-Gedaechtnis, CD8+ Effektor-gedaechtniszellen, CD28-CD57+ auf CD4+/CD8+ Effektorzellen, CD19+ -Zellen und Plasmablasten nach Umwandlung von mTORi reduziert wurden. Im Gegensatzdazu wurde eine Zunahme von CD4+ naiven Zellen und CD8+ Effektor-Gedaechtniszellen nach der Umstellung von CNI und von CD4+ / CD8+ naiven Zellen nach der Umstellung von mTORi beobachtet. Nach der Umstellung auf Belatacept wurden niedrigere Expressionsniveaus von Tregs, Th17-Zellen, CD8+ Zellen, CD4+ Zentralgedaechtniszellen, CD8+ Effektor Gedaechtniszellen und CD28-CD57+ an CD4+ naiven Zellen gefunden. Es wurden keine Veränderungen von Th1/Th2-Zellen nach der Umstellung auf Belatacept beobachtet. Die Umstellung auf Belatacept von CNI oder mTORi schien nach Nierentransplantation einen unterschiedlichen Einfluss auf den

Immunphänotyp von T- und B-Zellen zu haben.

Wir fanden auch, dass Effektor-T (Teff)-Zellen weniger Proliferation nach der Belatacept-Umwandlung von CNI zeigten. Tregs unterdrückten die Proliferation von Teff-Zellen mehr in Belatacept als in CNI-behandelten Patienten. Wir fanden eine abnehmende CD80 Expression auf CD19+ Zellen in der Belatacept-Umstellung von CNI. CD86 wurde in Belatacept staerker exprimiert verglichen mit einer angepaßten CNI-Kontrolle.

Schlussfolgerung: Diese Studien zeigen, dass Belatacept einen anderen Einfluss auf den Phänotyp und die Funktion von T- und B-Zell Subpopulationen bei Nierentransplantationspatienten hat, im Vergleich zu angepaßten CNI- oder mTORi-Patienten vor Umstellung. Die beobachteten Veränderungen des Phänotyps erfordern nachträgliche immunsuppressive Studien mit unterschiedlichen Aspekten. Auch sind weitere und umfangreiche Studien erforderlich, um eine Hemmung der B-Zell Proliferation durch Belatacept nachzuweisen.

1 INTRODUCTION

1.1 Renal transplantation

Chronic kidney failure is the consequence of widespread diseases, primarily diabetes mellitus (28%), chronic glomerulitis (34%), arterial hypertension (4%) and polycystic kidney disease (4%) (1). Renal insufficiency is grouped into different stages based on patient's glomerular filtration rate (GFR) (2). Depending on the clinical course of the underlying kidney disease, in case renal function fails below 15 ml/ min/1.73 m^2, renal replacement therapies have to be considered. Besides hemo- and peritoneal dialysis, renal transplantation is the best therapy for patients with end-stage renal disease (ESRD), it can improve patient survival rate and quality of life (3-5). In general, renal transplantation can be performed either from a living or deceased donor. In Germany, living donation can be performed only from relatives or close friends of the patients, after a suitable clinical and psychological evaluation. Alternatively, as a member of Eurotransplant (ET) system, patients with ESRD can receive a renal transplant in the ETKAS (ET Kidney Allocation System) and the ESP (European senior program) from a deceased brain death patient after an appropriate allocation.

Since 2006, the total number of renal transplant candidates on the kidney transplant waiting list has increased annually. Median waiting time to kidney transplant for adult patients has increased from 2.7 years in 1998 to 4.5 years in 2009 in the United States and 3.1 years in 2005 to 3.6 years in 2015 in the EU. A total of over 14,560 patients were placed on organs waiting lists on 31 December 2015 in the European Union (for comparison: 14,928 patients were placed on waiting lists in the EU on 31 December 2014), and over 10,400 patients were put on the kidney waiting lists on 31 December 2015 (for comparison: 10,689 patients were placed on waiting lists in the EU on 31 December 2014). Mortality on ET waiting list was approximate 4% per year from 2011-2015 (6-9). The discard rate of deceased donor kidneys has also increased, and the annual number of living donor transplants has decreased. Graft survival continues to improve for both adult and pediatric recipients because of optimized immunosuppressive strategies, but long-

term graft survival is still disappointing, due to increasing donor age, immunosuppression drug toxicities, opportunistic infections or over-immunosuppression (10). Five-year graft survival was highest for living donor recipients (89%) and lowest for deceased donor recipients (68%).

1.2 Mechanism of renal allograft rejection

Immune response to an allogeneic transplant involves both innate immunity and the adaptive (or acquired) immune response (11). Whereas acute rejection episodes can be prevented and treated with steroid pulse effectively, chronic rejection is still a common problem after transplantation and the major cause of graft loss. Kidney transplant rejections are classified into T-cell-mediated (acute cellular rejection; ACR) and antibody-mediated (humoral) rejection (AMR). Evidence from several transplant centers indicates that a substantial proportion of acute and chronic renal allograft rejection is caused by antibodies to donor antigens (12-15). AMR occurs in 20-30% of acute rejection cases, has a poorer prognosis than cellular rejection, and is refractory to conventional immunosuppressive therapy (16-17). Donor-specific antibodies (DSA) require expert care with human leukocyte antigen (HLA) matching and consideration of desensitization before transplantation (18-21). After transplantation, AMR occurs because of directed complement-mediated effects of DSA targeting the renal allograft (22). Acute AMR is most commonly observed within 3 months after transplant but it can also occur at a late stage, typically in response to excessive reduction in immunosuppression or non-adherence (19, 23).

ACR involves the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response to the antigen. Mitchison (24) showed that primed lymphocytes, subsequently shown to be T cells, could adoptively transfer rejection, as had previously been shown for delayed-type hypersensitivity. The immune response from the recipient to the allograft is termed an allo-immune response, which is initiated by T-cell recognition of alloantigen. Allo-recognition is the first step of a series of complex

events that leads to T-cell activation, antibody production, and allograft rejection (25).

1.3 Immunobiology in the context of renal transplantation

1.3.1 T-cells are key players in the adaptive immune response

T cells play a crucial role in the initiation and regulation of the adaptive immune response to antigen, be it foreign or native. Naive T cells require two signals for activation. Signal 1 is antigen-presenting cells (APCs) present the antigenic peptide complex with the major histocompatibility complex (MHC) to the T-cell receptor. Signal 2 is co-stimulation which leads to amplification of the T-cell response. If the co-stimulation is blocked, the T-cell becomes anergic and eventually undergoes apoptosis (26). T-cell activation is the key process of allograft rejection. T-cells recognize alloantigen through T-cell receptors (TCR). The activation of T-cell in renal transplantation have a three-signal model, Figure 1(27, 28):

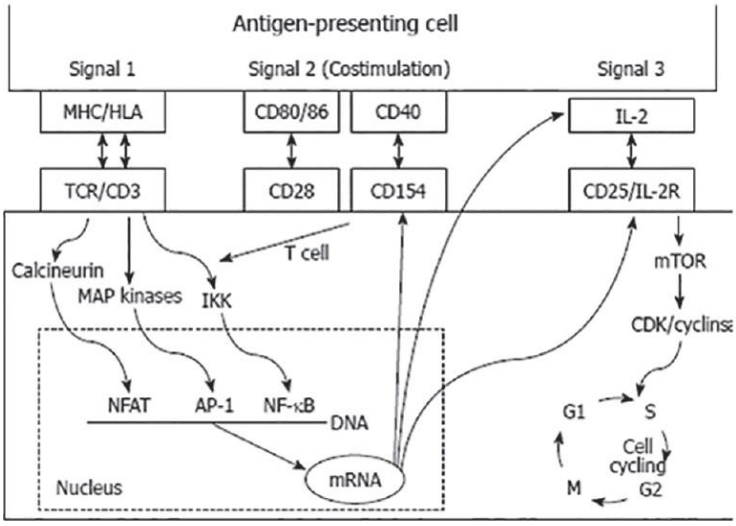


Figure 1: The three-signal model of T-cell activation. HLA, human leukocyte antigens; IL, interleukin; MHC, major histocompatibility complex; mTOR, mechanistic target of rapamycin; NFAT, nuclear factor of activated T cells; TCR, T-cell receptors. Figure taken from (29)

1.3.1.1 T-helper cells activate other immune cells

T-helper cells are a type of T cell that play an important role in the immune system, particularly in the adaptive immune system. They help the activity of other immune cells by releasing T cell cytokines, suppress or regulate immune responses and are also necessary in B cell antibody class switching, in the activation and growth of cytotoxic T cells, and in maximizing bactericidal activity of phagocytes such as macrophages. Depending on the cytokines produced and functions, CD4+ T-helper cells can be classified in subsets T-helper 1 (Th1), Th2, Th17 (30, 31). Th1 cells secrete interferon gamma (IFN-γ) and promote the cell-mediated immune response, whereas Th2 cells produce IL-4 and suppress Th1 cell-mediated response. Th17 cells produce IL-17A, IL-6, and TNF-γ involved in promoting inflammation in the pathogenesis of many diseases (32-34).

Rejection of transplanted tissues involves interplay between mechanisms that maintain tolerance to the graft and factors that accelerate rejection. While immunological factors are important for both, the process of rejection is very much an inflammatory one and, as a consequence, the production of many pro-inflammatory cytokines, such as IL-2, IL-6, IL-15 and IFN-γ, locally from infiltrating lymphocytes and resident cells, is increased during acute renal graft rejection (35–37). IL-17 protein is elevated in human renal allografts during borderline (subclinical) rejection together with detectable IL-17 mRNA in the urinary MNC sediment of these patients; in control (non-rejecting) patients, IL-17 is not detectable in either the biopsy sample or the urinary sediment (38).

1.3.1.2 Memory/Effector subset in CD4+ helper and CD8+ cytotoxic T cells

Immunological memory is a basic feature of the adaptive immune system. It enables the immune system to respond more rapidly and vigorously to infectious pathogens that have been encountered previously. A memory response differs both quantitatively and qualitatively from a primary response (39, 40). Within the overall memory T cell population, at least three distinct subpopulations have been described and can be recognized by the differential expression of chemokine receptors CCR7 and CD62L: central memory T cells,

effector memory and naive T cells (41).

Central memory T cells display a capacity for self-renewal due to high levels of phosphorylation of an important transcription factor known as STAT5 in mice (42). Central memory T cells have been shown to confer superior protection against viruses (42), bacteria (43), and cancer (44), compared with effector memory T cells. The enhanced functional properties and diversity of memory T cells discussed above suggest that memory T cells may potentially participate in early and late graft rejection by a number of different mechanisms (see Figure 2). Because effector memory T cells can recirculate in peripheral tissues, memory T cells may be rapidly recruited and initiate early responses directly at the graft site. These effector-memory T cells could immediately produce effector cytokines in situ that recruit additional immune cells for early transplantation damage (Figure 2). Alloreactive central memory T cells in lymphoid tissue may also be activated early after graft rejection and subsequently migrate to the graft site (45). Heeger and colleagues have directly demonstrated the presence of primed allo-specific memory T cells in transplant recipients using a sensitive ELISPOT assay based on cellular quantitation of effector cytokine producers in 2000 (46).

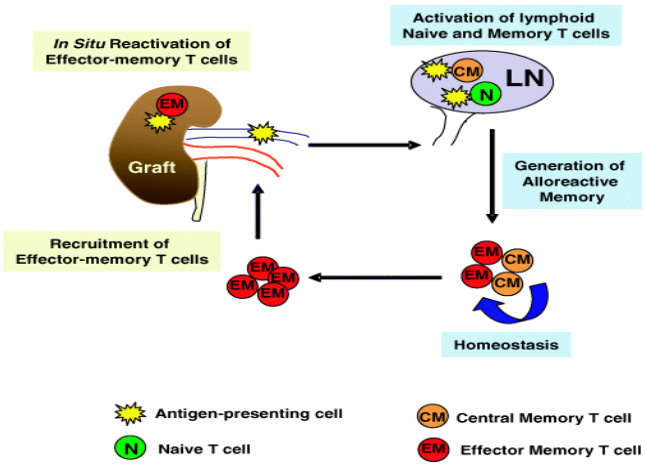


Figure 2: Schematic, showing potential roles of alloreactive memory T cells in both early and late allograft rejection. Yellow shading indicates early events and blue-shading represents later or long-term events in graft rejection. Figure taken from (45).

Memory CD8 T cells can be divided into two subsets: central and effector memory cells (47). Effector memory T cells could convert to central memory T cells in programmed differentiation within immunization. CCR7- cells display an immediate effector function and express receptors for migration to inflamed tissues. In contrast, CCR7+ cells express lymph-node homing receptors and lack an immediate effector function, but could stimulate dendritic cells efficiently and differentiate into CCR7- effector cells upon secondary stimulation (48). Wherry observed that central memory cells have a greater capacity than effector memory cells to persist in vivo, and central memory cells are more efficient in mediating protective immunity because of their proliferative potential (49). Activated CD8+ T cells are usually cytotoxic T lymphocytes responding to antigenic challenge by lysis of the target cells, while CD4+ T cells are helper cells that produce lymphokines and play a role in the activation of B cells, cytotoxic T lymphocytes and macrophages (50). Human T cells also adopt a CD28-CD57+ phenotype in chronic viral infections. This has been hypothesized to result from continuous stimulation, however this phenotype may be due to direct viral effects on T cells (51).

1.3.1.3 Regulatory T cells

Regulatory T (Treg) cells play an indispensable role in maintaining immunological unresponsiveness to self-antigens and in suppressing excessive harmful immune responses. Tregs are produced in the thymus as a functionally mature subpopulation of T cells and can also be induced from naive T cells in the periphery (52).

There are two categories of Tregs, which differ in their origin, phenotype, plasticity, mode of action and epigenetic modifications at the Foxp3 locus (53). Naturally Tregs (nTregs) develop from T-cell precursors during the normal process of T-cell maturation in the thymus and survive in the periphery poised for immuno-regulation (54). The other subset of induced Treg (iTreg) in the presence of TGF-β develops as a consequence of peripheral activation of classical naive CD4+CD25− T-cell populations under particular conditions (55). These 2 subsets work at the same time: nTreg cells are initially recruited

when iTreg cells are induced to further suppress the immune response and to achieve a fine homeostatic balance (56).

Moreover, Treg cells inhibit proliferation of effector T cell by secreting TGF-β and IL-10 to play its immunomodulatory effects (57). The imbalance between Treg and Th17 cells play a key role in inflammatory and autoimmune diseases (58–60). Treg cells can mediate donor nonreactivity in long-term immunosuppressed kidney allograft patients (61). The effects of conventional immunosuppressants on Tregs have been fairly well characterized. CNI inhibitors had less influence on Tregs by blocking IL-2 production which is most important for Treg development and survival (62). And anti-IL2R mAbs (e.g., basiliximab and daclizumab) also lead to a decrease in Treg numbers (63). MTOR inhibitors (sirolimus and everolimus) inhibit effector T cell responses by blocking IL-2 receptor signaling. Interestingly, they suppress effector T cell proliferation but spare Treg expansion, when mycophenolate mofetil seems to have little or no direct effect on Tregs (61).

1.3.2 B-cells are key players in the innate immune response

B cells are known to play a central role in humoral immunity and to boost cellular immunity, especially in acute and chronic graft rejection. Historically, the primary focus of research on B cells in transplantation has been on plasma cells and their role in antibody-mediated rejection (64). Standard immunosuppressant developed for blocking T-cell activity might also have direct or indirect effects on humoral immunity (65, 66). The inability to target B cells with standard immunosuppressive agents may result in a refractory rejection with poor graft outcome (67). Recent studies of acute rejection in pediatric (67–70) and adult (71, 72) renal transplant recipients showed that an incidence of 22% to 53% of acute rejection is associated with CD20+ B-cell clusters. An anti-CD20 monoclonal antibody (Rituximab) is specific for the CD20 molecule expressed on the surface of pre-B cells and mature B cells but not plasma cells. Rituximab has been used to successfully treat human steroid-resistant acute cardiac humoral rejection and to improve function in highly sensitized kidney transplant patients by depleting B cells and suppressing donor-specific

cytotoxic antibody production (73, 74).

1.3.2.1 Plasmablast cells

Plasmablasts are the rapidly produced and short-lived effector cells of the early antibody response. They result from T cell-independent activation of B cells or the extrafollicular response from T cell-dependent activation of B cells (75). Activated B cells participate in a two-step process that yields both short-lived plasmablasts for immediate protection and long-lived plasma cells mediators of lasting humoral immunity and memory B cells for persistent protection (76,77). Masson et al. found that CD27hiCD38hi plasmablast B-cell differentiation increased the frequency of IL-10-producing B cells in vitro (78). They also confirmed allogeneic transplant recipients had an impaired reconstitution of the memory B-cell pool. Chronic graft-versus-host disease (cGVHD) patients had less CD24hiCD27+ B cells and IL-10–producing CD24hiCD27+ B cells. Patients with cGVHD had increased plasmablast frequencies but decreased IL-10-producing plasmablasts (78).

1.3.2.2 Memory B cell and Naive B cell

Memory B-cells are activated and differentiate either into plasmablasts and plasma cells via an extra follicular response or enter a germinal center reaction where they generate plasma cells and more memory B cells (79, 80). Marc et al. assessed circulating memory B-cell frequencies against class I and II HLA antigens in highly sensitized and non-sensitized patients on the waiting list for kidney transplantation and transplanted patients. They showed that class I and II HLA-sp memory B-cell frequencies were identified in highly sensitized individuals but not in non-sensitized and healthy individuals, many years after first sensitization. Also, high donor-specific memory B-cell responses regardless of circulating DSA were clearly found both during antibody-mediated rejection and before transplantation. The higher the donor-specific memory B-cell response, the more aggressive the allograft rejection (81). Thus, assessing donor-specific memory B-cell frequencies may be relevant to patient risk of alloimmune stratification, and also provides

new insight into the mechanisms of the adaptive humoral alloimmune response which are taking place in kidney transplantation.

1.4 Immunosuppression

A life-long immunosuppressive therapy is required to avoid rejection processes leading to graft failure. Immunosuppression can be achieved by depleting lymphocytes, diverting lymphocyte traffic, or blocking lymphocyte response pathways. Currently available immunosuppressive agents can be classified into three categories: induction agents, maintenance therapy, and treatment for rejection. The objective of immunosuppression in kidney transplantation is to prevent acute or chronic rejection and to preserve renal function. On the other hand, side effects of current immunosuppressive regimen can lead to over-immunosuppression and cardiovascular events or malignancy that result in reduced patient and graft survival (82). Thus, minimization of given immunosuppression is the key principle to prevent side effects over the long-term course of transplantation. Current immunosuppressive regimens provide excellent one-year graft and patient survival rates; whereas five-year survival rates among recipients of kidneys from cadaveric donors and living related donors are only 66% and 79%, respectively (83, 84).

The most renal transplant recipients commonly used calcineurin inhibitors (CNI), like cyclosporin A (CsA) and tacrolimus, and mammalian target of rapamycin inhibitors (mTORi), like sirolimus and everolimus, anti-proliferative agents, like azathioprine and mycophenolic acid, and corticosteroids (85, 86), etc. all effective for immunosuppression. The introduction of cyclosporine, tacrolimus, and mycophenolate mofetil (MMF) reduced rates of acute rejection and improved short-term and midterm graft survival.

1.4.1 Calcineurin Inhibitors (CNI)

Cyclosporin binds to the cytosolic protein cyclophilin (immunophilin) of lymphocytes, especially T cells. This complex of cyclosporin and cyclophilin inhibits calcineurin, which,

under normal circumstances, is responsible for activating the transcription of interleukin 2 (IL-2). In T-cells, activation of the T-cell receptor normally increases intracellular calcium, which acts via calmodulin to activate calcineurin. Then calcineurin dephosphorylates the transcription nuclear factor of activated T-cells, which moves to the nucleus of the T-cell and increases the activity of genes coding for IL-2 and related cytokines. CsA prevents the dephosphorylation of NF-AT by binding to cyclophilin (87). It also inhibits lymphokine production and interleukin release, which therefore leads to a reduced function of effector T-cells. Cyclosporine and tacrolimus based on the calcium-dependent serine/threonine phosphatase calcineurin, and the activity of the NFAT are decreased, which is essential for the signal cascade leading to allograft rejection (88). These two drugs showed a significant reduction in acute rejection and improvement in 1-year allograft survival (89).

