Benefit of Belatacept in Cord Blood-Derived Regulatory T Cell-Mediated Suppression of Alloimmune Response

Xing He¹, Sang Li¹, Juan Zhang¹, Lu Cao¹, Cejun Yang¹, Pengfei Rong¹, Shounan Yi¹,², Kedar Ghimire², Xiaoqian Ma¹, and Wei Wang¹

Abstract
The role of Regulatory T cells (Tregs) in tolerance induction post-transplantation is well-established, but Tregs adoptive transfer alone without combined immunosuppressants have failed so far in achieving clinical outcomes. Here we applied a set of well-designed criteria to test the influence of commonly used immunosuppressants (belatacept, tacrolimus, and mycophenolate) on cord blood-derived Tregs (CB-Tregs). Our study shows that while none of these immunosuppressants modulated the stability and expression of homing molecules by CB-Tregs, belatacept met all other selective criteria, shown by its ability to enhance CB-Tregs-mediated in vitro suppression of the allogeneic response without affecting their viability, proliferation, mitochondrial metabolism and expression of functional markers. In contrast, treatment with tacrolimus or mycophenolate led to reduced expression of functional molecule GITR in CB-Tregs, impaired their viability, proliferation and mitochondrial metabolism. These findings indicate that belatacept could be considered as a candidate in Tregs-based clinical immunomodulation regimens to induce transplant tolerance.

Keywords
cord blood-derived tregs, belatacept, tacrolimus, mycophenolate, immunosuppressant

Introduction
Solid organ transplantation, a well-established clinical procedure is used to treat patients with end-stage organ failure and save lives every year. However, its frequent use in the clinical setting has been hampered by several issues, including shortage of organ donors, and organ rejection by recipients’ immune system, leading to unfavorable transplant outcomes. The use of immune inhibitors can effectively prevent acute rejection, but it has a marginal impact on long-term graft survival and may result in other adverse effects¹,². Therefore, special attention should be paid to the development of novel therapeutic strategies that can help achieve donor-specific hyporesponsiveness.

During the last two decades, many in vitro and in vivo experiments have demonstrated regulatory T cells (Tregs) as a promising cellular therapy which can play an important role in maintaining immunological tolerance in graft-versus-host disease³, autoimmune diseases⁴, and transplantation⁵. In humans, Tregs are commonly defined as a population of CD4⁺CD25hiCD127low T cells that depend on the Foxp3 transcription factor for their development and function⁶. Studies have shown that Tregs can suppress various immune cells including T effector cells (CD4⁺ and CD8⁺), natural killer cells (NKCs), B cells and dendritic cells (DCs) to prevent rejection by various immunosuppressive mechanisms⁷. Tregs’ immunosuppressive function do not only depend on the number but also rely upon the secretion of inhibitory cytokines (IL-10, TGF-β) and expression of function molecules⁸. Stability and function are important

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issues in Tregs therapy and can be influenced by cell metabolism which has been shown to play a critical role in T-cell activation, differentiation, and function. While effector T cells utilize oxygen-dependent metabolic pathway such as glycolysis when activated for energy, Tregs mainly rely upon mitochondrial oxidative phosphorylation to generate ATP.