Although both cyclosporine and tacrolimus are effective on the rejection, the use of CNI can cause severe side effects. CNI are nephrotoxic (90) and have adverse effects on blood pressure (91), lipid levels (92), and glucose homeostasis (91, 93). These side effects increase risks of developing diabetes, cardiovascular disease, cancer and chronic kidney transplant scarring.

1.4.2 Mammalian target of rapamycin inhibitors (mTORi)

In 1991, the mammalian target of rapamycin (mTOR) pathway and its importance in intracellular signaling were revealed. They inhibit proliferation of activated T cell via blocking its progression of the cell cycle from G1- to S-phase and proliferative responses induced by several cytokines, including interleukin 1 (IL-1), IL-2, IL-3, IL-4, IL-6, IGF, PDGF, and colony-stimulating factors (94). MTOR has two distinct multiprotein complexes, mTORC1 and mTORC2. MTORC1 is a complex of mTOR, Raptor and mLST8/GβL, which mediates interactions between mTOR and Raptor and is essential for mTORC1 activity and regulation. MTORC2 contains mTOR, GβL and mSIN1. By activation of AKT, mTORC2 seems to be an important regulator of the cytoskeleton. Activated mTORC1 phosphorylates two key enzymes of protein translation: 4E-BP1 and p70S6K1 (protein70-

S6-kinase-1), which control mRNA translation for protein biosynthesis and are responsible for growth, differentiation and proliferation as well as autophagy within the cell-cycle (95,96)

Sirolimus and everolimus are strongly antiproliferative immunosuppressive drugs approved for the prevention of kidney allograft rejection (97). Sirolimus has a high binding capacity to human plasma proteins and, in plasma, is mainly associated with serum albumin (97%). This capacity leading to impaired erythroid cell proliferation and anemia together with an erythrocyte microcytosis is observed frequently in sirolimus- treated patients (98). MTORi lack the high incidence of nephrotoxicity caused by CNI, but may lead to leukopenia, thrombocytopenia, hypercholesterolemia, proteinuria, stomatitis and diarrhea, and have also increased the risk of developing DSA (98, 99).

1.4.3 Mycophenolate mofetil (MMF)

Mycophenolate mofetil (MMF) is a prodrug of mycophenolic acid (MPA), an inhibitor of inosine-5'-monophosphate dehydrogenase, has several immunosuppressive actions. The development of MMF was the first application of human genetics to define a therapeutic target (100). This strategy was novel in 1981 but is now widely used. MPA inhibits the proliferation of human T and B lymphocytes, dGTP depletion is the most important mechanism by MPA suppresses DNA synthesis and proliferation of T lymphocytes. Unlike CNI, MPA does not inhibit the production of IL-2 or the expression of the IL-2 receptor, and IL-2-dependent T lymphocyte apoptosis also (101, 102), MPA specifically mediates with cytokine-dependent signals that block activated T lymphocytes at the early–to mid–G1 phase of the cell cycle (102). Based on this, MMF can induce the apoptosis and elimination of T lymphocytes responding to antigenic stimulation under some conditions. In addition, MPA also suppressed the proliferation of human B lymphocytes and immunoglobulin (Ig) production and even ongoing IgG responses (101,103). MMF treatment also decreased primary and secondary humoral responses to keyhole limpet hemocyanin, tetanus toxoid (104), and cytomegalovirus (CMV) (105).

MMF significantly decreases acute rejection rates after renal transplantation, but intolerance often occurs, like hematologic abnormalities and gastrointestinal (106-108). In phase III studies, between 12.7% and 37.3% of MMF-treated patients experienced diarrhea (106-108), and leukopenia was significantly more common, occurring in 10.9% to 35% in the MMF group (106, 107). These side effects often require MMF dose reduction or even discontinuation (108, 109).

1.4.4 Co-stimulation blocking via Belatacept

After more than one decade without new approved immunosuppressive compounds in the field of transplantation, the recombinant fusion protein belatacept, a highly specific blocker of the co-stimulatory signal, was approved in 2011. Because of its non-renal toxicities, belatacept provides a benefit in preserving renal function by avoiding calcineurin inhibitors and making for a better cardiovascular risk profile (110).

Belatacept, cytotoxic T lymphocyte-associated protein 4 (CTLA-4)-Ig (Nulojix®) is the first clinically relevant co-stimulation blocker, and is a high-affinity chimeric fusion protein that binds to CD80/CD86 on (antigen-presenting cells) APC (111). One of the best-characterized costimulatory reactions is between CD28/CTLA-4 on T cells and CD80/CD86 on APC. The interaction between CD28 and CD80/CD86 leads to T-cell activation (112). In general, CTLA-4 is a structural homolog of CD28, which binds to CD80/CD86 with higher avidity, and is a negative regulator of T cells. The receptor fusion protein belatacept (CTLA-4-Ig) comprising the extracellular-binding domain of CTLA-4 linked to the modified Fc domain of human immunoglobulin IgG1 inhibits costimulation and T-cell activation by binding to CD80/CD86 (113). Abatacept (approved for rheumatic arthritis) is the first-generation molecule, and belatacept is the second-generation molecule. The key alteration in contrast to abatacept, belatacept binds CD80 twice as well and CD86 four times as well, and provides 10-fold more potent T-cell inhibition (114).

The first clinical trial with belatacept in renal transplantation was designed with a regimen that supplied efficacy in a nonhuman primate model (115). The phase II multicenter

clinical trial compared the safety and efficacy of two dosing regimens (more or less intensive) of belatacept to CsA. At 6 months, the incidence of acute rejection was similar among the groups. The measured GFR (mGFR) at 12 months was significantly higher among patients who receiving the more intensive and less intensive belatacept compared with those receiving CsA (66.3, 62.1 and 53.5 ml/min per 1.73 m^2, respectively). Lipid levels and blood-pressure values were similar or slightly lower in the belatacept groups (6). Two phase III trials were subsequently undertaken and 7 years of data have been published for both trials. The first trial, belatacept evaluation of Nephro-protection and Efficacy as First-line Immunosuppression Trial (BENEFIT), is a 3-year randomized, active-controlled, parallel- group multicenter trial. At first year, the graft survival rates were 95%, 97%, and 93% with a mean mGFR of 65, 63.4 and 50.5 ml/min/1.73m^2 in the more intensive, less intensive, and CsA groups, respectively, with $p<0.0001$ for both more-intensive and less-intensive groups versus CsA. It continued by the end of third-year, the mGFR was higher in the belatacept-treated, 65.2±26.3 (more intensive) and 65.8±27.0 (less intensive) than CsA-treated group, 44.4±23.6 ml/173 m^2 (83). Belatacept is an effective maintenance immunosuppressive agent and is extraordinary for having no nephrotoxicity compared to CsA (116, 117). It is approved for use in kidney transplant recipients for rejection prophylaxis and is to be used in EBV seropositive individuals in order to reduce the risk of post-transplant lymphoproliferative disease (PTLD). As so, we designed this study that transferred patients to belatacept from conventional immunosuppressive therapy to evaluate the immunophenotyped.

2 AIMS AND OBJECTIVES

The main objective of this study was the identification of the different immunophenotypes of peripheral lymphocyte subsets in renal adult transplant patients after conversion from maintenance immunosuppressive therapy with CNI (either Ciclosporin A or Tacrolimus) or mTORi (either Everolimus or Sirolimus) to belatacept over a time period of 6 months in a prospective manner. Adequate matched-CNI or -mTORi control patients were investigated in this context.

1. To investigate the impact of T cells expression level (like regulatory T cells, Th1/2/17 T cells, effector/memory T subpopulations) after CNI or mTORi conversion to belatacept from CNI or mTORi treatment compared to pre-conversion or matched controls.

2. To identify and investigate the impact of B cells (like memory B cells and plasma blasts) after CNI or mTORi conversion to belatacept compared to pre-conversion or matched controls.

3. To study the impact of regulatory and effector T cells function after CNI or mTORi conversion to belatacept compared to pre-conversion or matched controls,

4. To monitor CD80/86 expression on CD19+ cells after CNI or mTORi conversion to belatacept compared to pre-conversion or matched controls.

5. To study CD19+ cells function after CNI or mTORi conversion to belatacept compared to pre-conversion or matched controls.

3 MATERIALS and METHODS

3.1 Materials

3.1.1 Reagents, medium and solutions

3.1.1.1 Chemicals

Ficoll-Paque™ PLUS	GE Healhcare
MACS Separation Buffer	Miltenyi Biotec
Bovine serum Albumin	Sigma
Phosphate Buffered Saline without calcium/magnesium	Gibco
Cell Trace Violet(CTV)	Life Technology
Dulbecco's Phosphate Buffered Saline (DPBS)	Gibco
Dimethylsulfoxid (DMSO)	Life Technology
GolgiPlug (Brefeldin A + Dimethylsulfoxid)	Becton Dickinson
Propidium-Iodid (PI), 1g/l	Sigma
Methanol	J.T. Baker
Ethanol	J.T. Baker
Nuclease-Free Water	Ambion
FACS Lysing Solution	BD Biosciences

3.1.1.2 Culture medium

RPMI 1640 Medium	Biochrom AG
L-Glutamin	Biochrom AG
Penicillin	Biochrom AG
Streptomycin	Biochrom AG
Fetal Bovine Serum	Biochrom AG

3.1.1.3 Solutions

RPMI-1640

RPMI-1640 culture medium worked with 2mM L-glutamine, 100U/ml penicillin and

100mg/ml streptomycin, and 10% FBS. RPMI-1640 were used for Treg suppression assay.

DMEM

DMEM culture medium worked with 100U/ml penicillin and 100mg/ml streptomycin, and 10% FBS. DMEM were used for B cell culturing.

Lysis Buffer

There are two steps for preparing the lysis buffer for ConA stimulated assays. First, pre-buffer consists of 8.29g NH_4Cl and 0.0372g Na_2-EDTA diluted in distilled water 1000ml; 10g $KHCO_3$ dissolved in 100ml distilled H_2O (100g/l$KHCO_3$) as stock solution at room temperature. Take 1ml 100g/l $KHCO_3$ stock solution into 100ml the pre-buffer before using the lysis buffer in 6 hours. The final working concentration is 8.29g/l NH_4Cl, 37.2mg/l Na_2-EDTA, 1g/l $KHCO_3$.

Permeabilizing Buffer

Dissolve 20mg saponin in 15ml PBS, and then add 0.2ml heat- inactivated FCS in solutions, filled up to 20ml. Aliquote the permeabilizing buffer in 0.33ml and store at -20℃.

Formalin solutions

0.5% (v/v) formalin are accordingly 497,5ml PBS with 2.5ml;

1% (v/v) Formalin are given 5ml formalin in PBS 495ml;

Formalin solutions are stored in the refrigerator at 4℃.

Propidium iodide solution

1% PI solution is used for staining, take PI stock solution(1mg/ml) ratio 1:100 to PBS. This solution is protected from light in a refrigerator at 4℃.

RNase A

20mg RNAse A is dissolved in a reaction vessel with 0.2ml of nuclease-free water. This solution is heated to 99℃ for 10 minutes to possibly contained DNase to inactivate. After cooling to room temperature 1.8ml PBS are added. The solution is aliquot 0.05ml stored at -20℃.

Cell trace violet(CTV)

Prepare a 2.5 mM CellTrace™ Violet stock solution by dissolving the contents of 1 vial of CellTrace™ Violet Reagent into 40 µl of DMSO. The CTV was aliquot with 1 µl into 1.5ml Eppendorf tubes stored at -20℃. The final working concentration was at 5 µM.

3.1.2 Kit

CD4+CD25+ Regulatory T Cell Isolation Kit, human	Miltenyi Biotec
CD19+ B Cell Isolation Kit, human	Miltenyi Biotec
Treg Suppression Inspector, human	Miltenyi Biotec
BD Multi-test™ 6-color TBNK	BD Biosciences

3.1.3 Antibodies

V450-conjugated mouse antibody against human CD3	BD Biosciences
PerCP-Cy5.5-conjugated mouse anti-human CD4	BD Biosciences
V500-conjugated mouse antibody against human HLA-DR	BD Biosciences
APC-H7-conjugated mouse antibody against human CD8	BD Biosciences
PE-Cy7-conjugated mouse antibody against human CD45RA	BD Biosciences
PE-conjugated mouse antibody against human CCR7	BD Biosciences
APC-conjugated mouse antibody against human CD28	BD Biosciences
APC-conjugated mouse antibody against human CD38	BD Biosciences
FITC-conjugated mouse antibody against human CD57	BD Biosciences
PE-conjugated mouse antibody against human CD25	BD Biosciences
APC-H7-conjugated mouse antibody against human CD45RO	Biolegend
APC-conjugated mouse antibody against human CD127	eBioscience
Alexa Fluor 405-conjugated mouse antibody against human CCR4	eBioscience
PE-Cy7-conjugated mouse antibody against human CCR6	BD Biosciences
FITC-conjugated mouse antibody against human CD38	BD Biosciences
Alexa Fluor 405-conjugated mouse antibody against human CCR4	BD Biosciences

PE-conjugated mouse antibody against human CXCR3	BD Biosciences
APC-conjugated mouse antibody against human CD27	BD Biosciences
PE-conjugated mouse antibody against human IgD	BD Biosciences
PE-Cy7-conjugated mouse antibody against human CD19	Biolegend
PerCP-conjugated mouse antibody against human CD20	BD Biosciences
APC-Cy7-conjugated mouse antibody against human HLA-DR	Biolegend
FITC-conjugated mouse antibody against human PCNA	BD Biosciences

3.1.4 Device

The QuadroMACS™ Separator	Miltenyi Biotec
The MiniMACS™ Separator	Miltenyi Biotec
Pre-Separation Filter, 30 μM	Miltenyi Biotec
MACS Separation LD Columns	Miltenyi Biotec
MACS Separation MS Columns	Miltenyi Biotec
MACS Separation LS Columns	Miltenyi Biotec
Vacutainer LH170 I.U. 10ml	Becton Dickinso
Vacutainer SST II Advance, 6ml	Becton Dickinso
Vacutainer K2E (EDTA), 6ml	Becton Dickinso
Falcon Tube, 15ml/50ml	Falcon
Serological Pipette, 5ml/10ml/25ml	Falcon
Transfer Pipette, 3.5ml	Sarstedt
Pipette tips, 10 μl/200 μl/1ml	Sarstedt
Pipette tips, 5ml	Eppendorf
Ficoll-Paque tubes	Becton Dickinson
Reaction vessels 3810X 1.5ml tubes	Eppendorf
Cleanroom work bench, Laminair 2000	Heraeus Instruments
Benchtop Centrifuge, 5451C Bio-fuge fresco	Heraeus Instruments

Centrifuge Megafuge 2.0R	Thermo Scientific
Microscope DMIL	Leica
Incubator, Cell House 200	Heraeus Instruments
Water, Master Shake	Heraeus Instruments
FACS Canto II with 405nm, 488nm, 632nm argon laser	Becton Dickinson
Electrical pipetting, Accu-Jet	Sigma
Pipette, 2.5 µl/10 µl/100ml/200ml/1ml	Eppendorf
Multi-pipette	Eppendorf
Vortex Mixer	Scientific Industries

3.1.5 Software

FlowJo 7.6.1	FLOWJO LCC
BD FACS Diva 8	Becton Dickinson
office software word/excel/access	Microsoft
SPSS for Windows, Version 19	IBM
Transplantation date bank, "T-Base"	Charite

3.2 Isolation of Peripheral blood mononuclear cells (PBMC)

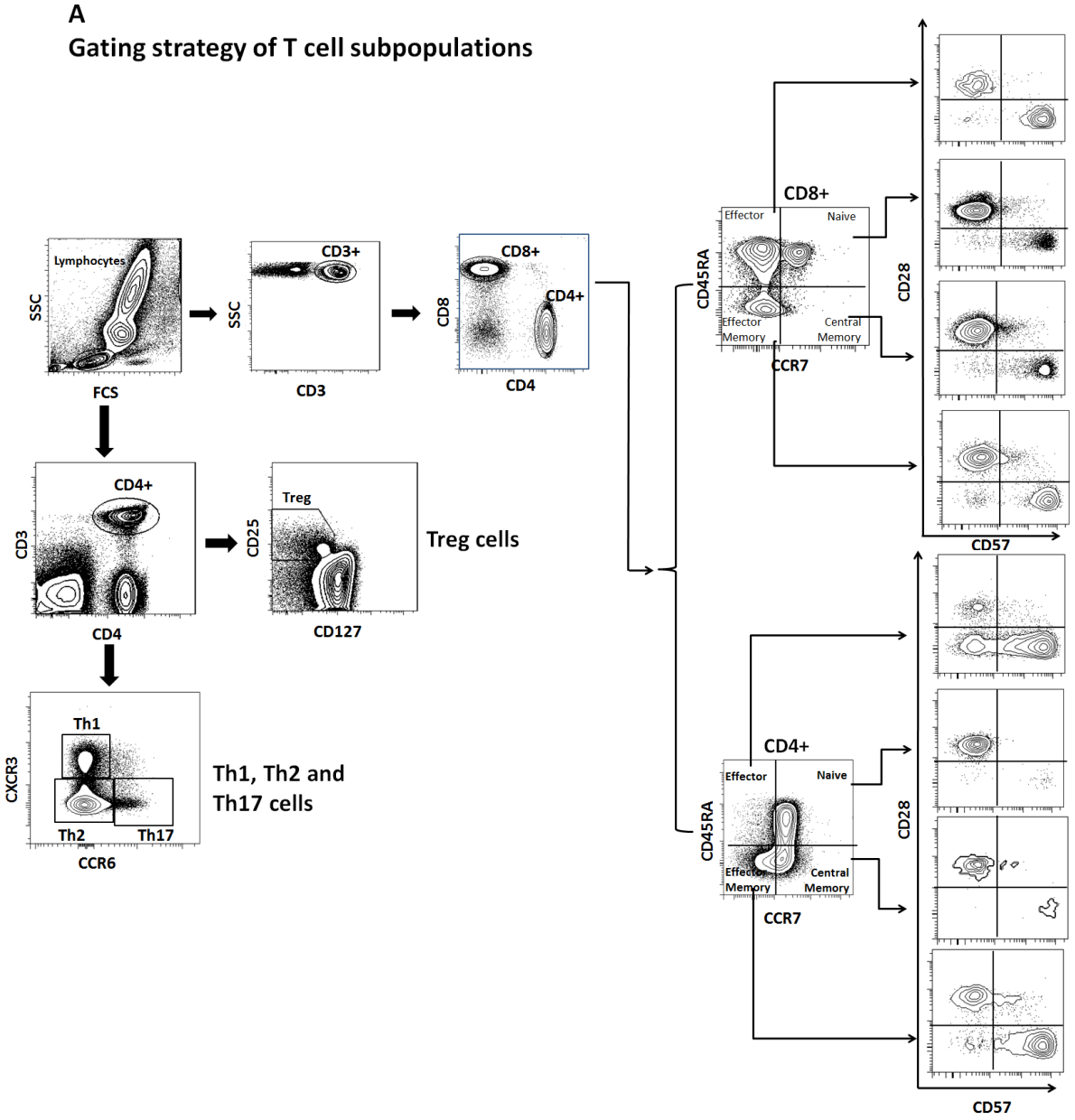
50ml peripheral blood were drawn into Li-Heparin (17IU/ml) tubes from each patient. PBMC were isolated by Ficoll-Paque™ Plus by density-gradient centrifugation at 1000g at 22°C for 20 min. The ring of PBMCs was collected into 15 ml Falcon-tubes, then filled to 13-14 ml with PBS and centrifuged again at 1300 rpm for 10 min. The supernatant was aspirated and the pellet was collected into one falcon-tube. The tube was then filled to 14 ml with cold PBS, the cells were counted and centrifuged at 1000 rpm for 10 min. After the removal of the supernatant, FACS buffer was added for biomarker analysis or MACS buffer for magnetic separation.

3.3 Biomarkers staining and Flow cytometric analysis

PBMCs were isolated immediately after blood draw. 50 µl FACS buffer and 2.5 µl antibody were added and stained for 30 min at 2-8°C. After that, 1mlFACS buffer was added and centrifuged at 1200 rpm for 5 min. The supernatant was aspirated and 150 µl - 300 µl FACS buffer was added: The measurement was performed on the Calibur or CANTO II FACS machine.

3.4 Gating strategy of T subpopulations and B cell subpopulations

The major subsets of T cells were defined by the expression of CD3, CD4 and CD8. For distinguishing naive, central memory, effector memory and effector CD4+ and CD8+ T cells, we stained the PBMCs with the following antibodies: CD3, CD4, CD8, CD45RA and CCR7, human T cells adopt a $CD28^{-}CD57^{+}$ phenotype in chronic viral infections, so, we added CD28 and CD57 in the tube also. Regulatory T(Treg) cells were defined by CD3, CD4, CCR4, CD25 and CD127, CD45RO added for defined memory and naive Treg cells. A panel containing CD3, CD4, CXCR3 and CCR6 were applied for T helper (TH) 1, 2, 17 cells. With the addition of activation markers, such as CD38 and HLA-DR, were used to defined activated subsets of each of these cell types as well. B cells staining were done with the following antibodies: CD3, CD19 and CD20 (to define B cells), CD38 (to identify plasmablasts), CD27 and IgD (for naive and memory B cell populations), HLA-DR as activation markers. Flow cytometry analysis was performed using a FACS Canto II flow cytometer (Becton Dickinson, CA) and analyzed using BD FACSDIVA 8 and Flowjo software. (Figure 3)


A
Gating strategy of T cell subpopulations
Lymphocytes
SSC
FCS
CD3+
SSC
CD3
CD8+
CD4+
CD8
CD4
CD4+
CD3
CD4
Treg
CD25
CD127
Treg cells
Th1
CXCR3
Th2
Th17
CCR6
Th1, Th2 and
Th17 cells
CD8+
Effector
Naive
Effector
Memory
Central
Memory
CD45RA
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CD57
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CD45RA
CCR7
CD28
CD57

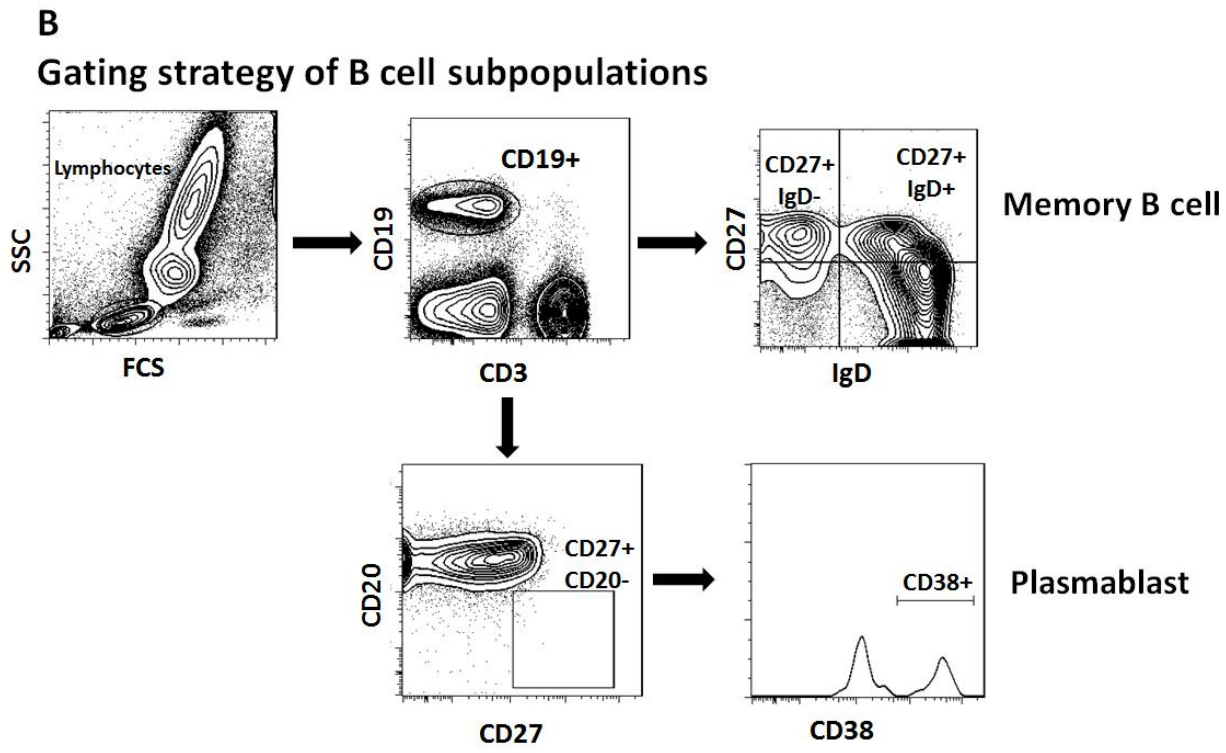


Figure 3: Gating strategy for cell subpopulations. (A) CXCR3+CCR6- Th1 cells, CXCR3-CCR6- Th2 and CXCR3+CCR6+ Th17 cells, CD4+CD25+CD127low Treg cells, CCR7+CD45RA+ Naive cells, CCR7+CD45RA- Central memory cells, CCR7-CD45RA+ Effector cells, CCR7-CD45RA- Effector memory cells and CD28-CD57+ cells in different CD4+ / CD8+ subpopulations; (B) CD19+ B cells, CD19+CD27+ memory B cells and CD27+CD20-CD38+ Plasmablasts.

3.5 Cell assays

Regulatory T cells (CD4+CD25+), effector T cells (CD4+CD25-) and CD19+ cells were purified with MACS magnetic cell sorter (Miltenyi, Germany) to ensure the cells viability.

3.5.1 Cell separation of Treg and effector T cells

(1), in-directed magnetic labelling of non-CD4+ cells with CD4+ T Cell Biotin-Antibody Cocktail and Anti-Biotin MicroBeads was performed with PBMCs. The cells were incubated with 10 µl cocktail per 10^7 total cells for 10 minutes at 2−8°C, then 20 µl anti-Biotin Micro-Beads were added per 10^7 cells and incubated for an additional 15 minutes at 2−8°C.

(2), magnetic separation with LD Columns was performed to deplete non-CD4+ cells. Magnet and columns were prepared and columns rinsed with 2 ml of buffer. The cells were washed with 4 ml cold MACS buffer and centrifuged at 1200 rpm for 5 min. The supernatant was aspirated and the volume was adjusted to 500 µl with MACS buffer. The cell suspension was poured into the column and unlabelled cells were collected. The cells passed through were Pre-enriched $CD4^+$ cells. The column was washed with 3×1 ml of MACS buffer. The total effluent was collected.

(3), positive selection of CD4+CD25+ T cells with direct magnetic labelling of $CD25^+$ T cells with CD25 Micro-Beads was performed. The cell suspension from the second step was centrifuged at 1200 rpm for 8 minutes at 4°C. The supernatant was aspirated completely. The pre-enriched CD4+ cell pellet was re-suspended in 60 µl MACS buffer per 10^7 total cells and 10 µl CD25 MicroBeads per 10^7total cells were added. An incubation step of 15 minutes in the dark at 2−8°C followed.

(4), pre-enriched CD4+ cells were washed by adding 2 ml buffer and centrifuged at 1200 rpm for 5 minutes at 10°C. The supernatant was aspirated and the pellet re-suspended in 500 µl MACS buffer. Then magnetic separation was performed by using 2 stacked MS Columns. After rinsing, the cell suspension was poured into the column and the eluted Teff cell fraction was collected for co-culture. The column was washed with 4×500 µl of buffer. The 1^{st} column was flushed with buffer and the magnetically labelled Treg cells were collected. The last step was repeated for the 2^{nd} column. Afterwards, the cell suspension was centrifuged at 1200 rpm for 8 min, and the pellet re-suspended in 1 ml PBS.

3.5.2 Treg suppression assay

Preparation of Treg Suppression Inspector (Beads) was performed by re-suspending 30 µl beads for 1 patient thoroughly in 1 ml culture medium. After centrifugation at 1200 rpm for 6 minutes, the supernatant was aspirated completely. The beads were re-suspended in suitable culture medium with a concentration of $1×10^7$ MACSiBead particles per ml and

a bead-to–cell ratio of 1:1.

For the Treg suppression assay, Treg cells and effector T (Teff) cells were isolated by magnetic separation. Cells were counted and re-suspended in 1640 culture-medium with 2×10^5 per ml. Treg cells as inhibitors were co-cultured with Teff cells responding to Treg suppression inspector (bead-to-cell ratio 1:1) stimulation for 4 days with CTV (Cells Trace Violet) labelling in a 96-well round bottom plate. Detailed cell number, medium volume, suppression inspector beads and CTV labelling are depicted in Table 2.

Ratio Treg:Teff	Treg cells/ Volume (µl)	Teff cells/ Volume (µl)	MACSiBead /volume (µl)	CTV-labeling	Cultured medium (µl)
0:1	-	2×10^4/100	2×10^4/2 µl	Teff	152
1:0	2×10^4/100	-	2×10^4/2 µl	Treg	152
1:1	2×10^4/100	2×10^4/100	4×10^4/4 µl	Teff	50
1:2	1×10^4/50	2×10^4/100	3×10^4/3 µl	Teff	101
0:1	-	2×10^4/100	-	Teff	154
1:0	2×10^4/100	-	-	Treg	154

Table 2: Number of Treg, Teff cells, Treg suppression inspector (MACSiBead) and cultured medium volume and CTV labelling per well.

Cells were incubated at 37°C in a 5% CO_2 atmosphere for 4 days. After 4 days, 100 µl supernatant was collected into 1.5ml Eppendorf tubes. The harvested cells were transferred into FACS tubes and washed 3× with PBS by centrifugation at 1200 rpm for 5 min. 50 µl FACS buffer was added followed by measurement with the FACS machine named CANTO II on excitation/emission in 405/450nm.

3.5.3 CTV labelling with final working concentration at 5 µM

We used CTV-labelled Teff cells, Treg cells and CD19+ B cells to trace the cell division cycle, the CTV is evenly distributed to their two daughter cells. Therefore, each peak in the histogram of flow cytometry represents cells from one division cycle. 500 µl PBS at room temperature was added to the 1.5ml Eppendorf tube which held 1 µl CTV. Teff cells, Treg cells and CD19+ B cells were re-suspended with the 0.5ml CTV solution for a final

working concentration of 5 µM. Cells were stained for 25 min at 37°C. After incubation, 3ml cold culture medium was added to stop the reaction and incubated for 5 min on ice. Centrifugation was performed at 1200 rpm for 5 minutes and the supernatant was aspirated. The pellet was washed with 2.5 ml culture medium, and CTV-Teff cells, CTV-Treg cells and CD19+ B cells were counted. After centrifugation, culture medium was added for a final concentration of 2×10^5 cells per ml for Treg suppression assay and B cell proliferation assay.

3.5.4 Cell separation of CD19+ B cells

CD19+ B cells were isolated by Magnetic separation with the CD19+ beads kit. Positive selection of CD19+ B cells with direct magnetic labelling of CD19+ micro-Beads was performed. PBMCs were centrifuged and a total of 10^7 total cells was suspended in 60 µl per MACS buffer. 20 µl CD19 MicroBeads per 10^7total cells was added, mixed well and incubated for 15 minutes in the dark at 2−8°C. Cells were washed by adding 2 ml buffer and centrifuged at 1200 rpm for 5 minutes at 4°C. After supernatant aspiration, up to 10^8 cells were re-suspended in 500 µl of MACS buffer. Then, magnetic separation by using one LS Column was performed. The elution fraction was collected and the magnetically labelled CD19+ B cells were flushed. The cell suspension was centrifuged at 1200 rpm for 8 min and the re-suspended pellet was used for functional characterization.

3.5.5 CD80 and CD86 expression on CD19+ B cells

CD19+ cells were isolated by Magnetic separation and then seeded at 1.5×10^5 / well in a 96-wells flat plate. The cells were stimulated with 2.5 µM CpG-ODN, 1 µg /ml anti-CD40 and 10 ng / ml IL-4 at 37°C with 5% CO2 for 3 days. After 3 days, cells were harvested into FACS tubes and washed 3×with PBS by centrifuging at 1200 rpm for 5 min. 50 µl FACS buffer was added and cells were stained with 2.5 µl antibody CD80 Pe-Cy7, CD86 APC and CD19 V450 for 20 min at 4°C. They were washed again with 1 ml FACS buffer and centrifuged at 1200 rpm for 5 min, the supernatant was aspirated. After adding 50 µl

buffer, measurement was performed with a CANTO II FACS machine.

3.5.6 B cell proliferation

After obtaining CD19+ cells by Magnetic separation, staining with CTV labeling as described above to trace CD19+ cells division cells at 1.5×10^5 /well in a 96-well flat plate were stimulated with 2.5 μM CpG-ODN, 1 μg /ml anti-CD40 and 10ng / ml IL-4 at 37°C with 5% CO2 for 7days.After 7 days, 100 μl supernatant was collected into 1.5ml Eppendorf-tubes. Cells were washed 3×with PBS and centrifuged at 1200 rpm for 5 min. 50 μl FACS buffer was added for staining with 2.5 μl antibody CD19 Pe-Cy7 for 20 min at 4°C. After washing again with 1 ml FACS-buffer by centrifugation at 1200 rpm for 5 min, all the supernatant was aspirated. 50 μl buffer was added and flow cytometry was performed with the FACS machine named CANTO II on excitation/emission in 405/450 nm.

3.6 Statistical analysis

Data were analyzed using the statistical software SPSS (SPSS version 19, IBM). Wilcoxon signed-rank test was used to compare continuous variables. $P<0.05$ was considered significant.

4 RESULTS

4.1 Assigning Subjects to Treatment

20 patients were enrolled in the study and converted either from CNI (n=10) or mTORi (n=10) to belatacept. All patients were converted for clinical reasons in the context of CNI- or mTORi-related toxicity or intolerance with a clinical indication of conversion to CNI- and mTORi-free therapy with belatacept. Conversion from either CNI or mTORi to belatacept was performed in a stepwise manner over a 4-week period. Patients received belatacept 5 mg/kg on baseline (day 0), week 2 (day 14), week 4 (day 28), week 6 (day 42), and week 8 (day 56), and then every 4 weeks thereafter until completion of the trial. In addition, immunosuppressive co-medication of steroids and Mycophenolate was continued unchanged in all study patients.

One matched control patient was identified and investigated for each renal transplant patient who was converted to belatacept. Control patients were matched by identical baseline immunosuppression, age (+/- 10 years), gender, renal function (+/- 1.5mg/dl creatinine) and time post-transplant (+/- 10 years) (Table 1). Controls were investigated at 3 time points over a 6-month period with a careful documentation of clinical follow-up. The biomarkers were measured at baseline (BL), month 1 (M1), month 3 (M3) and month 6 (M6) and cell function assays were performed at BL, M3 and M6.

	CNI conversion [belatacept(CNI)] n=10	CNI control n=10	p value	mTORi conversion [belatacept(mTORi)] n=10	mTORi control n=10	p value
Male	90%	90%	1	50%	50%	1
Age at conversion (years)	53.83±13.14	50.79±12.84	0.248	55.49±13.88	57.58±14.06	0.508
Time after transplant(years)	6.53±5.75	7.82±5.07	0.248	10.15±4.06	10.33±4.69	0.959
Creatinine (mg/dL)	2.68±0.75	1.99±0.35	0.007	1.68±0.48	1.11±0.29	0.009
Proteinuria (mg/L)	155.5±104.6	193.0±180.1	0.859	268.9±280.2	118.2±57.7	0.139
Reason for conversion, n						
CNI-induced toxicity	8	N/A		5 (history)	N/A	
DSA positvity	2			2		
Proteneuria				2		
underlying disease, n						
Chronic Glomerulonephritis	3	5		2	3	
Diabetes-Adult type	2	0		1	0	
Polycystic	3	1		2	1	
Hypertensive Nephropathy	0	1		0	0	
Hemolytic Uremic Syndrome	0	0		1	0	
Pyelonephritis	0	0		1	0	
IgA Nephropathy	0	1		1	0	
Reflux Nephropathy	0	0		1	1	
Alport Syndrome	0	1		0	0	
Interstitial Nephritis	0	0		0	1	
Immune Complex Nephritis	0	0		0	1	
Other	1	0		1	3	

Table 1: Patient characteristics of renal transplantation before conversion to belatacept showed as mean ± standard deviation.

4.2 T cell subpopulation and Function assay

4.2.1 CD4+ populations in CNI or mTORi conversion belatacept and matched control groups

We analysed CD4+ T cell frequency and absolute numbers by TBNK Kit (BD Bioscience). We found no significant changes in CD4+ T cell frequency after CNI (Figure 4A) or mTORi (Figure 4B) conversion to belatacept compared to BL at M1 (CNI: p=0.799, mTORi: p=0.646), M3 (CNI: p=0.477, mTORi: p=0.859), M6 (CNI: p=0.333, mTORi: p=0.678) and compared to the matched CNI or mTORi control group.

As shown in Figure 4C, absolute numbers of CD4+ T cells decreased after conversion to belatacept from CNI at M1 (Mean± standard deviation: 577.42μl ±260.61, p=0.050) compared to BL (740.11μl ± 319.87). No differences were observed after conversion to belatacept at M3 (p=0.314) and M6 (p=0.859) comparing to pre-conversion or matched CNI control. The CD4+ absolute numbers were not significantly after conversion to belatacept from mTORi-treated (Figure 4D) at M1, 3, 6 compared to pre-conversion and the matched mTORi controls.

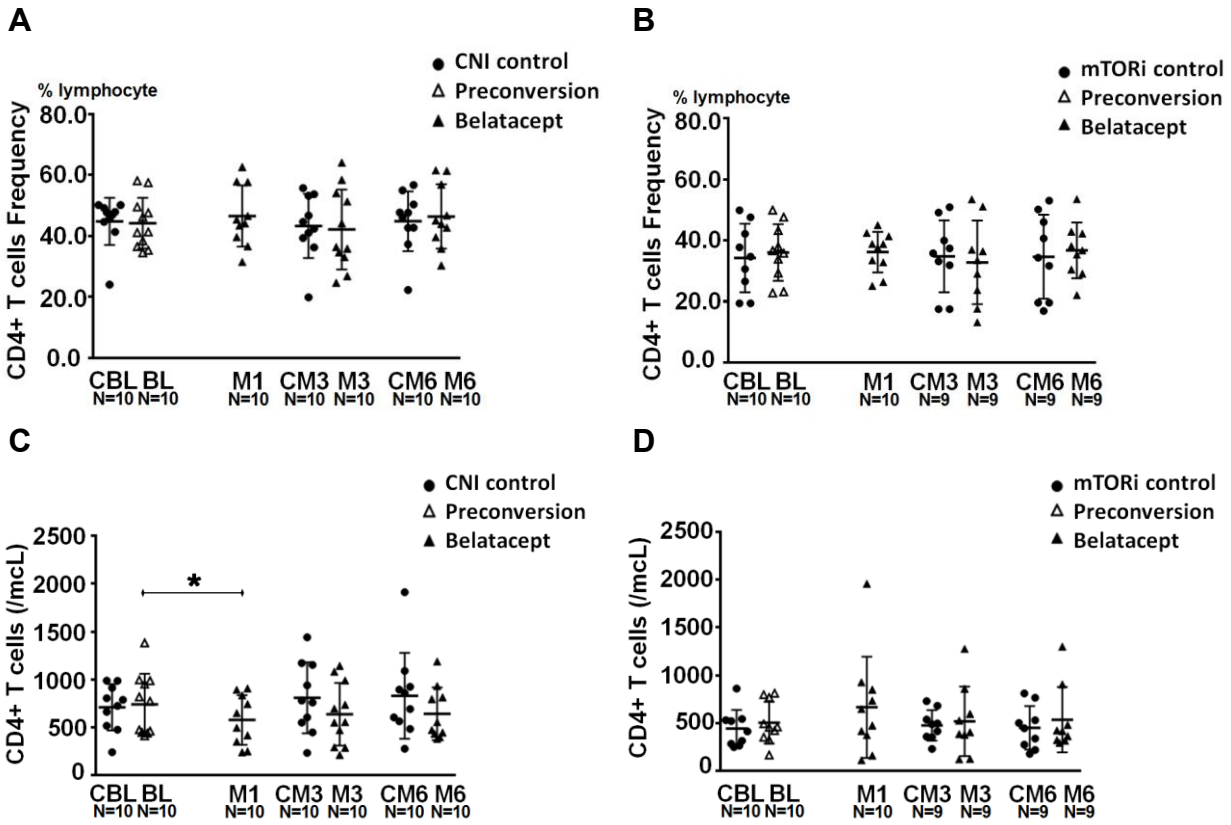


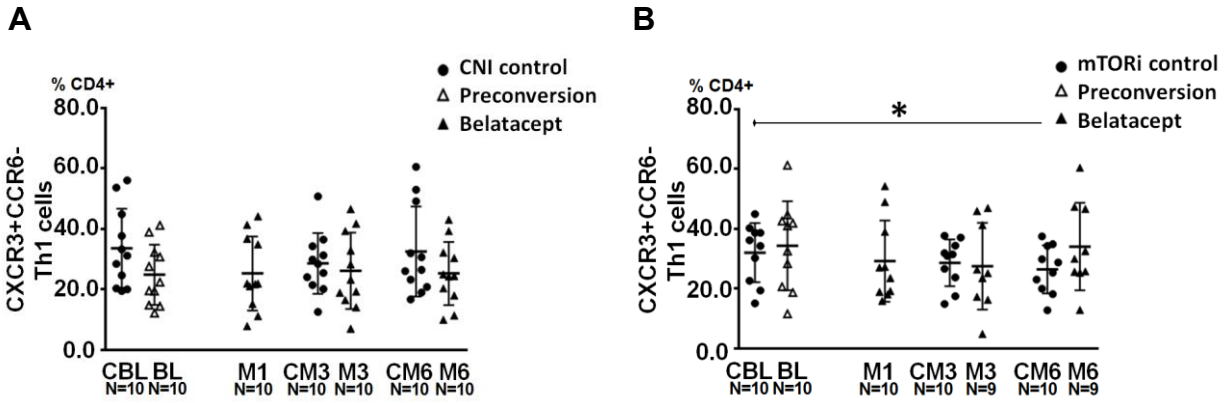
Figure 4: Frequency and absolute numbers of CD4+ T cells in the belatacept and matched control

groups. (A) Frequency of CD4+ T cells in belatacept and matched CNI control groups; (B) Frequency of CD4+ T cells in belatacept and their matched-mTORi control groups; (C) CD4+ T cells' absolute numbers in belatacept and the matched-CNI control groups; (D) CD4+ T cells' absolute numbers in belatacept and the matched-mTORi control groups. *, p<0.05, compared to BL.