Clinical graft tolerance is difficult to achieve with a single infusion of Tregs without immunosuppressants. Most immunosuppressive drugs not only inhibit effector T cells, but also negatively affect Tregs. Hence, immunosuppression strategies that selectively inhibit effector T cells while preserving or even enhancing Tregs may allow for the minimization of immunosuppressant-associated toxicities. The safety and validity of adoptive transfer of Tregs isolated from adult peripheral blood (APB) or umbilical cord blood (UCB), has been demonstrated in several phase I/II clinical trials. Such as, studies indicated that adoptive transfer of peripheral blood derived Tregs prevented GVHD and promoted immune reconstitution in hematopoietic stem-cell transplantation (HSCT). In addition, Brunstein et al have used KT64/86-expanded UCB Tregs for adoptive cell therapy in GVHD in humans. The study reported a decrease of acute GVHD, which presented evidence of the safety and promising efficacy of adoptive UCB Tregs therapy. Furthermore, Jennifer et al have observed expanded UCB Tregs that induced immune tolerance in two patients with COVID-19 ARDS. Compared to APB Tregs, UCB Tregs are mostly naive and have higher expression of CD25, providing stronger immunosuppressive function. This naive phenotype is associated with a significantly enhanced proliferation potential and naive Tregs under the influence of a specific immunological environment may develop into memory Tregs with the appropriate tissue-specific homing and functional molecules. The superior performance of UCB-derived over APB-derived Tregs has been shown in mice skin transplantation model and this may be attributable to the greater persistence of UCB-derived Tregs in the early phase after adoptive transfer. However, it is unclear whether immunosuppressive drugs exert any effect on UCB-Tregs, and if they do, what those effects are.

Through this study, we aim to demonstrate the effect of commonly used immunosuppressive drugs on UCB-Tregs’ viability, proliferation, metabolism, and immune suppressive function. It will deliver valuable information to considering while selecting the Tregs-friendly immunosuppressive regimens and provide guidelines for the use of improved cellular therapies in transplantation.

**Material and Methods**

**Blood Donors and IRB Ethics**

Ethical consent was obtained from the 3rd Xiangya Hospital, Central South University, Hunan province (China batch number: NO:2019-S550) for all experimental procedures. Cord blood (50-60 ml) was collected from the umbilical vein after the umbilical cord was detached from the infant and the placenta was removed from the uterus.

**Isolation and Ex Vivo Expansion of UCB-Tregs**

Cord blood monocyte cell (CBMC) were isolated from cord blood following established protocols, using Ficoll-Paque Plus (GE, USA) gradient centrifugation. CD4⁺CD25⁺ Tregs with purity >90% were isolated from CBMC using CD25 microbeads (BD Biosciences, San Diego, CA, USA) according to the manufacturers’ instructions. The CD4⁺CD25⁺ Tregs were expanded with CD3CD28 microbeads (Thermo Fisher Scientific, Lithuania) at a ratio of three cycles (7 days/cycle) in RPMI1640 media containing 10% Human serum (Villey Biomedical, USA), 800 IU/mL IL-2 (Chiron, Emeryville, CA), 2 mM glutamine, 25 mM HEPES, 50 U/mL penicillin, 50 μg/mL streptomycin, 100 μL 2-mercaptoethanol, and 100 nM rapamycin (Sigma-Aldrich, San Luis, MO USA) at 37°C and 5% CO₂. Tregs were collected after expansion for 14–21 days for use in all subsequent experiments.

**Flow Cytometry for Tregs Characterization**

Fluorochrome-coupled antibodies specific for human antigens: anti-CD4-FITC, anti-CD127-PE-Cy7, anti-CD62L-PE, anti-CCR4-PE, anti-CCR7-PE, anti-CCR10-PE, anti-CXCR3-PE-Cy5, anti-HLA-DR-FITC, anti-CD25-PE-Cy7, anti-CD39-PE, anti-CD44-PE-Cy7, anti-CD45RA-PE-Cy5, anti-GITR-PE, anti-CD45RO-PE, and anti-CD28-PE (all from eBioscience, USA) were used for cell surface staining. Monoclonal antibodies (mAbs) anti-Foxp3-PE, anti-CTLA-4-PE-Cy7, anti-Helios-FITC, and anti-IDO (indolamine-2,3-dioxygenase)-PE-Cy7 (all from BD Pharmingen, USA) were used for intracellular staining. Quantitative analysis was performed using FlowJo software.

**In Vitro Proliferation and Viability Assays**

Tacrolimus (TAC) and mycophenolic acid (MPA) (Sigma-Aldrich, USA) were diluted in dimethyl sulfoxide (DMSO) and belatacept (BEL) (NulojixTM, USA) diluted in saline were used at different concentrations (see figure legends for details). To assess viability, expanded Tregs were co-cultured in different concentrations and combinations of immunosuppressive drugs (TAC, MPA, BEL) in complete RPMI 1640 medium (10% Human serum, 20 IU/ml IL-2, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) for 5 days, after which MTS (Sigma-Aldrich, USA) was added to the medium. After 3 hours of co-culture, the absorbance was measured at 450 nm. In addition, the AO/PI kit (LUNA-FL, Korea) was also used to detect cell viability according to manufacturer’s instruction. The proliferation capacity was measured by a CellTrace™ CFSE Cell Proliferation Kit (CFSE) (Invitrogen, USA). Expanded Tregs or

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human PBMC (1 × 10⁵/well) stained with CFSE were cultured with irradiated allo PBMC (ratio 1:3) or CD3CD28 microbeads with different immunosuppressive drugs in 96-well plates (Corning Costar, USA) in complete RPMI 1640 medium for 5 days. They were then harvested and analyzed by flow cytometer.

**Tregs Suppressive Function Assay**

The suppressive capacity of Tregs was assessed by allo-MLR assays. CFSE-labeled responder cells (human PBMC 1 × 10⁵/well) were cocultured with 3 × 10⁵ irradiated allo PBMC. Tregs were titrated into different MLR cultures at different ratios. After 7 days, proliferation of responder cells (CFSE-positive cells) was evaluated based on the percentage of proliferating responder cells cultured in the absence of Tregs compared to the percentage cultured in the presence of Tregs. The percentage of proliferating responder cells in the absence of Tregs was considered as proliferating at 100% and 0% of suppression. The supernatant of the MLR medium was harvested for CBA (Human Th1/Th2/Th17 Cytometric bead array kit, BD Pharmingen, USA) assay according to the manufacturer’s instructions.

**Tregs Stability Assay**

Expanded Tregs were cultured with a single immunosuppressive agent (BEL, TAC or MPA) or without drug (control) for 5 days in the presence of pro-inflammatory cytokine cocktails which consisted of IL-2 (10 IU/mL, R&D Systems, Minneapolis, MN, USA), IL-6 (10 ng/mL, R&D Systems, Minneapolis, MN, USA), IL-21 (25 ng/mL, R&D Systems, Minneapolis, MN, USA), IL-1β (10 ng/mL, R&D Systems, Minneapolis, MN, USA), and or IL-23 (25 ng/mL, R&D Systems, Minneapolis, MN, USA). Cells cultured in complete medium supplemented with IL-2 (10 IU/mL) were used as controls to ensure their survival throughout the 6-day stability assay. For intracellular cytokine analysis, cells were stimulated with phorbol myristate acetate (PMA) 50 ng/mL and ionomycin 1 μg/mL (Sigma-Aldrich, USA) in the presence of protein transport inhibitor (GolgiPlug™, BD Biosciences, USA) for 4 h. Subsequently, cells were analyzed for IL-17 and IFN-γ (BD Biosciences, USA) expression by flow cytometer.

**Extracellular Metabolic Flux Analysis**

The metabolic profile of Tregs treated with different drugs and cultured for 5 days was evaluated. For this, harvested cells were plated in XF-24 plates (Agilent, USA) at the concentration of 6 × 10⁵ cells/well and cultured in XF media (non-buffered RPMI 1640 containing 25 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate). Real-time measurements of oxygen consumption rates (OCR) of the cells were analyzed under basal conditions and compared with their response to mitochondrial inhibitors: 1 μM oligomycin, 2 μM of carbonyl cyanide-4-(trifluoromethoxy) -phenylhydrazone (FCCP), 0.5 μM of antimycin and 100 mM rotenone (Agilent, USA) on the XF-24 extracellular flux analyzer ²⁴(Agilent, USA).