4.2.2 Th1/Th2/Th17 subpopulations

Identification of T-helpers is shown in Figure 3A. As shown in Figures 5A, 5C and 5E, no differences were observed in the expression level of Th1 (CXCR3+CCR6-), Th2 (CXCR3-CCR6-) and Th17 (CXCR3-CCR6+) markers after patients were converted to stable Th1, Th2, and Th17 populations compared to control baseline (CBL). Interestingly, Th17 cells were lower in patients at M6, compared to matched-CNI control (p=0.033) (Figure 5E), whereas Th1 (Figure 5A) and Th2 (Figure 5C) were not different compared to the matched-CNI control group.

The frequency of Th1(Figure 5B), Th2(Figure 5D) and Th17(Figure 5F) cells did not change significantly in patients after conversion from mTORi to belatacept compared to pre-conversion or matched-mTORi controls at M1, M3 and M6. Only mTORi-control group patients had decreased in Th1 expression at M6 (26.49±8.03, p=0.017) (Figure 5A, right) compared to CBL (32.07±9.91).



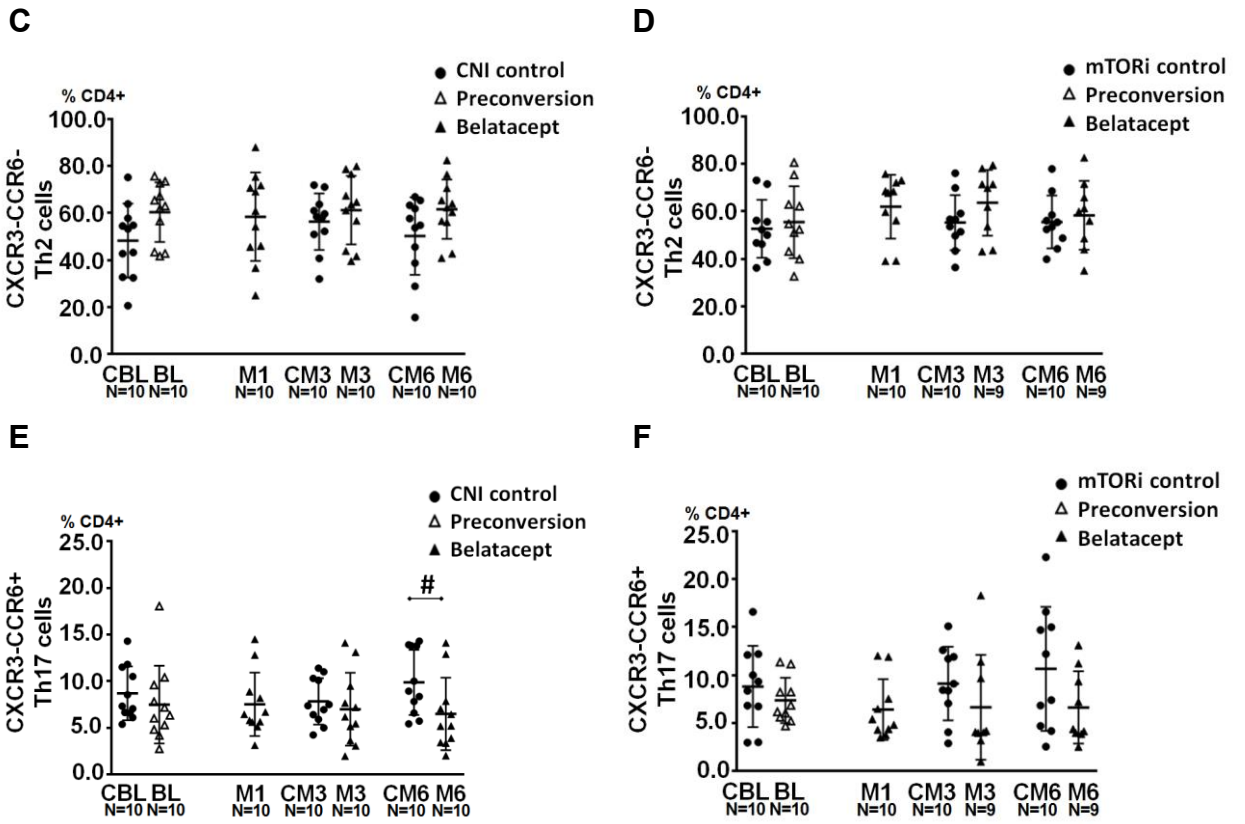
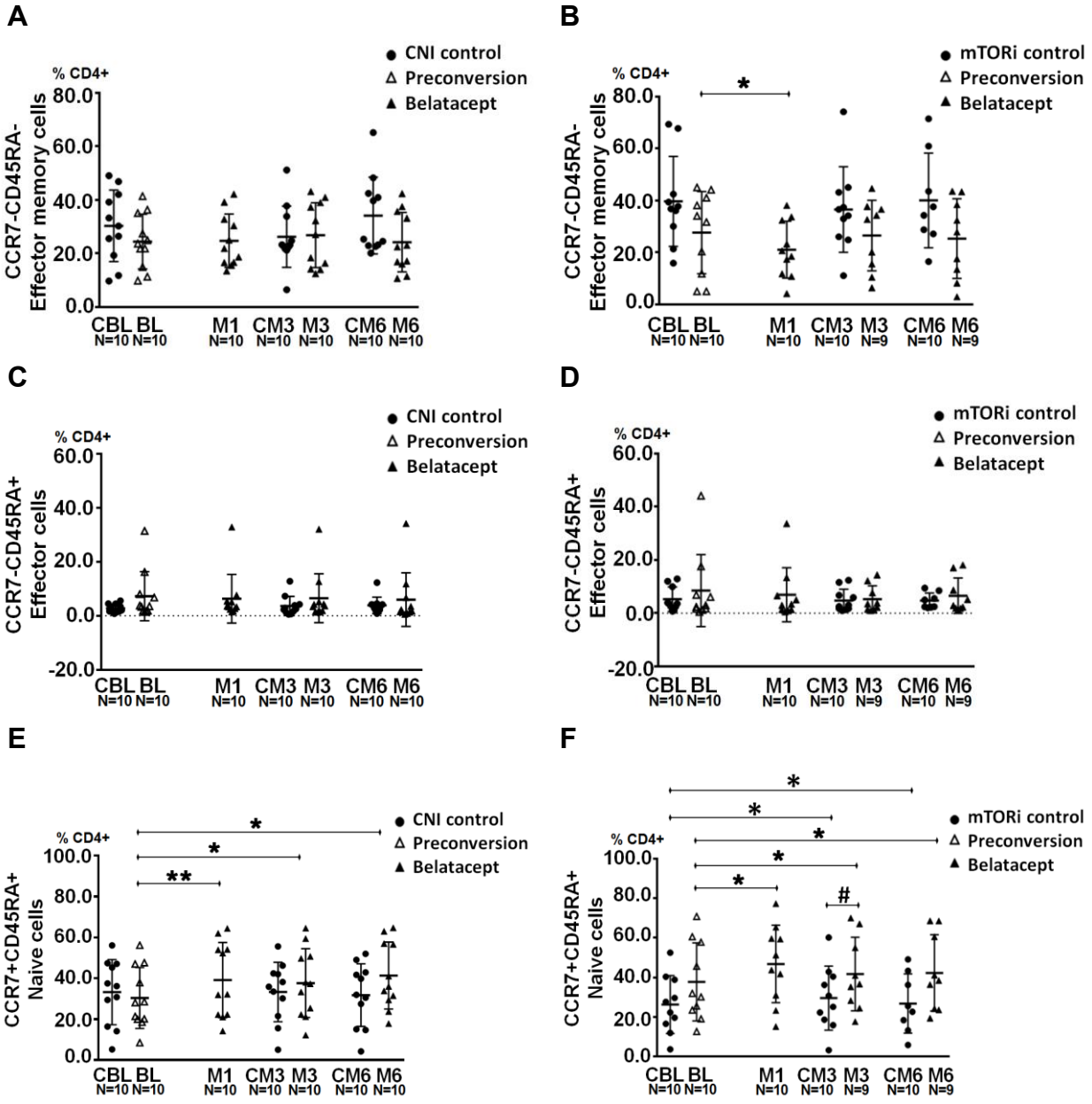


Figure 5: The level expression of T-help cells shown in CNI-, mTORi- and belatacept groups. (A) Th1 expressed in belatacept and matched-CNI control groups; (B) Th1 expressed in belatacept and matched-mTORi control groups; (C) Th2 expressed in belatacept and matched-CNI control groups; (D) Th2 expressed in belatacept and matched-mTORi control groups; (E) Th17 expressed in belatacept and matched-CNI control groups; (F) Th17 expressed in belatacept and matched-mTORi control groups. *, $p<0.05$, compared to CBL; #, $p<0.05$, compared to matched control.

4.2.3 CD4+ Effector / Effector memory / Naive / Central memory subpopulations

CD4+ T cell subsets are gated as shown in Figure 3A. The percentage of effector memory cells (CD4+CCR7-CD45RA-) (Figure 6A), effector cells (CD4+CCR7-CD45RA+) (Figure 6C) and central memory cells (CD4+CCR7+CD45RA-) (Figure 6G) did not change significantly after conversion from CNI to belatacept over 6 months compared to pre-conversion. The naïve (CD4+CCR7+CD45RA+) cells had increased significantly compared to pre-conversion after CNI patients converted to belatacept at M1 (p=0.003), M3 (p=0.026) and M6 (p=0.010) (Figure 6E).

After mTORi patients converted to belatacept, CD4+ naïve cells increased also at M1 (p=0.011), M3 (p=0.011) and M6 (p=0.015) (Figure 6F), whereas the percentage of CD4+ effector memory T cells significantly decreased at M1(p=0.028) (Figure 6B), and the central memory CD4+ T cells decreased significantly at M6 (p=0.008) (Figure 6H). Terminally differentiated effector CD4+ T cells did not change significantly after mTORi patients converted to belatacept over 6 months. (Figure 6D)



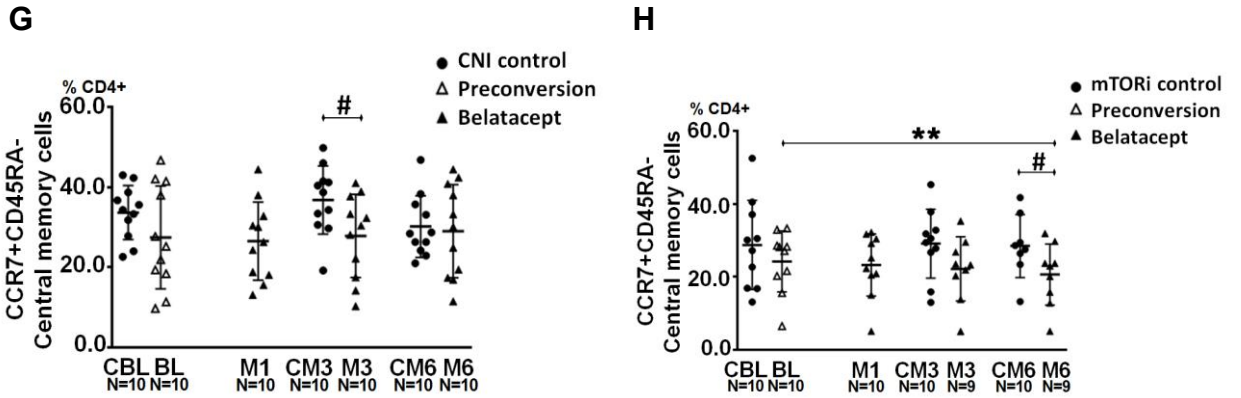


Figure 6: Expression of CD4+ T-cell subpopulations after conversion to belatacept. (A) Percentage of effector memory cells in belatacept and matched-CNI control groups; (B) Percentage of effector memory cells in belatacept and matched-mTORi control groups; (C) Percentage of effector cells in belatacept and matched-CNI control groups; (D) Percentage of effector cells in belatacept and matched-mTORi control groups; (E) Percentage of Naive cells in belatacept and matched-CNI control groups; (F) Percentage of Naïve cells in belatacept and matched-mTORi control groups; (G) Percentage of central memory cells in belatacept and matched-CNI control groups; (H) Percentage of Central memory cells in belatacept and matched-mTORi control groups. **, p<0.01; *, p<0.05, compared to BL; *, p<0.05, compared to CBL; #, p<0.05, compared to matched control.

4.2.4 CD28-CD57+ out of CD4+ T subpopulations

CD28−CD57+ CD4+ subpopulations are gated as shown in Figure 3A. The percentage of CD28-CD57+ showed significant differences between CBL and CM3 on CD4 effector memory cells (p=0.021, Figure 7A) and CD4+ effector cells (p=0.037, Figure 7C) of CNI control group. CD28-CD57+ expressed significantly lower levels at M1 on naive (p=0.008, Figure 7E) and central memory (p=0.021, Figure 7G) CD4+ T cells after patients conversion from CNI to belatacept. And CD28-CD57+ frequency out of CD4+ naive T cells had lower level in belatacept which converted from CNI compared to matched-CNI control at M6 (p=0.041, Figure7E).

The expression of CD28-CD57+ out of CD4+ effector T cells had significantly decreased after mTORi patients conversion to belatacept at M3 (p=0.021, Figure 7D). There was no significant change with CD28-CD57+ expressed on CD4+ effector memory cells (Figure 7B), CD4+ Naïve cells (Figure 7F) and CD4+ central memory cells (Figure 7H) in mTORi control and belatacept groups, when comparing to Baseline or matched control at different time points.

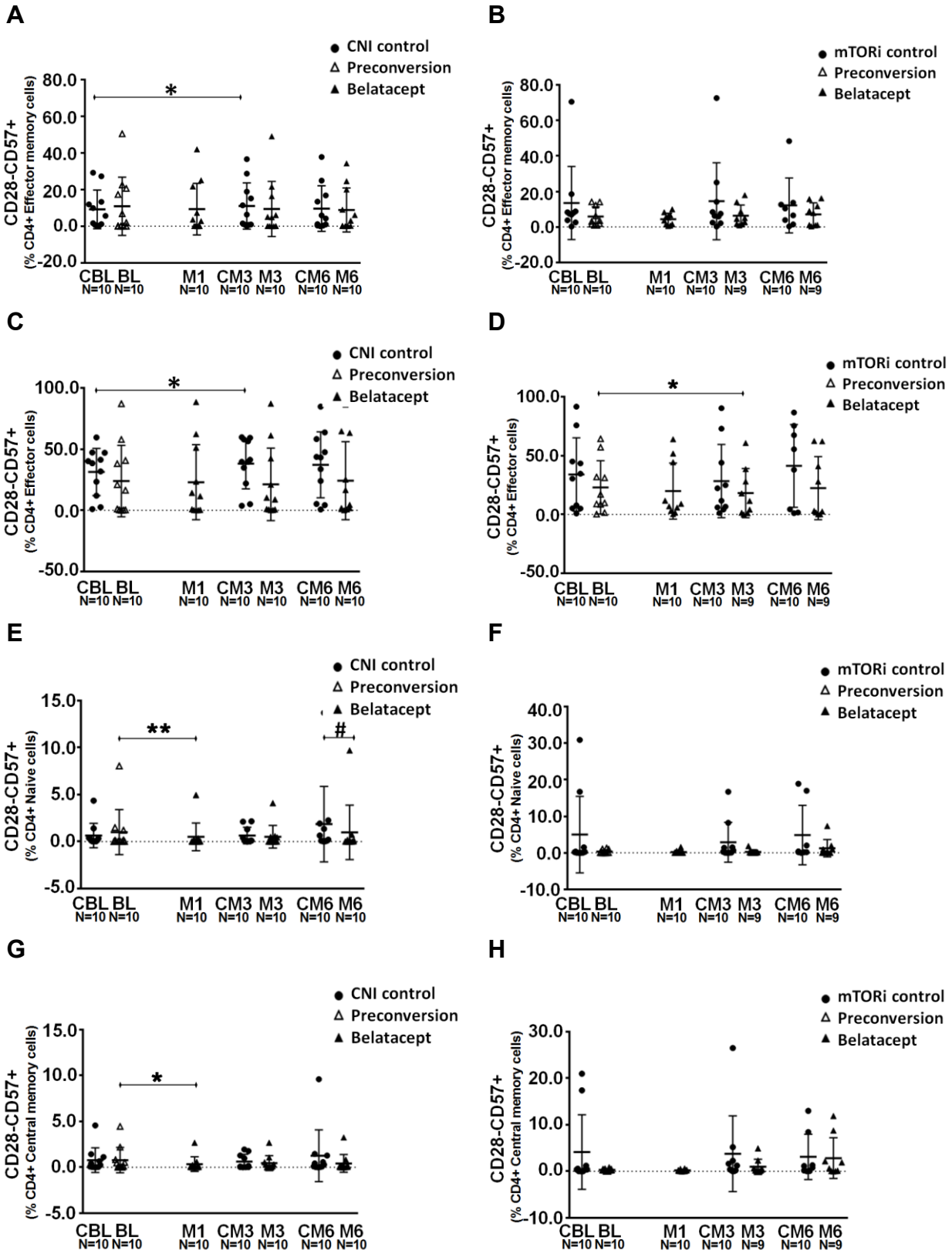


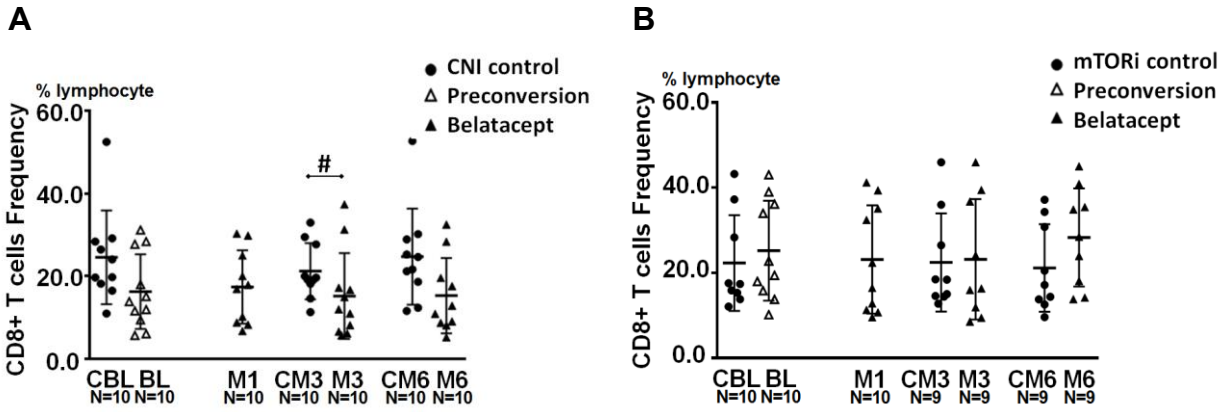
Figure 7: Percentage of CD28-CD57+ expressed on different CD4+ T subpopulations. (A) Percentage of CD28-CD57+ expressed on CD4+ Effector memory cells in belatacept and matched-CNI control groups; (B) Percentage of CD28-CD57+ expressed on CD4+ effector memory cells in belatacept and

matched-mTORi control groups; (C) Percentage of CD28-CD57+ expressed on CD4+ effector in belatacept and matched-CNI control groups; (D) Percentage of CD28-CD57+ expressed on CD4+ effector cells in belatacept and matched-mTORi control groups; (E) Percentage of CD28-CD57+ expressed on CD4+ Naive cells in belatacept and matched-CNI control groups; (F) Percentage of CD28-CD57+ expressed on CD4+ Naïve cells belatacept and matched-mTORi control groups; (G) Percentage of CD28-CD57+ expressed on CD4+ central memory cells in belatacept and matched-CNI control groups; (H) Percentage of CD28-CD57+ expressed on CD4+ Central memory cells in belatacept and matched-mTORi control groups. **, p<0.01; *, p<0.05, compared to BL; *, p<0.05, compared to CBL; #, p<0.05, compared to matched control.