**JC-1, Apoptosis and Mitotracker Staining**

Mitochondria of Tregs were stained with MitoTracker Red CMXRos (Invitrogen, USA) for 20 min. Then, the cells were washed twice with PBS and fixed using formaldehyde. Next, the fixed cells were stained for 15 min with DAPI (Invitrogen, USA). Images were taken using a confocal laser scanning microscope. To analyze the mitochondrial mass, Tregs were stained with Mitotracker green (Invitrogen, USA) for 30 min. Then, the cells were washed twice with PBS and were analyzed by flow cytometer. JC-1 probe (Invitrogen, USA) was employed to measure mitochondrial depolarization in Tregs. Briefly, cells cultured in 96-well plates after indicated treatments were incubated with an equal volume of JC-1 staining solution (5 μg/ml) for 20 min and washed twice with PBS. Mitochondrial membrane potentials were monitored by flow cytometer. Mitochondrial depolarization is indicated by an increase in the green to red fluorescence intensity ratio. Apoptotic Tregs were analyzed by Annexin V/PI double dye methods. After washed 2 times with PBS, Tregs in each group were labeled with Annexin V-FITC and PI following the instructions of Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, USA).

**Statistical Analysis**

Data are presented as the mean ± SD (unless otherwise indicated). Comparisons between groups were analyzed using unpaired Student’s t tests or Mann-Whitney U non-parametric tests. Statistical significance was established at the levels of *P < 0.05, **P < 0.1, ***P < 0.001, ****P < 0.0001; where a P < 0.05 was considered statistically significant.

**Results**

Treatment with MPA but not BEL or TAC, impaired CB-Tregs viability and proliferation capacity. To investigate whether immunosuppressive drugs (ISDs) affected the viability of Tregs in vitro, we tested their influence using a range of concentrations (TAC: 0–20 ng/mL; MPA: 0–3 μg/mL; BEL: 0–20 μg/mL) including their maximum therapeutic efficacy on the viability of Tregs in culture for 5 days. The MTS results showed that MPA significantly impaired the viability of Tregs in a dose-dependent manner, however BEL and TAC did not affect it significantly (Fig. 1A–C). We also detected that if the maximum concentration of DMSO (0.08%) which used to dissolve MPA and TAC would affect the viability of Tregs and found no negative effect on Tregs (Fig. 1D). To explore whether the
immunosuppressive drugs could suppress the proliferation of Tregs, CFSE assay was utilized to detect the Tregs expansion. For this, we selected the doses that lead to 50% (LD50) of Tregs viability, that is, (0.3 μg/mL MPA, 10 μg/mL BEL, and 10 ng/mL for TAC). We observed that BEL and TAC did not affect the proliferation of Tregs at their clinically relevant doses while MPA significantly inhibited it regardless of the stimulator: allo PBMC or CD3/CD28 microbeads (Fig. 2A). To clarify these results further, direct effect of ISDs on human PBMC stimulated with CD3/CD28 microbeads or allo PBMC was also examined. Our findings show BEL, TAC, and MPA could inhibit the proliferation of human PBMC stimulated by allo PBMC. However, BEL had no effect on human PBMC proliferation, activated by CD3/CD28 beads while TAC and MPA did affect it (Fig. 2B).

**TAC but not BEL or MPA Treated CB-Tregs showed Reduced Expression of Functional Molecule GITR.**

To investigate whether the immunosuppressive drugs affected the Tregs phenotype stability and their homing ability, we collected previously expanded Tregs that were cultured with TAC, MPA, or BEL for 5 days. In our study, we observed that the level of expression of critical molecules (CD25, CD127, FOXP3) on Tregs was not affected by the treatment with TAC, MPA, or BEL. Moreover, BEL and MPA did not affect the expression of Tregs functional molecules (CTLA-4, GITR, ICOS, IDO, CD39, CD73), and only TAC showed negative effect on functional molecule GITR on Tregs at therapeutically relevant concentrations. In addition, BEL, TAC, and MPA did not affect Tregs activation and memory marker (CD45RA, CD45RO, HLA-DR, CD44, and CD27) expression and they also did not change the expression of chemokine receptors (CD62 L, CCR4, CCR6, CCR7, CCR10, CXCR3, and CLA) of Tregs (Fig. 3B).

**BEL, TAC, and MPA did not Impact CB-Tregs Stability Under an Inflammatory Environment**

Published information indicate that Tregs are recruited directly to the site of inflammation25. Thus, the CB-Tregs treated with different drugs were tested for their plasticity towards effector Th17 or Th1 cell phenotype under pro-inflammatory conditions. Tregs under ISDs treatment were stimulated with a combination of pro-inflammatory cytokines for 5 days prior to detecting the proportion of CD4+Foxp3+ Tregs co-expressing IL-17 or IFN-γ. After stimulation, there was no significant change in frequency of IL-17 and IFN-γ producing T cells in either Tregs treated with or without ISDs (Fig. 4A–E). Furthermore, stabilized Foxp3 expression is also important for Tregs in inflammatory environment. Our FACS results show that Helios and
Foxp3 were still highly expressed in all of the Tregs group (Fig. 4F).

CB-Tregs Suppressive Capacity in the Allogeneic Response Was Enhanced Only in the Presence of BEL but Not MPA or TAC

The immunosuppressive potential of Tregs is commonly assessed in the Tregs suppression assay which measures the suppression of proliferation of responder cells by Tregs. Suppression is also determined by evaluating the ability of Tregs to repress cytokine production by the responder cells. Thus, allo MLR assay and CBA were used to detect if Tregs combined with ISD would enhance the suppression of activated PBMC. Results (Fig. 5A) showed that at the ratio of Tregs: PBMC (0:1), ISD (BEL, TAC, or MPA) can significantly suppress the proliferation of Teff compared to the control group. While in Tregs: PBMC (1:1) ratio, Tregs showed strong suppressive capacity with or without ISD. But when Tregs: PBMC ratio was low (1:64), the combination of Tregs and ISDs showed stronger suppression ability in MLR than Tregs alone. However, this Tregs suppressive ability at a low ratio to PBMC (1:64) was not as strong as one achieved with TAC or MPA treatment alone. The ISD alone (TAC or MPA) treated group and the group treated with the combination of Tregs with ISD (BEL, TAC, or MPA) had similarly suppressive capacity. More interestingly, treatment with the combination of Tregs and BEL had stronger suppression than one treated with either Tregs or BEL alone, which suggested a synergistic effect of BEL and Tregs. These results were supported by the results of CBA assay. The production of IL6, IFN-γ, and TNF-α was much lower in a group of Tregs treated with BEL compared to Tregs alone or only BEL-treated group (Fig. 5B). Moreover, the combination of Tregs with MPA or TAC did not show the synergistic effect compared to MPA or TAC alone-treated group. We further decreased the concentration of Tac and MPA in MLR assay and found that at the ratio of Tregs: PBMC (0:1), low dose of ISD (TAC, or MPA) can still suppress the proliferation of Teff to some degree compared to the control group. In Tregs: PBMC (1:1,1:4,1:16,1:64) ratio, the group treated with TAC had similarly suppressive capacity compared to the control group. However when cocultured with MPA, the proliferation of PBMC was even higher than control group at the ratio of Tregs: PBMC (1:64) (Fig. 5C).

In polyclonal MLR assay we found at all ratios of Tregs: PBMC (from 1:1 to 1:64), the group treated with the combination of Tregs with BEL had similarly suppressive capacity on the proliferation of human PBMC stimulated by CD3CD28 microbeads compared to the control group, which showed no synergistic effect of BEL and Tregs (Fig. 5D).