4.2.5 CD8+ cytoxic T cell in CNI or mTORi conversion belatacept and matched-control groups.

Percentages and absolute numbers of CD8+ T cell were analysed on FACS. The frequency of CD8+ T cell out of lymphocytes was at a lower level in belatacept compared to matched-CNI group at Month3 (p=0.012, Figure 8A). Absolute numbers of CD8+ T cells had decreased when comparing belatacept with matched-CNI patients at Month 3 (p=0.038, Figure 8C).

CD8+ T cells Frequency (Figure 8B) and CD8+ absolute numbers (Figure 8D) did not change after mTORi patients' conversion to belatacept over 6 months compared to pre-conversion and matched-mTORi control group.



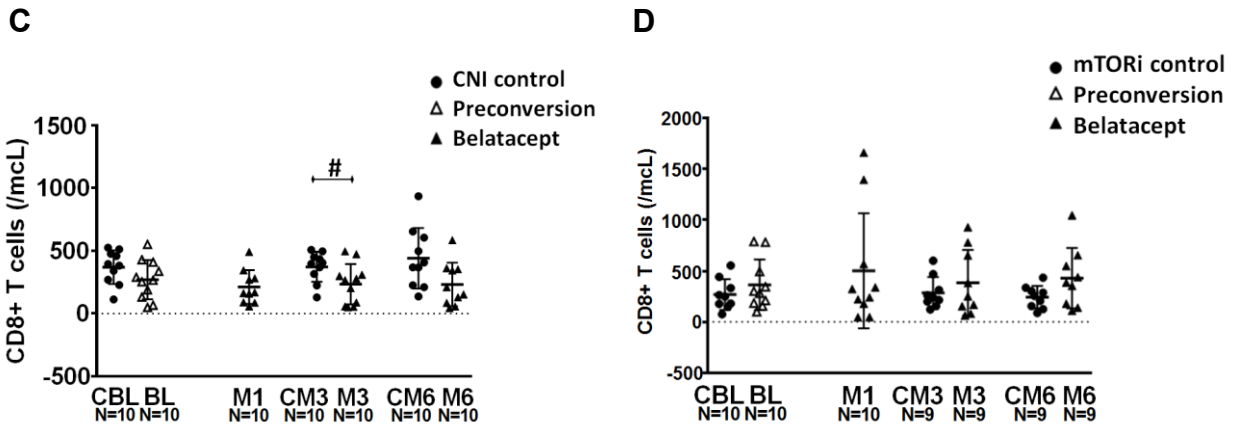


Figure 8：Frequency and absolute numbers of CD8+ T cells in the belatacept and matched-control groups. (A) Frequency of CD8+ T cells in belatacept and matched-CNI control groups; (B) Frequency of CD8+ T cells in belatacept and their matched-mTORi control groups; (C) CD8+ T cells' absolute numbers in belatacept and the matched-CNI control groups; (D) CD8+ T cells' absolute numbers in belatacept and the matched-mTORi control groups. #, $p<0.05$, compared to matched control groups.

4.2.6 CD8+ Effector/ Effctor memory/Naive/Central memory subpopulations

CD8+ T cell subsets are gated as shown in Figure 3A. The percentage of the CD8+ effector (CD8+ CCR7-CD45RA+, Figure9C), naive (CD8+CCR7+CD45RA+, Figure9E) and central memory (CD8+CCR7+CD45RA-, Figure9G) cells did not significantly change after CNI patients' conversion to belatacept over 6 months, when the CD8+ effector memory (CD8+CCR7-CD45RA-, Figure 9A) cells had increased significantly at M3 (p=0.050) compared to pre-conversion.

The CD8+ effector memory cells decreased (p=0.038) at M1 after mTORi patients conversion to belatacept, and it expressed much lower levels at M3 (p=0.018) and M6 (p=0.018) compared to matched-mTORi control (Figure 9B). The CD8+ naive cells (p=0.021, Figure 9F) increased in mTORi patients converted to belatacept for 1 month. when the percentage of CD8+ effector (Figure 9D) and CD8+ central memory (Figure 9H) cells did not significantly change compared to pre-conversion and matched-mTORi control.

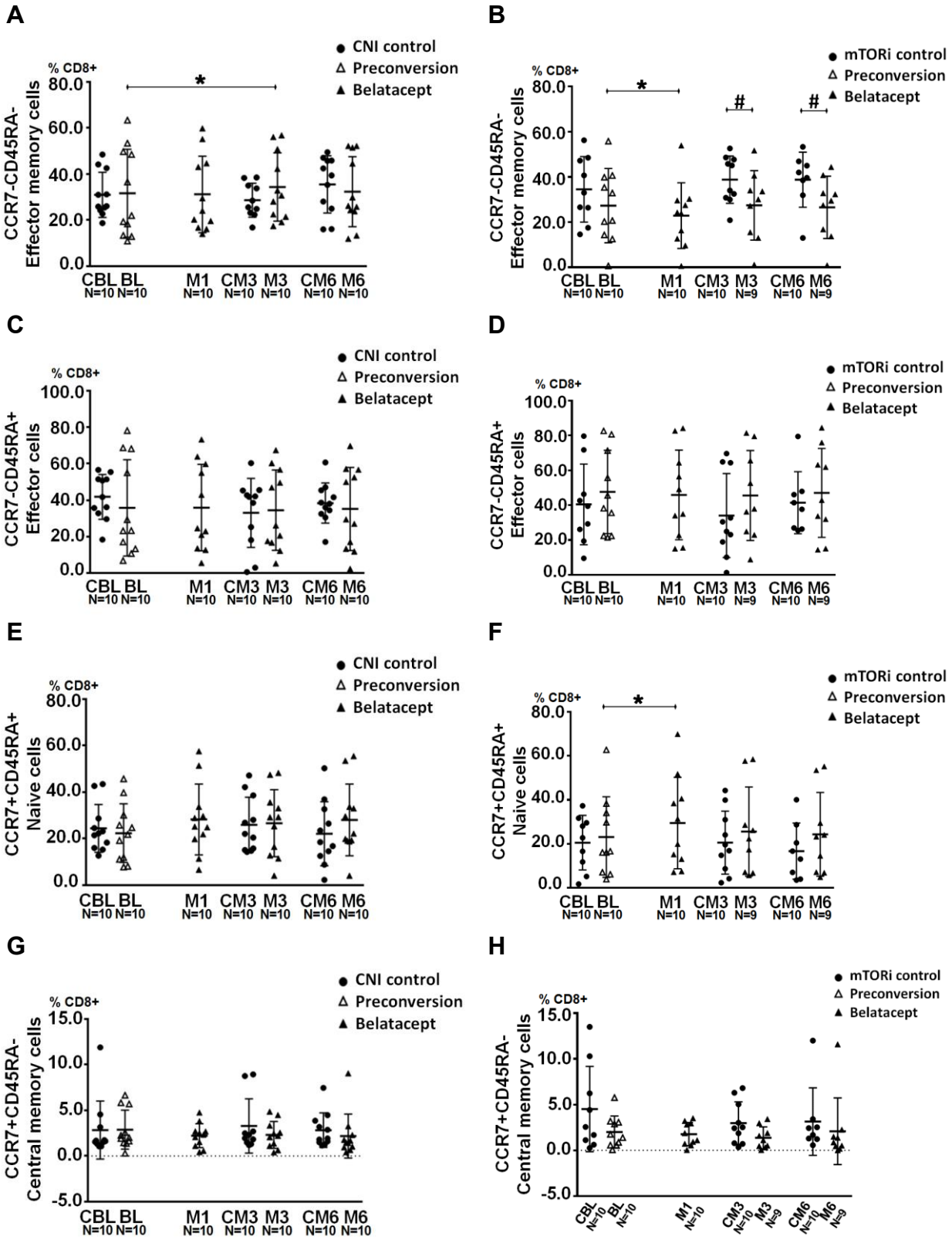


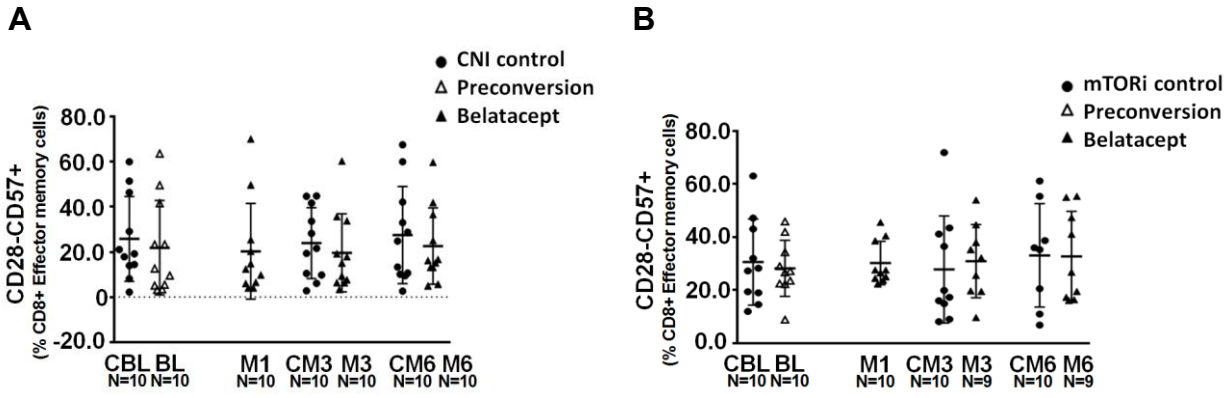
Figure 9: Expression of CD8+ T-cell subpopulations after conversion to belatacept. (A) Percentage of CD8+ effector memory cells in belatacept and matched-CNI control groups; (B) Percentage of CD8+ effector memory cells in belatacept and matched-mTORi control groups; (C) Percentage of CD8+ effector in belatacept and matched-CNI control groups; (D) Percentage of CD8+ effector cells in

belatacept and matched-mTORi control groups; (E) Percentage of CD8+ Naive cells in belatacept and matched-CNI control groups; (F) Percentage of CD8+ Naive cells belatacept and matched-mTORi control groups; (G) Percentage of CD8+ central memory cells in belatacept and matched-CNI control groups; (H) Percentage of CD8+ Central memory cells in belatacept and matched-mTORi control groups. **, p<0.01; *, p<0.05, compared to BL; #, p<0.05, compared to matched control. *, p<0.05, compared to B.

4.2.7 CD28-CD57+ in CD8+ T subpopulations

The gating of CD28−CD57+ in CD8+ subpopulations is shown in Figure 3A. There was no difference in expression of CD28-CD57+ on CD8 effector memory (Figure 10A) and effector cells (Figure 10C) in belatacept compared to pre-conversion and matched-CNI control group. CD28−CD57+ on CD8+ Naive cells (Figure 10E) had decreased significantly after CNI patients conversion to belatacept for 1 month (p=0.003) and 3 months (p=0.041) compared to pre-conversion. There was also a decrease in central memory cells (p=0.010, Figure 10G) in belatacept at M1 compared to pre-conversion, but no difference when comparing to CNI controls.

No significant difference in expression was observed on CD8+ effector memory (Figure 10B), CD8+ naive (Figure 10E) and CD8+ central memory cells (Figure 10H) in conversion from mTORi to belatacept compared to pre-conversion and matched-mTORi control group at different time points. CD28−CD57+ cells expressed on effector CD8+ T cells decreased significantly after conversion to belatacept at M3 (p=0.015) compared to pre-conversion, but there was no difference compared to matched-mTORi control (Figure 10D).



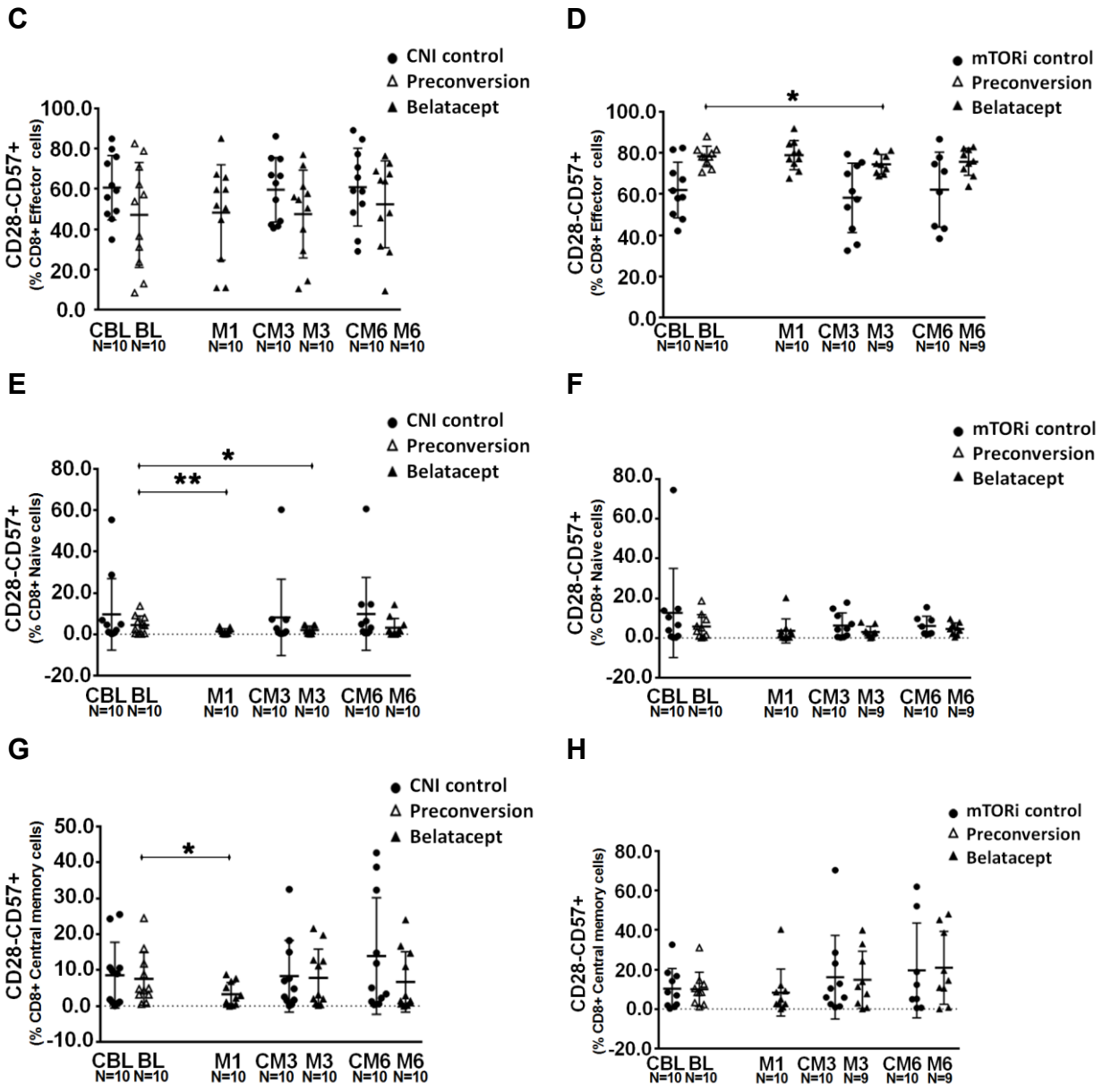


Figure 10: Percentage of CD28-CD57+ expressed in different CD8+ T subpopulations. (A) Percentage of CD28-CD57+ expressed on CD8+ Effector memory cells in belatacept and matched-CNI control groups; (B) Percentage of CD28-CD57+ expressed on CD8+ effector memory cells in belatacept and matched-mTORi control groups; (C) Percentage of CD28-CD57+ expressed on CD8+ effector in belatacept and matched-CNI control groups; (D) Percentage of CD28-CD57+ expressed on CD8+ effector cells in belatacept and matched-mTORi control groups; (E) Percentage of CD28-CD57+ expressed on CD8+ Naive cells in belatacept and matched-CNI control groups; (F) Percentage of CD28-CD57+ expressed on CD8+ Naive cells in belatacept and matched-mTORi control groups; (G) Percentage of CD28-CD57+ expressed on CD8+ central memory cells in belatacept and matched-CNI control groups; (H) Percentage of CD28-CD57+ expressed on CD8+ Central memory cells in belatacept and matched-mTORi control groups. **, $p<0.01$; *, $p<0.05$, compared to BL; *, $p<0.05$, compared to CBL; #, $p<0.05$, compared to matched control.

4.2.8 Regulatory T-cell subpopulation

CD4+CD25+CD127low Regulatory T-cells are gated as shown in Figure 3A. Tregs among CD 4+ cells decreased significantly after CNI conversion to belatacept at M1 (p=0.026), M3 (p=0.033) and to their minimum at M6 (p=0.006) compared to pre-conversion. There was no difference compared to CNI control group (Figure 11A). In addition, Tregs' absolute number dropped which was coincident with frequency of Tregs at M1 (p=0.008), M3 (p=0.011) and M6 (p=0.028) compared to pre-conversion. Again no significant change was observed in belatacept compared to matched-CNI control (Figure 11C).

In mTORi conversion to belatacept group, the Tregs among CD4+ cells decreased significantly at M3 (p=0.008) and M6 (p=0.011) compared to pre-conversion, and had a lower level compared to matched-mTORi control at M3 (p=0.017) and M6 (p=0.036, Figure 11B). Treg absolute numbers decreased significantly at M3 (p=0.025), no difference was observed at M6 compared to pre-conversion and matched-mTORi control (Figure 11D).