Mitochondrial metabolic status of CB-Tregs affected by MPA, but not BEL and TAC

Cell metabolism plays a critical role in T-cell activation, differentiation, and function. Tregs are thought to primarily rely on oxidative phosphorylation (OXPHOS) for energy need. Thus, we measured the bioenergetics of ex vivo expanded CB-Tregs which was cultured with medium alone or in the presence of single immunosuppressive agent (BEL, TAC or MPA) for 5 days. We found that compared to the control group, BEL and TAC treatment groups did not show any change compared to the control group. While MPA-treated Tregs exhibited increased OXPHOS, including higher baseline levels (43.15 ± 2.687 vs. 93.35 ± 6.160 pmol/min), ATP-linked oxygen consumption rates (30.98 ± 3.485 vs. 80.48 ± 6.184), attenuated maximal OCR (229.3 ± 26.76 and 478.2 ± 45.26 pmol/min) after FCCP injection, and the corresponding spare respiratory capacity (SRC) levels (186.1 ± 24.72 and 384.9 ± 50.96 pmol/min). (Fig. 6A–E.)
To determine whether these differences were the consequence of different mitochondrial contents between the groups, we stained all the Tregs with Mitotracker Red CMXRsos, a fluorescent, cell-permeable dye that stains mitochondria providing valuable information on mitochondrial mass and is dependent on mitochondrial membrane potential. Our confocal microscopy and FACS results indicate that MPA-treated cells had more mitochondrial mass...
Compared to control treatment, which suggested the cells were more prone to death. Comparable results were achieved with JC-1 and Apoptosis staining, MPA treated Tregs had lower mitochondrial membrane potential and expressed higher levels of the apoptosis rates than control group (Fig. 7C, D).

**Discussion**

Tregs therapy is already in clinical trial in renal transplant patients (the One Study) showing its safety and fewer infection-related complications. However at present, in all transplantation-related immunosuppression design, immunosuppressive drugs are unavoidable. Previously, several studies have focused on the viability and function of Tregs affected by the immunosuppressant. However, it was not clear if the stability and metabolism play a crucial role for Tregs function. Thus, to better understand the effects of commonly used immunosuppressive drugs on Tregs and to select a Tregs friendly immunosuppressant, we tested the viability, proliferation, stability, function and metabolism of Tregs treated with the calcineurin inhibitor (tacrolimus), mainstay of immunosuppression regimen (mycophenolate, a de novo purine synthesis inhibitor) and the first B7/CD28 co-stimulatory blocking agent (Belatacept).

Belatacept is a recombinant protein containing the ectodomain of human CTLA4 that interrupts the CD28-B7(CD80-CD86) co-stimulatory pathway, thus preventing effective T cell activation and proliferation. Our results are consistent in that belatacept inhibited the proliferation of human PBMC stimulated by allo antigen (Fig. 2A) but not CD3CD28 microbeads (Fig. 2B). Belatacept was approved by the U.S. Food and Drug Administration (FDA) in 2011 as an immunosuppressive agent for renal transplantation. However, despite promising results in preclinical and clinical trials and FDA approval a decade back, clinical use of belatacept still remains limited, in part due to severe acute rejection. Previous studies have shown that in comparison to the calcineurin inhibitor (CNI)-based immunosuppressive regimens, although rejection risk increased with the new belatacept-based regimens; reduced side effects and diminished long-term cardiovascular risk remain as major advantages of these regimens. One vital aim remains, which is to reduce rejection rates under belatacept-based therapy. If a
Figure 5. BEL but not MPA or TAC, combined with Tregs enhances the suppression of activated PBMC compared to BEL alone or Tregs alone. (A) CFSE-labeled human PBMC as responder activated by irradiated allo peripheral blood mononuclear cells (PBMC) were cultured with Tregs (Tregs: responder cell ratio at 0:1, 1:1, 4:1, 1:6, 1:64) in the presence of medium (control), or single immunosuppressive agent (TAC (10 ng/ml), BEL (10 μg/mL), or LD50 MPA (0.3 μg/mL)), for 7 days. The proliferation of activated human PBMC was determined by flow cytometric analysis. (B) The supernatants were collected to test the concentration of IL-6, TNF-α, and IFN-γ by human CBA assay. (C) CFSE-labeled human PBMC as responder activated by irradiated allo peripheral blood mononuclear cells (PBMC) were cultured with Tregs (Tregs: responder cell ratio at 0:1, 1:1, 1:4, 1:16, 1:64) in the presence of medium (control), or single immunosuppressive agent (TAC (5 ng/ml), MPA (0.15 μg/mL)), for 7 days. The proliferation of activated human PBMC was determined by flow cytometric analysis. (D) CFSE-labeled human PBMC as responder activated by CD3CD28 microbeads were cultured with Tregs (Tregs: responder cell ratio at 0:1, 1:1, 1:4, 1:16, 1:64) in the presence of medium (control), or single immunosuppressive agent (BEL (10 μg/ml)), for 7 days. The proliferation of activated human PBMC was determined by flow cytometric analysis. Data are from three independent experiments with Tregs and responder PBMC from three individual donors and presented as mean ± SD. *P < 0.05, **P < 0.01.
strategy can be developed to enhance the immunosuppressive function of belatacept without increasing the side effects, it would be a promising therapy in transplantation tolerance induction.