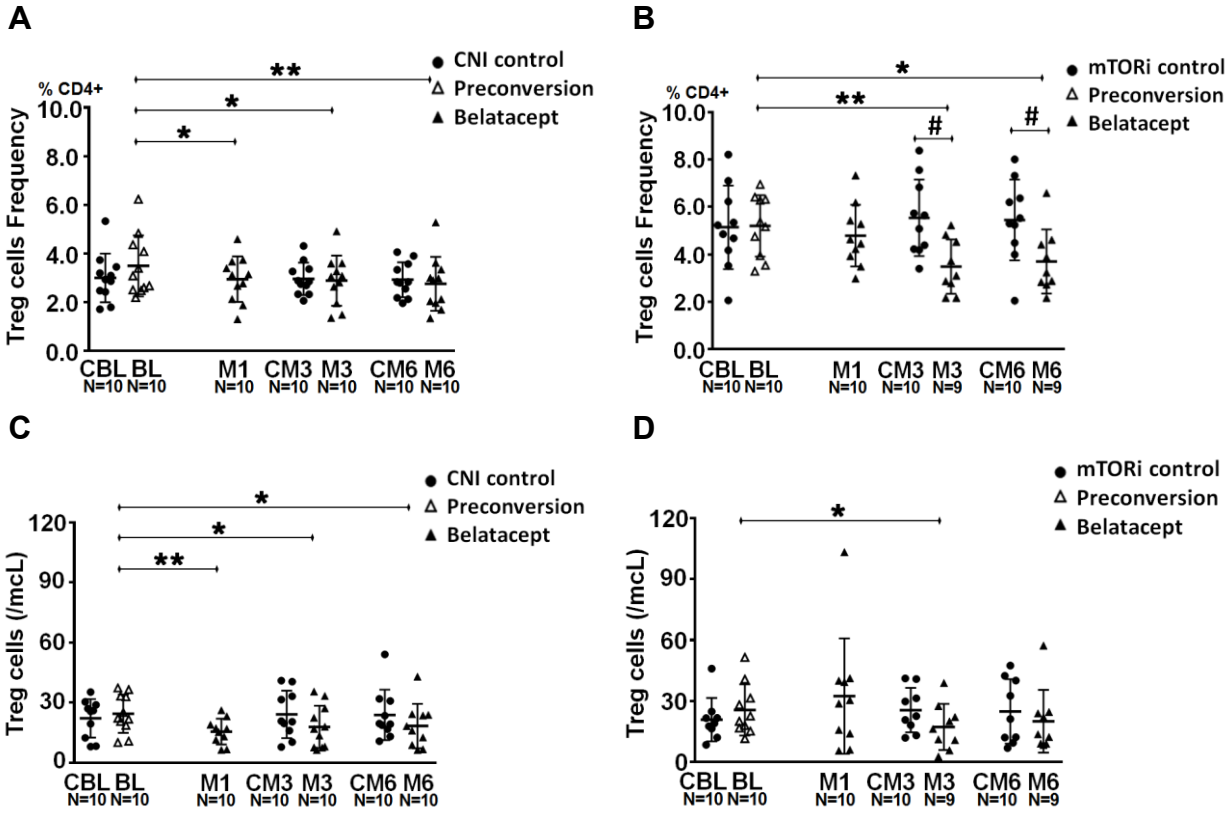


Figure 11: Frequency and absolute numbers of Tregs in belatacept and matched control groups. (A) Frequency of Tregs among CD4+ cells in belatacept and matched-CNI control groups; (B) Frequency of Tregs among CD4+ T cells in belatacept and matched-mTORi control groups; (C) Tregs' absolute

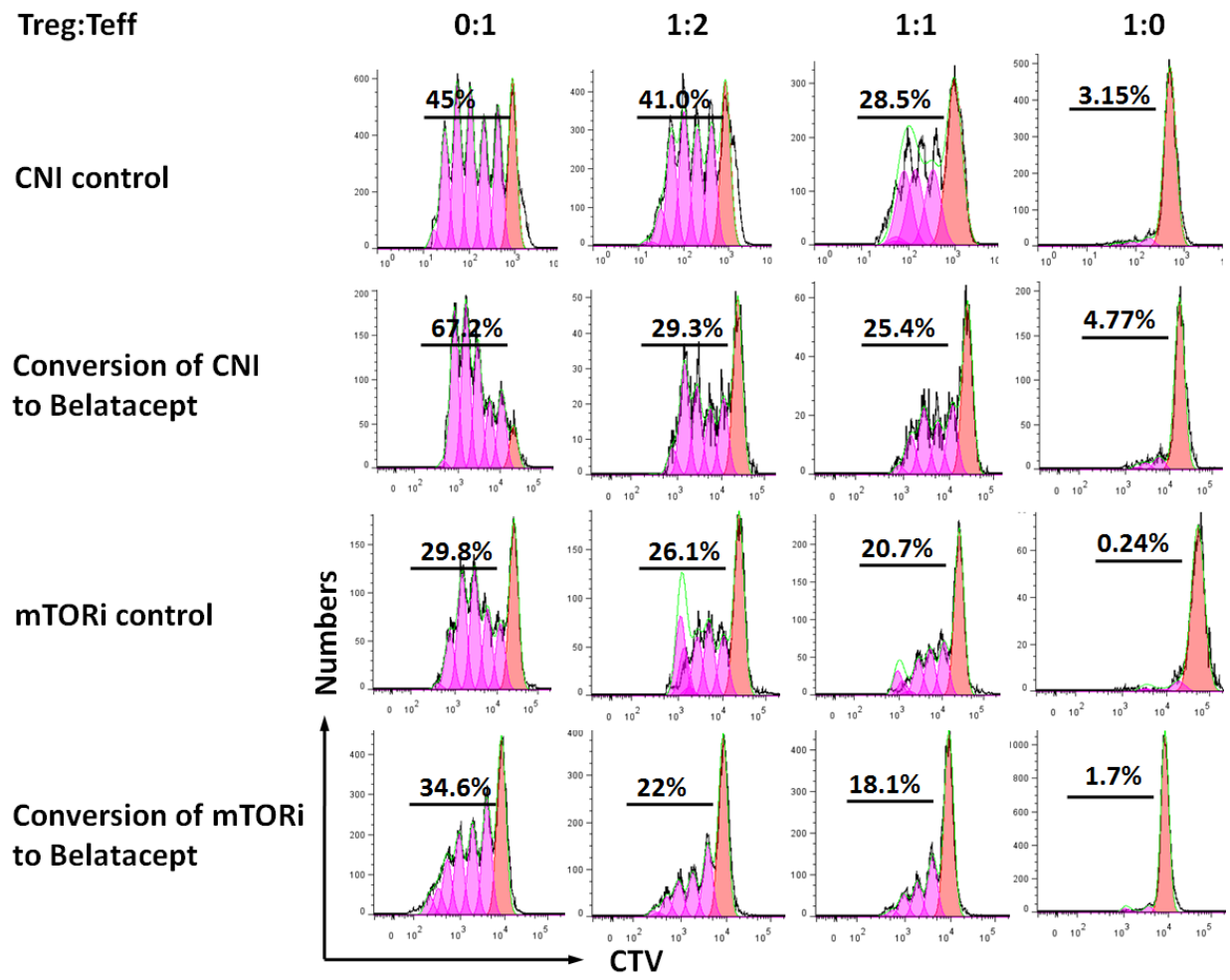
numbers in belatacept and the matched-CNI control groups; (D) Tregs' absolute numbers in belatacept and the matched-mTORi control groups. **, p<0.01, *, p<0.05, compared to BL;

4.2.9 Treg suppression assay

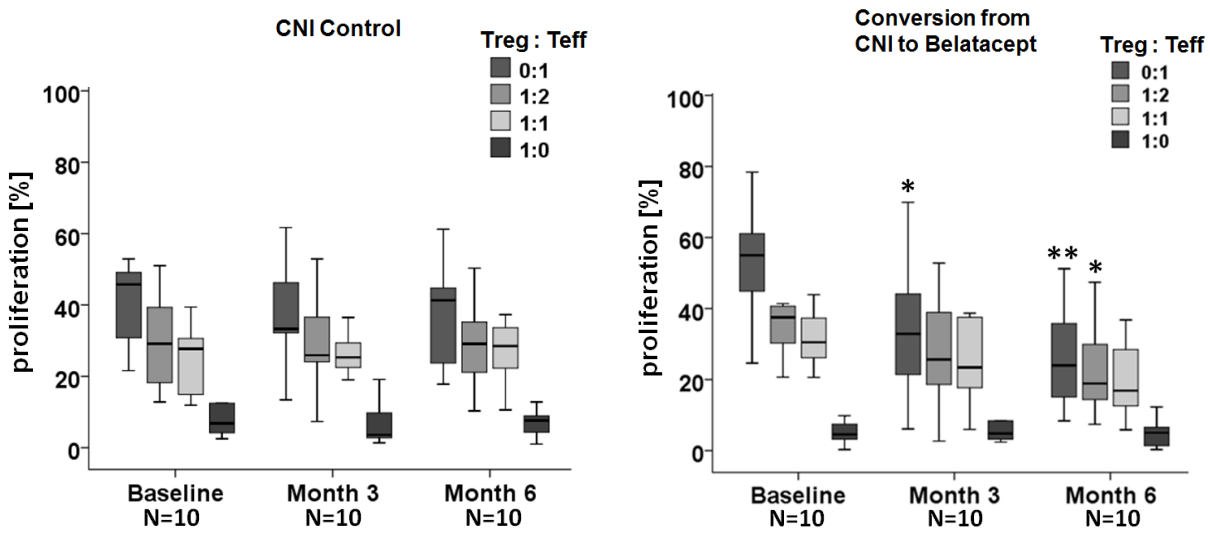
In order to assess the suppression capability of Treg cells, we isolated effector T cells (CD4+CD25-, Teff) and Treg cells by Magnetic sorting. The purity of Tregs and Teff cells was over 90% (data not shown). After Tregs were co-cultured with Teff cells for 4 days, CTV labelled cells showed the division cycle with each peak in the FACS histogram represented one cell division. Figure 12A showed a representative patient for this experiment of each group. All 4 groups of Tregs cultured alone showed a hypo-proliferative response (Figure 12A, 1:0).

Proliferation of Teff cells cultured alone were significantly lower after CNI patients conversion to belatacept at M3 (p=0.016) and M6 (p=0.008) compared to pre-conversion (Figure 12B). Tregs co-culture with Teff cells resulted in reduced proliferation of Teff cells with ratios 1 to 2 and 1 to 1, and co-cultured Teff cells proliferation in ratio 1 to 2 had reduced significance at M6 (p=0.033) in the CNI conversion belatacept group compared to pre-conversion (Figure 12B, right), but there were no significant changes when compared to the matched-CNI control group at different time points. No significance was observed in belatacept compared to pre-conversion and matched-mTORi control group at different time points (Figure 12C).

A


Treg:Teff
0:1
1:2
1:1
1:0
CNI control
45%
41.0%
28.5%
3.15%
Conversion of CNI to Belatacept
67.2%
29.3%
25.4%
4.77%
mTORi control
29.8%
26.1%
20.7%
0.24%
Conversion of mTORi to Belatacept
34.6%
22%
18.1%
1.7%
Numbers
CTV

B


CNI Control
Treg : Teff
0:1
1:2
1:1
1:0
proliferation [%]
Baseline N=10
Month 3 N=10
Month 6 N=10
Conversion from CNI to Belatacept
Treg : Teff
0:1
1:2
1:1
1:0
proliferation [%]
*
**
*
Baseline N=10
Month 3 N=10
Month 6 N=10

C

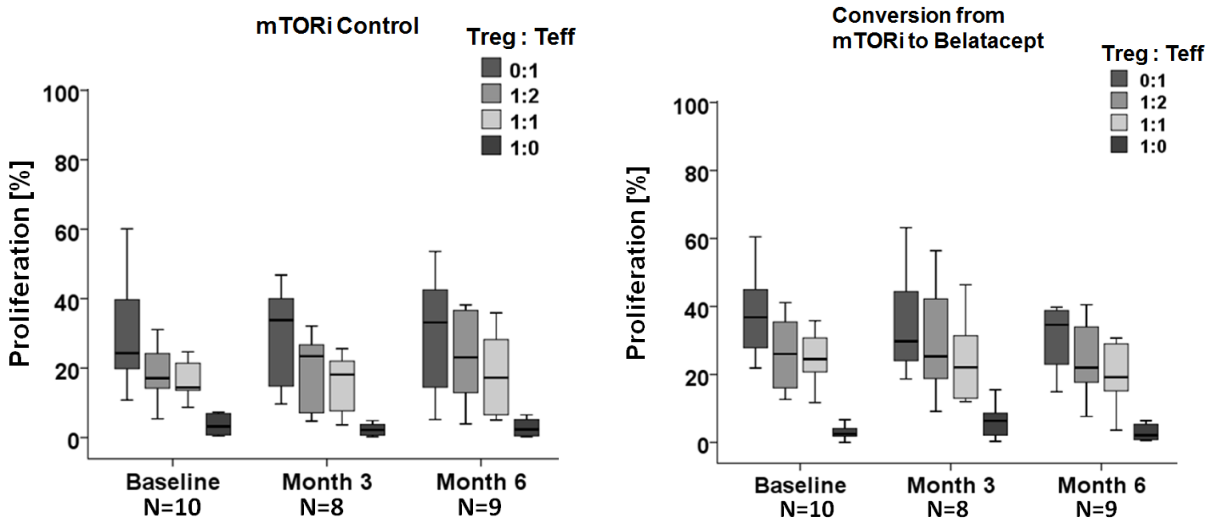


Figure 12: Suppressive function of Treg cells. (A) Proliferation characteristics of Treg cells that were co-cultured with T effector cells by CTV labeled at the ratios of 0:1, 1:2, 1:1 and 1:0 in belatacept and matched control groups, respectively; (B) The proliferation of Teff cells in CNI control and belatacept conversion from CNI groups; (C) The proliferation of Teff cells in mTORi control and belatacept conversion from mTORi groups. *, $p<0.05$, compared to Baseline.

4.3 B cell subpopulation and Proliferation assay

4.3.1 The impact of CD19 cells after conversion to belatacept

The CD19+ B-cells among lymphocytes did not change significantly after CNI patients conversion to belatacept over 6 months compared to pre-conversion and matched-CNI controls, but decreased at M6 ($p=0.047$) in the CNI control group (Figure 13A). The frequency of CD19 cells decreased significantly compared to pre-conversion after mTORi patients conversion to belatacept at M3 ($p=0.038$, Figure 13B), but there was no change compared to the matched-mTORi control group.

Absolute numbers of CD19+ cells are determined by per microliter peripheral blood (/mcL).

The absolute numbers of CD19+ cells did not change after conversion to belatacept for 6 months compared to pre-conversion and matched-CNI or mTORi control groups, respectively. (Figure 13C, 13D)

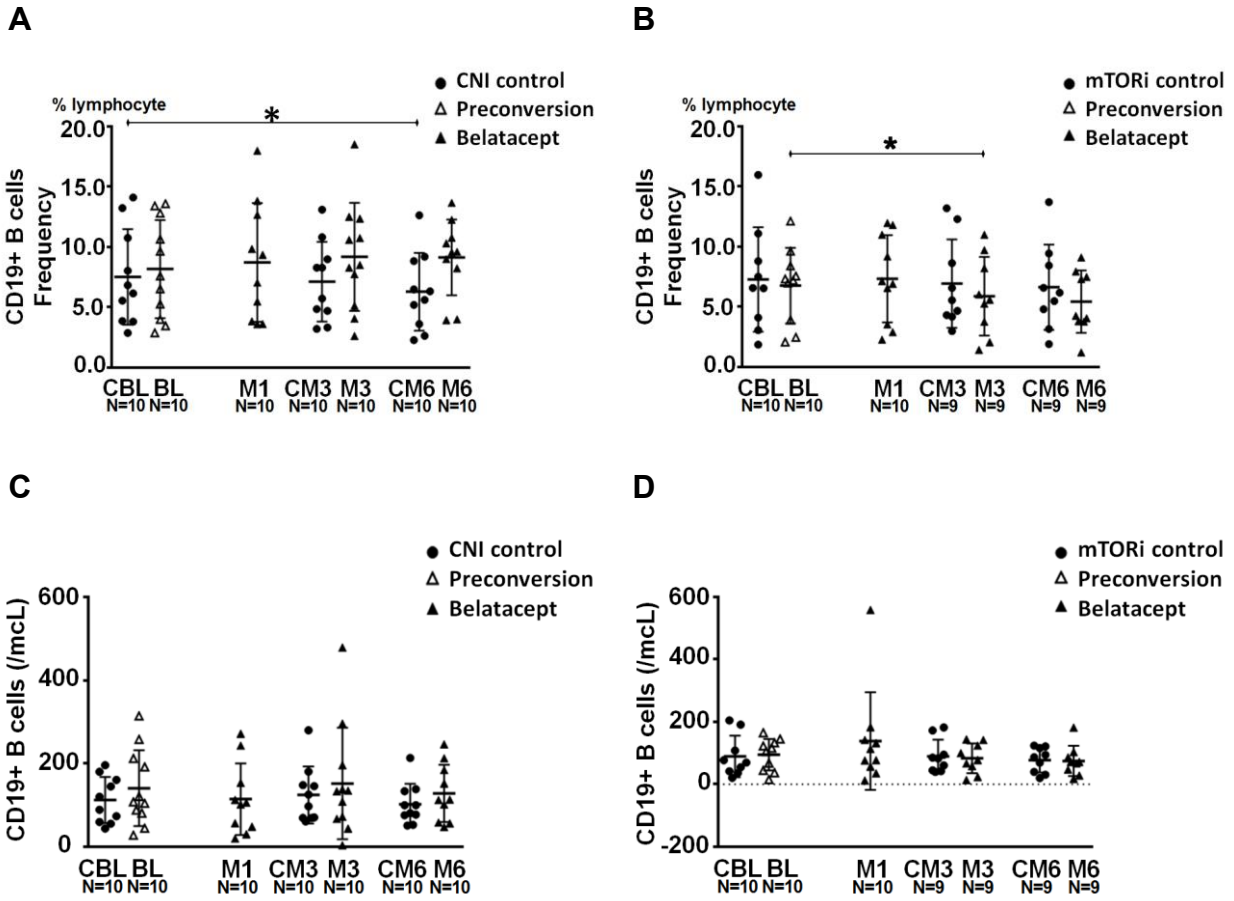


Figure 13: Impact of CD19+ cells after conversion to belatacept. (A) CD19+ cells' frequency shown in belatacept and matched-CNI control groups; (B) CD19+ cells' frequency shown in belatacept and matched-mTORi control groups; (C) CD19+ absolute numbers shown in belatacept and matched-CNI control group; (D) Absolute number shown in belatacept and matched-mTORi control group. *, $p<0.05$, compared to CBL; *, $p<0.05$, compared to BL.

4.3.2 Plasmablast cells CD27+CD20-CD38+

CD19+CD20-CD27+CD38+ cells were defined as plasmablasts and plasmablasts showed very low numbers in the whole CD19+ population in general. The level of plasmablasts among CD19+ cells decreased after CNI conversion to belatacept at M1 ($p=0.018$). Belatacept-treated patients were not different compared to matched-CNI control (Figure 14A).

Plasmablast cells had decreased after mTORi conversion to belatacept at M1 ($p=0.024$), but no obvious difference was observed in belatacept compared to the matched-mTORi control group, and mTORi control had higher levels at M6 compared to CBL ($p=0.015$,

Figure 14B).

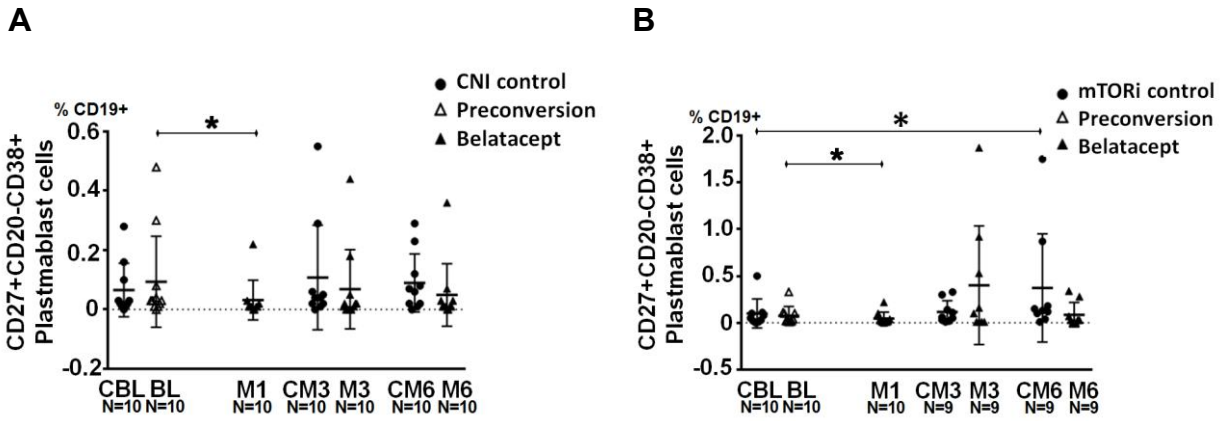


Figure 14: CD27+CD20-CD38+ plasmablast cells among CD19+ cells. (A) Percentage of plasmablast cells in CD19+ cells shown in belatacept and matched-CNI control group; (B) Percentage of plasmablast cells in CD19+ cells shown in belatacept and matched-mTORi control group; *, p<0.05, compared to CBL; *, p<0.05, compared to BL.