The adoptive transfer of Regulatory T cell represents a promising therapeutic strategy for immunomodulation in transplantation. Umbilical cord blood is an exceptional source for Tregs, due to convenient obtaining procedure and relatively low exposure of the fetus, which has been successfully tested in GvHD disease clinical trial and several other positive characteristics. Studies have been shown that multiple cellular signals control the development, differentiation and function of Tregs. Induced Tregs have inherent plasticity which is prone to destabilize in strongly inflammatory environment. Thus, careful consideration of the effects of immunosuppressive drugs on the stability, viability, proliferation capacity, migrating ability and phenotype of Tregs is important for their function. Our findings demonstrate that clinically relevant dose of immunosuppressive drug (BEL) and LD₅₀ MPA did not affect the expression of functional molecules and chemokine receptors of Tregs while TAC affected the GITR expression of Tregs. Groups treated with TAC or MPA alone and the one with Tregs combined with ISD (BEL, TAC, or MPA) had similar suppressive capacity. We also noted that BEL combined with Tregs significantly enhances the suppression of antigen-stimulated PBMC and reduces the inflammatory cytokines secretion, indicating that belatacept combined with Tregs has potential synergistic effect to promote allo-tolerance. However in polyclonal MLR assay, we found at all ratios of Tregs: PBMC (from 1:1 to 1:64), the group treated with the combination of Tregs and BEL had similarly suppressive capacity on the proliferation of human PBMC stimulated by CD3CD28 microbeads compared to the control group (Fig. 5D). Similarly, a recent study reported that in an ex vivo-generated alloantigen-specific immunomodulatory cells test, belatacept-treated groups produced lower IFN-γ and higher interleukin-10 levels in response to donor-antigens and increased the frequency of CD4⁺CD25⁺CD127lowFOXP3⁺T cell and FOXP3 mRNA expression. Furthermore, another study

![Figure 6](image)

**Figure 6.** Effect of immunosuppressive agent (BEL, TAC, or MPA) on the oxygen consumption rates of Tregs. Ex vivo expanded Tregs were cultured with IL-2 (100 IU/mL) alone or in the presence of single immunosuppressive agent (BEL 10 µg/mL, TAC 10 ng/ml, MPA 0.3 µg/mL) for 5 days. (A) OCR of indicated cells at baseline and in response to oligomycin (Oligo), FCCP, and rotenone plus antimycin A (R+ A). (B–E) Baseline OCR, Maximal OCR, SRC and ATP production of indicated cells from three independent experiments with Tregs from three individual donors and presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Figure 7. Effect of immunosuppressive agents (BEL, TAC or MPA) on the mitochondrial integrity and apoptosis of Tregs. Ex vivo expanded Tregs were cultured with IL-2 (100 IU/mL) alone (control) or in the presence of single immunosuppressive agent (BEL, TAC and MPA) for 5 days. (A) Mitochondrial morphology of Tregs treated with BEL, TAC, MPA or control and stained with Mitotracker Red CMXRos (red) and DAPI (blue). (B) Flow cytometry was performed to determine mitochondrial mass using Mitotracker Green in Tregs. (C) Mitochondrial membrane potential was detected through JC-1 staining. Red fluorescence, which indicated normal mitochondrial potential, was converted into green fluorescence after a reduction in mitochondrial potential; Quantitative analysis was performed using FlowJo software. (D) The percentages of apoptotic cells in Tregs assessed on the basis of annexin V expression. Scale bar = 5 μm. Data are from three independent experiments with Tregs from three individual donors and presented as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
also demonstrated that CTLA4Ig does not inhibit Tregs proliferation and promotes Tregs survival in vitro. In addition, another study\(^4\) showed that BEL could maintain a high level of intracellular FOXP3 and did not alter surface phenotype expression of Tregs. This is consistent with our findings where BEL did not exert any effect on phenotype of Tregs. However, some studies\(^41,42\) have indicated that kidney transplant patients with long-term belatacept treatment displayed an altered phenotype of Tregs and impaired suppressive function compared to Tregs from healthy controls. Interestingly, BEL-treated patients also demonstrated an enrichment of the stable CD45RA\(^+\)FOXP3\(^+\)Tregs and lower FOXP3 TSDR demethylation compared to healthy control which is suitable for UCB derived Tregs.

Cellular metabolism is also crucial to regulate T cells activation, proliferation, differentiation and function\(^9\) however comprehensive study has been missing in this area. The proliferation and differentiation of activated effector T cells rely primarily on glycolysis in a Warburg-like manner typical of tumor cells to provide energy. Unlike Teff cells, Tregs rely mainly on oxidative phosphorylation (OXPHOS) for energy production to maintain differentiation and function\(^10\). Taken together, the thorough understanding of the effects of the current immunosuppressors on Tregs metabolism not only provides valuable information on how their adverse effects may occur but also offers new opportunities to improve therapeutic strategies. In this study, we demonstrated that BEL and TAC did not change the metabolism of Tregs, but MPA treated Tregs exhibited increased OXPHOS. To understand whether this was because of the increased spare respiratory capacity (SRC) of Tregs or some other reason, we tested the mitochondrial mass of Tregs in different treatment groups. Using confocal imaging and FACS, we observed MPA-treated Tregs have higher mitochondrial mass compared to other ISD-treated groups and the control group. A recent study\(^26\) has shown that the cellular mitochondrial content determines the cell fate and variability in cell death. Cells with higher mitochondrial content are more vulnerable to death. We used JC-1 assay to detect the mitochondrial membrane potential for all the Tregs under treatment of different immunosuppressive drugs. Our results show that MPA-treated Tregs were highly prone to apoptosis which supports the idea that increased SRC might be due to higher mitochondrial content. This finding corroborates our results that MPA inhibited Tregs proliferation and viability in a dose-dependent manner.

In summary, our study shows that different immunosuppressors can have different effects on Tregs. In order to select an appropriate immunosuppressive drug that is Tregs-friendly, a set of well-designed criteria has to be established. Testing under these criteria, our study identifies belatacept to be an immunosuppressive drug that may benefit human regulatory T cell therapy in maintaining immune homeostasis and inducing immune tolerance. Nevertheless, a well-designed in vivo study would be required in a humanized transplant mouse model to evaluate the benefit of the combined regimen of belatacept and Tregs for the development of one of optimized Tregs therapy strategies to be applicable in clinical transplantation. This could have significant implications for potential drug repurposing which is a more cost-effective measure compared to the process of a novel drug development, testing, and progression to clinical trials.

**Declaration of Conflicting Interests**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Ethical Approval**

The study was approved by the Ethics Committee of the Third Xiangya Hospital, Central South University, Hunan province (China batch number: NO:2019-S550).

**Statement of Human and Animal Rights**

All procedures in this study were conducted in accordance with the Ethics Committee of the Third Xiangya Hospital, Central South University, Hunan province (China batch number: NO:2019-S550).

**Statement of Informed Consent**

Verbal informed consent was obtained from the patients for their anonymized information to be published in this article.

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