4.3.3 Memory B Cells Frequency

CD19+CD27+IgD+ and CD19+CD27+IgD- cells were both defined as memory B cells, when CD19+CD27- cells were defined as Naive cells. Memory B cell gating strategy is shown in Figure 3B. The percentage of CD27+ memory cells in CD19+ B cells decreased significantly at M6 (p=0.005) in CNI patients converted to belatacept group compared to pre-conversion, but it also decreased in CNI control at M3 (p=0.005). There was no difference between belatacept and matched-CNI control (Figure 15A).

CD27+ memory B cells were higher at M6 (p=0.015) in mTORi control group when no significant differences were observed after mTORi conversion to belatacept over 6 months and also compared to matched-mTORi control (Figure 15B).

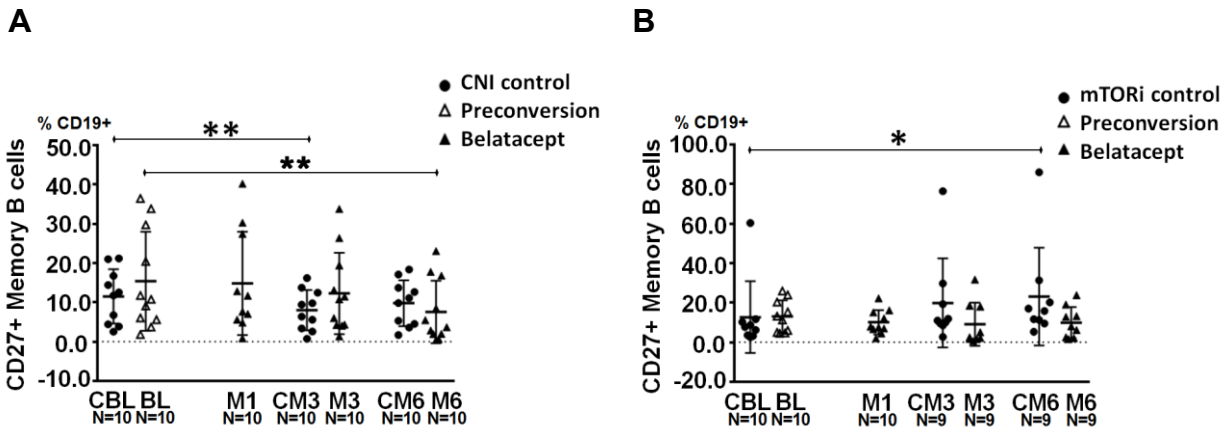


Figure 15: Percentage of CD27+ memory B cells in CD19+ cells. (A) Percentage of memory B cell in CD19+ shown in belatacept and matched-CNI control groups; (B) Percentage of memory B cell in CD19+ shown in belatacept and matched-mTORi control groups. **, p<0.01 and *, p<0.05, compared to CBL; **, p<0.01, compared to BL.

4.3.4 Expression of CD80 and CD86 on CD19+ B cells

After stimulating CD19+ cells with CpG-ODN, anti-CD40 and IL-4 for 3 days, CD80 expression levels had decreased significantly at M3 (p=0.036) and M6 (p=0.025) in belatacept conversion from CNI compared to pre-conversion, and did not show a difference compared to matched-CNI control (Figure 16A). The level of CD 80 expression was not different in belatacept conversion from mTORi compared to pre-conversion and matched-mTORi control (Figure 16B).

CD86 expression had a higher level at M6 (p=0.046) in belatacept compared to matched-CNI control (Figure 16C). It did not change significantly after both CNI and mTORi patients conversion to belatacept compared to pre-conversion over 6 months, not did it show any differences compared to matched-mTORi control (Figure 16C, 16D).

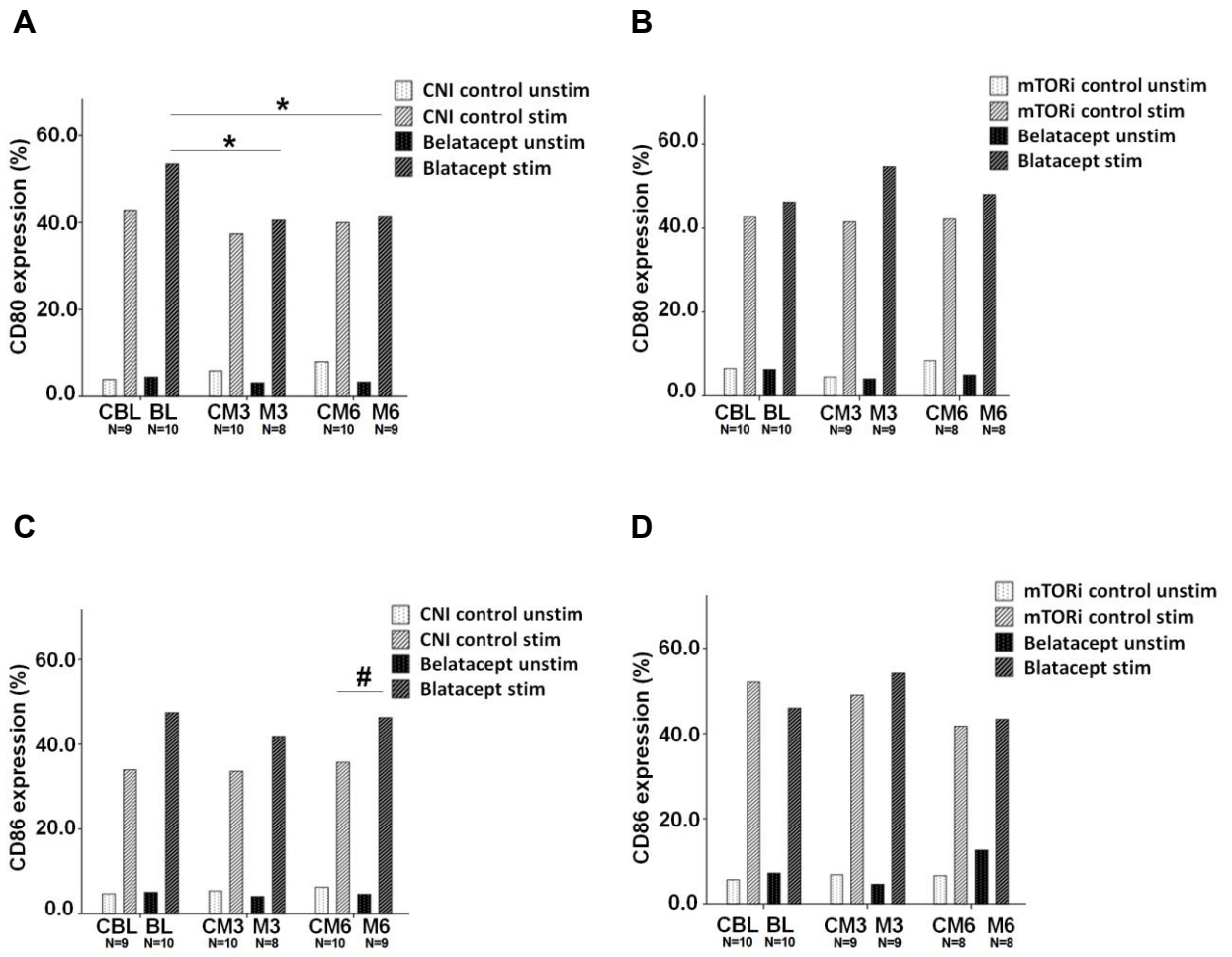


Figure 16: Expression of CD80/86 on CD19+ cells stimulated with CpG-ODN, anti-CD40 and IL-4. (A) CD80 expressed in belatacept and matched-CNI control; (B) CD80 expressed in belatacept and matched-mTORi control; (C) CD86 expressed in belatacept and matched-CNI; (D) CD86 expressed in belatacept and matched-mTORi control. *, $p<0.05$, compared to BL; #, $p<0.05$, compared to matched control.

4.3.5 Proliferation of CD19+ B cells

We found that there were not enough CD19+ cells for a proliferation assay after CNI- and mTORi- treated conversion to belatacept at M3 and M6, only 4 proliferation data sets were available at M3, 3 data sets at M6 in CNI-treated conversion to belatacept group, 5 data sets at M3 and 3 data sets at M6 in mTORi-treated conversion to belatacept. From one representative patient, we could see direct inhibition of their proliferation after 6 months (Figure 17A). After CD19+ B cells were labeled by CTV to trace the division cycle, and then after stimulated with 2.5 μM CpG-ODN, 1 μg /ml anti-CD40 and 10ng / ml IL-4

for 7 days, the proliferation of B cells had no significance in either group compared to pre-conversion or control, respectively, due to insufficient data on the patients (Figure 17B, 17C).

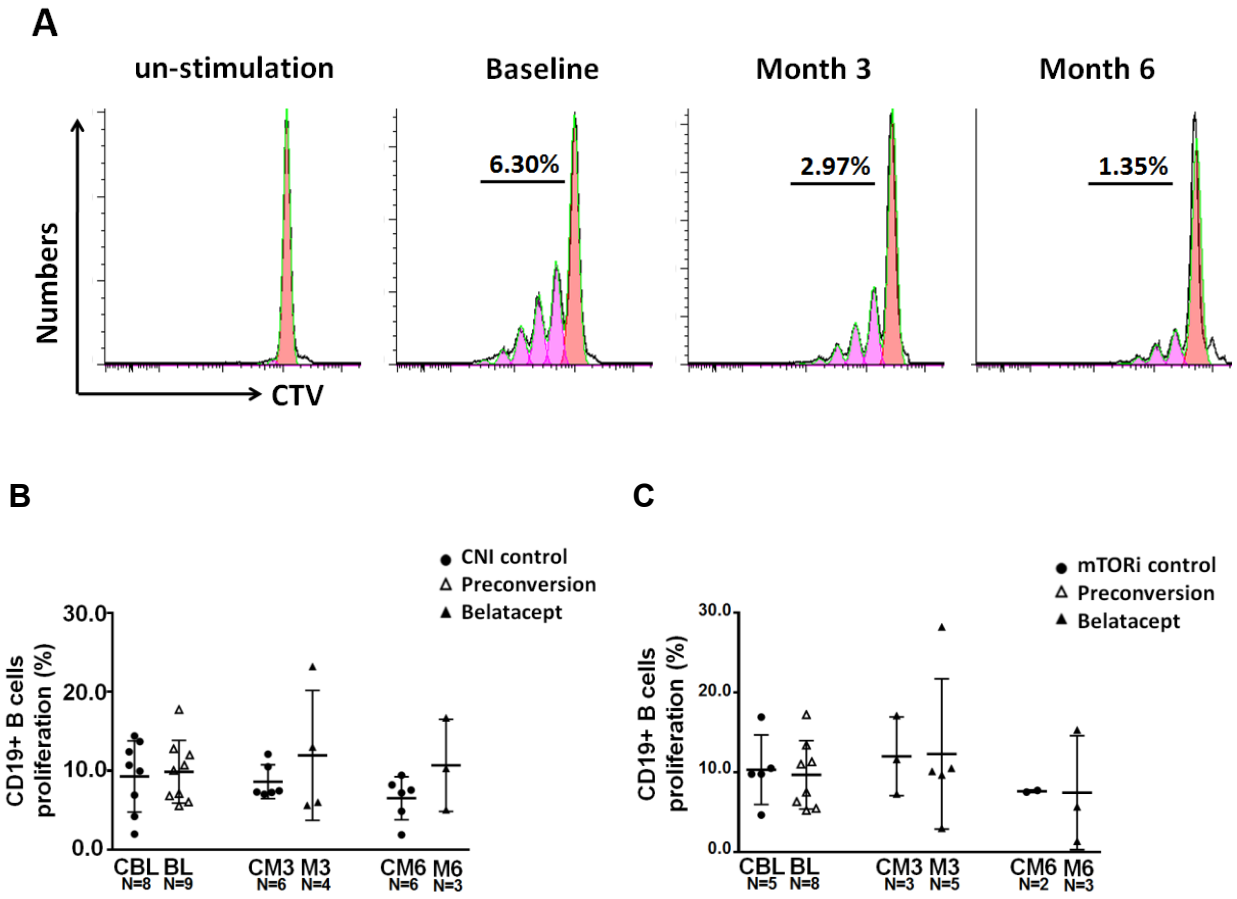


Figure 17: Proliferation of CD19+ cells stimulated for 7 days. (A) Proliferation characteristics of CD19+ cells labeled by CTV, showed a representative mTORi conversion patients. (B) Proliferation of CD19+ cells showed in belatacept and matched-CNI control conversion groups; (C) Proliferation of CD19+ cells showed in belatacept and matched-mTORi control conversion groups.

5 DISCUSSION

The success of solid-organ transplantation depends on the continuous administration of nonspecific immunosuppressive drugs, which provide effective protection against renal allograft rejection. However, their use is complicated by serious side effects. CNI-based immunosuppressive therapy causes nephrotoxicity and has adverse effects on blood pressure, lipid levels, and glucose homeostasis (29, 93), mTORi increase the risk of developing DSA, and may lead to leukopenia, thrombocytopenia, hypercholesterolemia, proteinuria, stomatitis and diarrhea (66, 74). All these side effects may cause eventually non-compliance and deterioration of graft function.

The first selective co-stimulation blocker belatacept represents a new option for a CNI-free regimen, avoids nephrotoxicity and preserves renal function over the long-term course. Because only little data is available on the immunological phenotype of belatacept treated patients, the aim of this study was to investigate and understand the diversity of different T- and B- cell subpopulations under the influence of three different immunosuppressive regiments, especially belatacept in renal adult transplant patients in a prospective manner. Until now, only very limited data on this important question exist in the literature, therefore, our study is one of the first systematic investigation on this topic.

5.1 Impact of T cells' expression level in renal transplantation after conversion to belatacept

T cells can contribute in multiple ways to early and late graft rejection in transplantation. The critical role of T cells in rejection has been established by the demonstration that T cells are involved in the rapid rejection of secondary allografts, T-cell activation is necessary to provide 'help' as a condition for B-cell activation and subsequent antibody production (45). Concerning T cells as one of the primary mediators of rejection processes, we were interested in investigating of T cell subpopulations and T cell function in renal transplant patients after conversion from either CNI or mTORi to belatacept. Unfortunately,

we didn't observe any significant changes in frequencies or absolute numbers of CD4+ cells after conversion from either CNI or mTORi to belatacept compared to pre-conversion time points and matched controls (Figure 4A, 4B). No changes for CD4+ frequency and absolute counts may indicate a stable immunological stimulate without increasing the risk for rejection after conversion to belatacept from either CNI or mTORi.

Regarding T-helper subsets, Th1 cells secrete IFN-γ and promote the cell-mediated immune response, whereas Th2 cells produce IL-4 and suppress Th1 cell-mediated response. Th17 cells produce IL-17A, IL-6, and TNF-γ involved in promoting inflammation in the pathogenesis of many diseases (32-34). Th17 cells represent a subset of T helper cells that can potentially lead or contribute to allograft rejection. In our study, Th1 and Th2 cell numbers were stable before and after conversion to belatacept from either CNI or mTORi over 6 months, respectively. But Th1 cells decreased slightly in the mTORi control group 6 months after conversion, in contrast to stable frequencies in CNI control group (Figure 5). These results were consistent with those of other groups, who found that belatacept-based regimen had only a limited or no effect at all on Th1 and Th2 (120). Belatacept may affect Th17 cells by decreasing secretion of IL17-A and TNF-γ (121,122). CTLA-4/B7 downregulate Th17 development in human naive CD4+ T-cells by blocking T cell differentiation into IL-17 and IL-22 producing cells (123). Furuzawa-Carballeda found that the amounts of Th17 markers were not higher in the group of patients receiving belatacept than in the cyclosporine treatment group (120). This observation is consistent with our results, where Th17 cells were decreased and showed significantly lower levels in belatacept at M6 compared to matched-CNI control (Figure 5E). This decrease in Th17 cells could contribute to an altered immune response, which may lead to less allograft rejection after conversion from CNI to belatacept, but theoretically could increase the susceptibility to infection.

Activated CD8+ T cells are usually cytotoxic T lymphocytes responding to antigenic challenge by lysis of the target cells (50). Donor-specific cytoxic T lymphocyte precursor frequency significantly decreased after CNI withdrawal (P=0.0001) (125). In our study, CD8+ cytoxic T cell frequencies were lower in belatacept treated patients, compared to

matched-CNI controls (Figure 8A). Interestingly, they showed no difference compared to matched-mTORi controls (Figure 8B).

Memory is the hallmark of the acquired immune system. Central memory T cells have little or no effector functions, but readily proliferate and differentiate into effector cells in response to antigenic stimulation. Effector memory T cells have a powerful and direct anti‐viral capacity e.g. to migrate to both nonlymphoid and lymphoid tissue (48) and during acute human CMV infection (126). Longer duration of human CMV replication is associated with a higher percentage of Human CMV-specific CD45RA expression on effector memory CD8+ T cells after transplantation (127). We used a panel containing CD3, CD4, CD8, CCR7 and CD45RA to identify naïve, central memory, effector memory and effector CD4+ and CD8+ T cells. Our data indicated that CD4+ effector memory, effector and central memory cells (Figure 6), and CD8+ effector, naïve and central memory cells (Figure 9) were not influenced by the conversion from CNI to belatacept over 6 months compared to pre-conversion. However, CD4+ central memory cells had lower expression compared to matched-CNI control at M3 ($p=0.041$). CD4+ Naïve cells increased after conversion from either CNI or mTORi to belatacept at M1, M3 and M6 compared to pre-conversion (Figure 6E, 6F). This increase may indicate that T cell differentiation is altered under co-stimulation blockade with belatacept compared to standard CNI therapy.

Interestingly, the amount of CD4+ naïve cells in mTORi control group was a little higher also compared to pre-conversion at M3 and M6 (Figure 6F). CD4+ effector memory cells decreased at M1 after mTORi patients conversion to belatacept compared to pre-conversion (Figure 9A), and CD4+ central memory cells decreased at M6 compared to pre-conversion and matched-mTORi control (Figure 9G). CD8+ effector and central memory cells were similar in belatacept converted from mTORi over 6 months (Figure 9D, 9H), but CD8+ effector memory cells decreased significantly at M3 and M6 in belatacept compared to the matched-mTORi group (Figure 9B).

CD28-CD57+ expressed on CD4+ central memory/ naive cells at M1, CD8+ central

memory at M1 and CD8+ naïve cells at M1 and M3 decreased after CNI patients conversion to belatacept compared to pre-conversion (Figure 10). CD28-CD57+ expression on CD8+ naïve cell had a lower level at M6 compared to matched-CNI control. The CD28 receptor lowers the threshold for T-cell activation, leads to IL-2 mRNA stabilization and to T-cell proliferation via binding to the ligands CD80 and CD86 on APCs (51). Memory cells could immediately produce effector cytokines in situ that recruit additional immune cells for early transplantation damage (45).A memory cell decrease and a naive cells increase could indicate that kidney transplant patients have less HLA antibody production, as evidenced by the result of the clinical trial (81).

Moreover, the percentage of Treg cells in the peripheral blood increased after CNI withdrawal (125). We analyzed the level of Treg cells defined as CD4+CD25+CD127low. Our data show that the percentage of Treg cells in CD4+ decreased in patients after conversion from CNI- or mTORi- treatment to belatacept-treatment over 6 months. Moreover, absolute numbers of Treg cells is in line with decreasing percentage of Treg cells in CD4+ cells compared to pre-conversion in belatacept (Figure 11). Grimbert et al. showed significantly lower ($p<0.001$) intra-graft expression levels of the mRNAs for Treg (FOXP3) in the belatacept group than the CNI group (128). These results are in agreement with our work. In vitro co-cultures using patients' Tregs and B cells found that Tregs could reduce IgG production and enhance B cell apoptosis, suggesting that transplant patients may produce less HLA-Ab (129). Only limited data on the relevance and functional capacity of Treg are available. We also analysed Treg function among the transplant patients (see below).

Our immunophenotyping study demonstrates that the conversion from either CNI or mTORi to belatacept affected the function of T-cells as important mediators of transplant rejection processes. The expression of activation markers when measured ex vivo is influenced. This is the first prospective investigation on this important topic in transplant patients.

5.2 Impact of B cells' expression level in renal transplantation after conversion to belatacept

As anti-HLA antibody is the major barrier in renal transplantation, it is highly important to note that B-cells and plasma cells are currently the major targets of treatment. B lymphocyte activation is mediated mainly by innate immune cells that bind to its receptor on B-cell and plasma cell. They are the main source of DSA in sensitized renal transplant recipients leading to chronic allograft dysfunction, and alloantibody levels often persist in parallel with clinical improvement after standard humoral rejection therapy (27, 130-131). Recent research found that the increase in B cell numbers reflects a specific expansion of transitional B cells (132, 133) and B cells that express inhibitory receptors (134), suggesting that these B cells may actively regulate the immune response to the transplanted kidney.

Our present study showed that CD19+ B cells didn't change after CNI conversion to belatacept over 6 months in frequency or in absolute number compared to pre-conversion or matched controls (Figure 13A, 13C), but CD19+ B cell frequency in lymphocyte was decreased at M6 in CNI conversion group ($p=0.047$, Figure 13A). In mTORi conversion group, CD19+ B cell percentage had decreased at M3 ($p=0.038$) after conversion to belatacept compared to pre-conversion (Figure 13D), when mTORi control showed no difference over 6 months in absolute number or percentage. Our data confirm previous findings from Chesneau et al. (135) that tolerant recipients had an elevated frequency of transitional and naive B cells and a decreased frequency of plasma cells, and moreover that B cells from tolerant patients produced more IL-10 and were less likely to differentiate into plasma cells in vitro than did B cells from patients receiving standard immunosuppression. They also found that B cells from tolerant recipients can suppress effector T cell responses in vitro in a granzyme B–dependent fashion (136). Furuzawa-Carballeda also found the frequency of CD19+/CD24high/ CD38high/CD27+ was lower in Belatacept-treated patients compared to CNI treated kidney transplanted patients (120). The results are in agreement with our study, that plasmablast B cell frequency of CD19+ cells had decreased after conversion from CNI to belatacept for 1 month. The observation

that at other time-points no significant difference was noticed suggests control B cell regulation (Figure 14A). Interestingly, the frequency increased also in the matched-mTORi group at M6 compared to baseline, however it was not significant in mTORi conversion group compared to pre-conversion or matched-mTORi control. At present, it is unclear whether these are normal fluctuations in stable patients or whether these changes represent true changes of the immunophenotyped B cell.

Assessing memory B cell frequencies against both class I and class II HLA antigens may be clearly detected in peripheral blood in patients with obvious allogeneic sensitization background, irrespective of the presence of circulating antibodies (81). It provided new insight into the mechanisms of the adaptive humoral alloimmune response taking place in kidney transplantation. In our study, after conversion from CNI to belatacept, the percentage of memory B cells had decreased at M6 compared to pre-conversion, however results were not significant compared to the matched-CNI group, which it decreased at M3 in CNI control group (Figure 15A). Belatacept converted from mTORi had no significant difference compared to pre-conversion or matched-mTORi group respectively, when matched-mTORi increased at M6 (Figure 15B). These data showed belatacept may decrease the memory B cell frequency and compared to the patients before conversion from CNI and mTORi immunosuppressive drugs. In fact, the analysis of the memory B cells culture supernatants confirmed the presence of the target HLA-specific antibodies, even in some patients with low HLA-specific antibody titers in the serum (81). Interestingly, a low but positive correlation between the frequency of HLA-sp memory B cells and the antibody MFI in the serum was observed, suggesting a direct contribution of the peripheral memory B cells compartment maintaining the levels of circulating antibodies within highly HLA-sensitized individuals (137-138). Therefore, the alterations of B memory compartment may partly explain lower DSA in belatacept treated patients.

5.3 Impact of Treg cell function in renal transplantation after conversion to

belatacept

Suppression of T-cel-mediated responses by Treg cells is fundamental to immune homeostasis and control of autoimmune disease (57, 71). T cells that were isolated from peripheral blood long term after transplantation were hypo responsive to alloantigens in Campath-1H and sirolimus or cyclosporine A-treated patients (139). Belatacept is a co-stimulator blocker, and is thought to interrupt the interaction between CD28 and CD80/86, thus preventing T cell activation by blocking the co-stimulatory second signal is necessary for autoimmunity in renal transplantation. Treg cells could suppress the functional ability of other cells, like preventing differentiation, activation and proliferation of effector T cells by secreting TGF-β and IL-10 (61). Notably, there was no evidence of specific interactions between T-effector (Teff) and regulatory T-cells (Treg) in vivo, based on these, we isolated Treg and Teff cells by magnetic sorting, the purity average is nearly 90% (data not shown), and then co-cultured Treg and Teff cells in different ratios. Teff cells had a significantly lower proliferation cultured alone after CNI conversion to belatacept at M3 and M6 compared to pre-conversion when no change was observed in the matched-CNI group (Figure 12B). When Teff cells were co-cultured with Treg cells, the proliferation of Teff cells was suppressed significantly, with a higher ratio of Treg/Teff cells in both belatacept and their matched-control groups. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent IL-2 production (82，140). We also found, that Teff cells display much lower proliferation in mTORi-based conversion and controls than CNI-based conversion and control groups, respectively (Figure 12). Interestingly, Treg cells function showed no significant difference after mTORi conversion to belatacept over 6 months. The data demonstrated that Tregs have the same suppressive capacity under belatacept as under CNI or mTORi. This may be caused by the fact that belatacept interrupts the interaction between CD28 and CD80/86, blocking the signal two. In vitro, it is clear that activated Treg can suppress Teff proliferation and cytokine production in the absence of any other cell types via direct cell-cell contact (141). CD25− to CD25+ conversion has been demonstrated in mice to generate fully functional Tregs (57, 142). The conversion requires IL-2 or antigen-stimulation and the ratios are determined by competition between

the two populations (57). These findings might give potential mechanism how Treg suppressed Teff cells and might lead into a new direction for future studies of immunosuppressive drugs like belatacept to benefit renal transplantation.

5.4 Impact of CD80 and CD86 expression in CD19+ B cells in renal transplantation after conversion to belatacept

CD80 and CD86 were found on activated B-cells, providing a co-stimulatory signal necessary for T-cell activation and survival. They belong to the B7 family of regulatory ligands and bind particularly to CD28 and CTLA-4 on T-lymphocytes (143, 144), an interaction classically considered as the main co-stimulatory and co-inhibitory leading for example to chronic rejection processes (145). Our data showed that CD80 expression on B cells decreased clearly compared to pre-conversion at M3 and M6 in belatacept converted from CNI (Figure 16A). Its decreasing expression prevented a co-stimulatory signal necessary for T- cell activation and survival. Interestingly, CD86+ expressed a higher level in belatacept at M6 compared to matched-CNI control. There is no change of CD80 and CD86 after conversion from mTORi patients compared to pre-conversion or matched-mTORi controls. Latek et al. found that whole blood from belatacept-treated patients had significantly lower levels of free CD86 receptors versus pretransplant levels, healthy volunteers, or cyclosporine-treated patients. CD86-receptor saturation correlated with belatacept dose/dose frequency and remained consistently more than 80% (114). These findings might give an additional role for B7 signaling in B-cell activation and might be of interest for future studies regarding any influence of belatacept as a potent B7-1/2 antagonist on immune cells in the transplant setting.

5.5 Impact of CD19+ B cell proliferation in renal transplantation after conversion to belatacept

B cells play an important and necessary role in humoral immunity and cellular immunity,

especially in acute and chronic graft rejection. This may affect the immune system through antigen presentation, cytokine production, immune regulation and differentiation into memory cells. The presence of functional T cells is crucial for B-cell inhibition, and cell-cell contact between mesenchymal stromal cells (MSCs) and T cells, but not between MSCs and B cells. (146). In our study, after conversion to belatacept, it was much more difficult to isolate enough CD19+ B cells for proliferation research, there are only 4 proliferation data at M3, 3 data at M6 in belatacept converted from CNI, 5 data at M3, 3 data at M6 in belatacept converted from mTORi, and we only get 2 full B cells proliferation data over 6 months from same patients, and it was decreased from some representative patients. Due to the low number of CD19+ B cells for study (Figure 17), we need more evidence to investigate inhibition of B cell proliferation by belatacept.

5.6 Limitation of the study

This is the first study about the change of immunophenotype after conversion to belatacept in renal transplanted patients. There is very limited data available in the literature to date about this subject. Nevertheless, there are several limitations of the present study.

There was a high diversity in patient demographics. Also, the data would have to be validated with higher numbers of patients to make a statistical power calculation possible. Also, different methods should be applied for validation in addition to the flow cytometry.

The data of the functional assays should be extended with analyses of cytokine production or proliferation capacity of different cell types. This would broaden the understanding of the underlying mechanisms triggered by conversion to belatacept.

5.7 Summary

Despite the fact that conventional immunosuppressive therapy after kidney transplantation had been optimized and excellent short-term outcomes achieved, the

effect on long-term allograft survival is still disappointing. Reasons for this are that long-term use of CNI causes some degree of nephrotoxicity, mTORi are associated with other side effects and might also increase de novo DSA production.

Belatacept is a selective co-stimulation blocker, which inhibits the interaction between CD28 and CD80/CD86. The high-affinity CTLA-4-Ig chimeric fusion protein therefore prevents T-cell activation. In the BENEFIT study, the results at 7-year post-transplantation showed that, compared with CsA, the risk of death or graft loss was significantly lower for belatacept-treated patients and the long-term renal function was significantly improved, thereby increasing the half-lives of transplanted kidneys (83, 116). Except for the overall clinical outcome of belatacept treatment, its influence on some aspects of T cell has been analyzed only in a few small studies (114, 120). CTLA-4/B7 negatively regulated Th17 development in human naive CD4+ T-cell differentiation into IL-17- and IL-22-producing cells (120). The amounts of Th17 markers were higher in the group of patients receiving cyclosporine than in the belatacept treatment group (121). In our study, Th17 cells showed significantly lower levels at M6 compared to matched-CNI controls, it also supported previous work, whereas Th1 and Th2 showed no difference. In the mTORi control group, Th1 expression had decreased significantly at M6, but no change was observed after conversion to belatacept. Donor-specific cytoxic T lymphocyte precursor frequency significantly decreased after CNI withdrawal (P=0.0001) (125). Our result showed that CD8+ cytoxic T cells expressed a lower level at M3 in belatacept compared to matched-CNI controls and no difference compared to matched-mTORi control. The decrease in cytoxic CD8+ T cells may be one of the reasons that benefit long-term life qualities for renal transplant patients after conversion to belatacept.

We found CD4+ central memory cells frequency is to be found at lower levels in belatacept compared to matched-CNI controls. CD4+ naive cells increased in belatacept conversion from either CNI or mTORi at M1/3/6 compared to pre-conversion. There was also higher expression in belatacept compared to matched-mTORi control at M3. CD8+ effector memory cells decreased at M1 and lower level was observed compared to matched-mTORi control group at M3 and M6. In contrast, CD8+ naïve cells increased at

M1 in belatacept conversion from mTORi compared to pre-conversion. The decrease in memory cells and increase in naive cells may indicate some potential effect in long-term treated by belatacept. Because effector memory T cells can recirculate in peripheral tissues, memory T cells may be rapidly recruited and initiate early responses directly at the graft site. They could immediately produce effector cytokines in situ that recruit additional immune cells for early transplantation damage. Alloreactive central memory T cells in lymphoid tissue may also be activated early after graft rejection and subsequently migrate to the graft site (44, 45).

Treg cells had lower expression in the belatacept group compared with pre-conversion and matched-CNI or mTORi control group, Grimbert showed significantly lower ($p<0.001$) intra-graft expression levels of the mRNAs for Treg (FOXP3) in the belatacept group than the CNI group (128). In vitro, it is clear that activated Treg can suppress Teff proliferation and cytokine production in the absence of any other cell types via direct cell-cell contact (142). Due to Treg cells' indispensable role in immune response, we performed a Treg suppression assay. The suppression assay investigated the capacity of Tregs to suppress Teff cell proliferation. The result showed significant lower proliferation of Teff cells cultured alone after CNI conversion to belatacept at M3 and M6 compared to pre-conversion. The frequency and absolute number of Treg cells both decreased after conversion to belatacept, but Teff cells proliferation was also suppressed even cultured alone after conversion to belatacept from CNI or mTORi. This means the belatacept may effect Teff cell function directly or indirectly, the underlying mechanism needs to be further investigated and explored.

B cells play a central role in humoral immunity and in boosting cellular immunity, especially in acute and chronic graft rejection. Compared to pre-conversion, plasma blast cells at M1 and memory B cells at M6 decreased also in belatacept patients after conversion from CNI. CD19+ cells at M3 and plasmablast cells at M1 decreased after mTORi conversion to belatacept, CD19+ cells and memory B cells had a lower expression at M6 in the CNI control group, when plasmablast and memory B cells had significantly higher frequencies at M6 in the mTORi control group. The lower CD19+ cells, plasmablast

and memory B cells in belatacept compared to pre-conversion may suggest a lower risk for chronic humoral rejection.

CD80/CD86 provide a co-stimulatory signal necessary for T-cell activation and survival and binds particularly to CD28 and CTLA-4 on T-lymphocytes (114, 143). In our study, CD80 showed a lower level after conversion from CNI to belatacept at M3 and M6 compared to pre-conversion by stimulated pure CD19+ cells. CD86 showed a slightly higher level at M6 in belatacept compared to matched- CNI controls. And our data showed less or not impact at all on CD80/CD86 expression after mTORi conversion to belatacept. These findings might suggest an additional role for B7 signaling in B-cell activation and might be of interest for future studies regarding any influence of belatacept as a potent B7-1/2 antagonist on immune cells in the transplant setting.

To evaluate the function of CD19+ cells, only a small complete set of data from patients in belatacept and CNI or mTORi control groups was available for analysis. It seems that the proliferation decreased at M3 and M6 in belatacept conversion from mTORi. One limitation of our study was that we didn’t get enough CD 19+ cells after conversion to belatacept. More evidence is needed for exploration of B cells under belatacept therapy.

All these studies indicate that belatacept has complex effects on different immune cells in patients after renal transplantation. In conclusion, renal transplant patients assigned to belatacept conversion from either CNI or mTORi had alterations in T- and B- cell subpopulations, phenotype and functions, as compared with pre-conversion and matched-CNI or mTORi control patients.

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7 ABBREVIATIONS

CNI	Calcineurininhibitor
mTORi	Inhibitor of the mammalian target of rapamycin
GRF	Glomerular filtration rate
ESRD	End-stage renal disease
ET	Eurotransplant
ETKAS	ET kidney allocation system
ESP	European senior program
ACR	Acute cellular rejection
AMR	Antibody-mediated (humoral) rejection
DSA	Donor-specific antibody
HLA	Human leukocyte antigens
cGVHD	Chronic graft-versus-host disease
APCs	Antigen-presenting cells
MHC	Major histocompatibility complex
TCR	T-cell receptors
IL	Interleukin
NFAT	Nuclear factor of activated T cells
IFN-γ	Interferon gamma
Tregs	Regulatory T cells
nTregs	Naturally Tregs
iTregs	Induced Tregs
Teff	Effector T cells
Con A	Concanavalin A
CsA	Cyclosporin A
CTLA-4	Cytotoxic T lymphocyte-associated protein 4.
CpG-ODN	CpG oligodeoxynucleotides
IL-2	Interleukin-2

FDA	Food and Drug Administration
MMF	Mycophenolate mofetil
PTLD	Post-transplant lymphoproliferative disease
APC	Antigen Presenting Cell
BMS	Bristol-Myers Squibb
CMV	Cytomegalovirus
$KHCO_3$	Kaliumhydrogencarbonat
FCS	Fetal Calf Serum
FACS	Fluorescence activated cell sorting
SRL	sirolimus
TAC	tacrolimus
PBS	Phosphate Buffered Saline
PCNA	proliferating cell nuclear antigen
PI	Propidiumiodid
PMA	12-Phorbol-13-Myristat-Acetat
Na_2-EDTA	Dinatrium-Ethylendiamintetraacetat
CTV	Cell trace violet
CBL	Control baseline
CM3	Control month 3
CM6	Control month 6
BL	Baseline
M3	Month 3
M6	Month 6
MSCs	Mesenchymal stromal cells

8 Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

9 PUBLICATIONS

1. Matz M, Fabritius K, **Liu J**, Lorkowski C, Brakemeier S, Unterwalder N, Dürr M, Mashreghi MF, Neumayer HH, Budde K. Conversion to Belatacept based regimen does not change T-cell phenotype and function in renal transplantation. Transplant Immunology. 33.3 (2015): 176-184.
2. **Liu, J**., He, J., Huang, L., Dou, L., Wu, S., & Yuan, QL. Neuroprotective effects of ginsenoside Rb1 on hippocampal neuronal injury and neurite outgrowth. Neural regeneration research, 2014, 9(9), 943-950.
3. **Liu J**, Qiu Y, He J, Huang L, Wen L, Guan XF, Yuan QL. Effects of ginsenoside Rb1 and Rg1 on hippocampal neurons. Journal of Tongji University (Medical science).2011, 32(3):1-7
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5. Gao XQ, Yang CX, Chen GJang, WGY, Chen B, Tan SK, **Liu J**,Yuan QL. Ginsenoside Rb1 regulates the expressions of brain-derived neurotrophic factor and caspase-3 and induces neurogenesis in rats with experimental cerebral ischemia. Journal of Ethnopharmacol. 2010. 132(2):393-399

10 Affidavit

“I, Juan, Liu certify under penalty of perjury by my own signature that I have submitted the thesis on the topic Early detection of the immunophenotyping after conversion to Belatacept in comparison to conventional immunosuppressive therapy in renal transplant patients I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

Signature, date and stamp of the supervising University teacher

_____

Signature of the doctoral candidate

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11 ACKNOWLEDGEMENT

It is a big project to finish the dissertation, even only my name is shown in the dissertation, actually many persons supported me in different ways. I really want to send my acknowledgement to them for helping me during my doctoral work in Germany.

In the first and the most important, I would like to thanks my supervisor **Professor Dr. med. Klemens Budde**, particularly thanks him to give me this cherished opportunity to join in the research lab. Please let me express my deepest gratitude to Professor Budde. He supported my idea and helped me a lot in the research and in my life in Berlin. And I was so happy and lucky to meet all the people in our lab.

I am very grateful to **Dr. Michael Dürr** and **Dr. Mareen Matz**, Firstly, thanks them for trusting me to perform the collaboration research work of belatacept. Secondly, thanks for their patience and instructions all the time. As a learner in the field of transplantation and Immunology, **Dr. Dürr** taught me not only experiment research but also related scientific theories, which make me think more deep and wide in the research work. **Dr. Matz** taught me how to figure out the problems which I met during my study, her kind continuous encouragement always touched me whenever I occasionally frustrated by research work.

I am so happy and lucky that I could work in the most comfortable research environment in the research lab with department of Nephrology. I appreciate **Katharina Fabritius** greatly for teaching experiment skills and helping me a lot in my whole research work in Berlin. Thank her for taking care patients' schedule, sharing my heavy work and helping me in the weekend work, she also encouraged me a lot when I frustrated by research and life problems. Thank **Marco Mai** and **Christine Lorkowski** always for helping me do the work during my holidays, and I would never forget one scene that both them persuaded me to go home for rest when I was sick. Thanks **Dr. Pia Hambach**, **Dr. Petra Glander**, **Olesja Rissling**, **Alma Hadzic-Rustemi**, **Julie Künzel**, **Laili Anwari**, **Luisa Deutsch** supported my work and kind helps.

I want to acknowledge any other persons, thanks friends **Dr. Kaiying Wu**, **Raluca Fleischer**, **Dr. Ye Zhu** for their kind helps and **Dr. Hui Li**, **Dr. Nan Zhu**, **Mufang Yang**, **Jingyi Zhang and Qiang Zhang** sharing happy time in Berlin. Thank **Dandan Luo**, **Weigui Zhou**, **Wenjie Han**, **Zongjin Feng and Huanhuan Jiang** for deepest supporting and encouragements in research area and life all the time over past years.

The most importantly, I am indebted to **my mother** for her deepest love and understanding. I feel very sorry that I spent too less time with her since I was learning and doing research. And she always told me good news, never told me she was sick and unhappy, especially only she and I in our small family. I am very grateful for her supporting and understanding.

Finally, I enjoyed the research time under such good environment in Berlin. I would never forget the days I stayed with the whole lab members together. These years gave me invaluable and unforgettable experience in my life. I appreciate greatly for all supports, helps and encouragements, thanks all the other people who have contributed to the success of my dissertation.