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Alloresponses of Mixed Lymphocyte Hepatocyte Culture to Immunosuppressive Drugs as an *In-Vitro* Model of Hepatocyte Transplantation

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Hepatocyte transplantation (HCTx) has the potential for the treatment of end-stage liver disease. However, failure of engraftment and the long-term acceptance of cellular allografts remain significant challenges for its clinical application. The aim of this study was to investigate the efficacy of the immunosuppressive agents, Cyclosporine, Everolimus, and Belatacept to suppress the alloresponse of primary human hepatocytes in a mixed lymphocyte-hepatocyte culture (MLHC) and their potential hepatotoxicity *in vitro*.

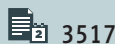
Material/Methods: Primary human hepatocytes were co-cultured with allogeneic peripheral blood mononuclear cells (PBMCs) in an MLHC. Proliferative alloresponses were determined by flow cytometry, and cytokine secretion was measured using Luminex-based multiplex technology. Using an MLHC, the alloresponses of primary human hepatocytes were compared in the presence and absence of Cyclosporine, Everolimus, and Belatacept. Cultured primary human hepatocytes were assessed for the production of albumin, urea, aspartate transaminase (AST) and DNA content. Metabolic activity was determined with the MTT assay.

Results: Immune responses induced by primary human hepatocytes were effectively suppressed by Cyclosporine, Everolimus, and Belatacept. Everolimus significantly reduced the metabolic activity of primary human hepatocytes *in vitro*, suggesting impairment of cell viability. However, further functional analysis showed no significant differences between treated and untreated controls.

Conclusions: Cyclosporine, Everolimus, and Belatacept suppressed the alloresponse of primary human hepatocytes in an MLHC without significant cytotoxicity or functional cell impairment.

MeSH Keywords: **Cell Transplantation • Hepatocytes • Immunosuppression**

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Background

Hepatocyte transplantation (HCTx) is a promising therapeutic approach for the treatment of end-stage liver disease. In selected cases, HCTx may be considered as an alternative for orthotopic liver transplantation due to the high patient mortality rate while on the waiting list for liver transplantation, as well as during the perioperative period. However, despite the encouraging results found in some patients after HCTx, the long-term success of this approach is still limited by failure of engraftment of transplanted cells into the recipient's liver and chronic rejection of allogeneic hepatocytes [1–3].

Clinical experience of orthotopic liver transplantation has shown that the liver is an immunologically privileged organ that requires less immunosuppression following transplantation than other solid organs, and in selected cases, there is the possibility to withdraw immunosuppression due to spontaneous development of graft tolerance [4,5]. However, hepatocytes do not show the same low alloreactivity found in orthotopic liver transplantation, as transplanted hepatocytes show rapid rejection *in vivo* and *in vitro* [2,6,7]. The differences in the immune response after orthotopic liver transplantation and HCTx could be explained by the absence of donor antigen-presenting cells in the liver, such as hepatic stellate cells, liver sinusoidal endothelial cells, and Kupffer cells. In HCTx, the different immune environment from that found in orthotopic liver transplantation, and the isolation process may have a negative impact on the immune tolerance to allogeneic hepatocytes [6–9].

The innate and adaptive immune systems can be involved in hepatocyte rejection [7]. In the adaptive immune response, both CD4⁺ and CD8⁺ T cells have been shown to independently induce a strong cell-mediated immune response in mice following HCTx [10]. The contribution of the humoral immune responses may also play a role after HCTx, as recently Jorns et al. published the first report of donor-specific antibodies associated with graft loss following HCTx in humans [11]. Gupta and colleagues previously described a strong reaction of the

innate immune system and demonstrated that the majority of hepatocytes (>70%) were eliminated by phagocytosis or macrophage responses irrespective of an allogeneic or syngeneic origin of the transplanted hepatocytes [12].

Currently, there are no clinical guidelines or standards for immunosuppressive therapy after HCTx, and despite the differences between orthotopic liver transplantation and HCTx described above, most clinical transplant groups apply immunosuppressive protocols used in orthotopic liver transplantation for patients following HCTx [13–17].

In contrast to calcineurin inhibitors and Everolimus that suppress the nuclear factor of activated T cells (NFAT) and mammalian target of rapamycin (mTOR) signaling pathways, respectively, the biologic immunosuppressive drug, Belatacept, is a fusion protein of the mutated cytotoxic T lymphocyte-associated protein 4 (CTLA-4) extracellular domain with the Fc part of IgG4. However, there has been no previously reported experience of the use of Belatacept in the context of HCTx.

Therefore, the aim of this study was to investigate the efficacy of the immunosuppressive agents, Cyclosporine, Everolimus, and Belatacept to suppress the alloresponse of primary human hepatocytes in a mixed lymphocyte-hepatocyte culture (MLHC) and their potential hepatotoxicity *in vitro*. Also, the study aimed to determine whether these immunosuppressive drugs had different mechanisms from those in solid organ transplantation and to assess their toxicity in primary human hepatocytes that may impair cell engraftment *in vivo*.

Material and Methods

Hepatocyte isolation and culture

Liver tissue was obtained from six patients who underwent partial hepatectomy. All patients provided written, informed consent to provide tissue. This study was approved by

Table 1. Demographic and clinical data of hepatocyte donors.

Donor #	Age	Sex	Diagnosis	Histology*
1	67	M	Caroli Syndrom	Steatosis
2	40	M	IgG4 cholangitis	Steatosis
3	52	F	CRLM**	Unaltered
4	50	M	pCC***	Fibrosis
5	57	F	pCC***	Cirrhosis
6	35	M	HCC****	Steatosis

* Liver tissue used for hepatocyte isolation; ** colorectal liver metastasis; *** perihilar cholangiocarcinoma; **** hepatocellular carcinoma.

Professor Troeger, Chairman of the Ethics Committee, who signed an approval statement (#252-2008) from Hannover Medical School. Relevant demographic and clinical data of the hepatocyte donors are shown in Table 1. Hepatocytes were isolated by a modified two-step collagenase perfusion method, as previously described [18]. Primary human hepatocytes were cultured using 6-well plates that were pre-coated with collagen. To allow the formation of a confluent monolayer of hepatocytes and to remove dead cells, the culture medium was changed after 16–18 hours.

Mixed lymphocyte-hepatocyte culture (MLHC)

Based on a previously reported *in vitro* model [19], a recently described modified approach for mixed lymphocyte-hepatocyte culture (MLHC) was used [20]. Briefly, primary human hepatocytes were cultured as monolayers and were used as stimulator cells. Allogeneic peripheral blood mononuclear cells (PBMCs) from healthy donors (n=14) were isolated from whole blood by density gradient centrifugation and used as responder cells following staining with the red fluorescent dye, PKH26, which binds to cell membranes (Sigma-Aldrich, St. Louis MO, USA). MLHC was performed in 6-well plates supplemented with 2 ml of Williams' Medium E (Merck, Germany) with daily change of medium using a volume of 0.5 ml. Primary human hepatocytes were seeded at 1.5×10^6 /well and 5×10^6 naïve responder PBMCs were added on day 0 or cultured alone, as applicable. The concentrations of the immunosuppressive agents used were determined from a previous pilot study that used a range of concentrations (data not shown) and that matched the blood concentrations observed in patients receiving solid organ transplantation [21–23]

The experimental groups were as follows: PHH+PBMC; PHH+PBMC+Cyclosporine (1,000 ng/ml); PHH+PBMC+Everolimus (100 ng/ml) and PHH+PBMC+Belatacept (1 µg/ml); the PHH control; and the PBMC control. Culture supernatants were stored at -80°C for cytokine analysis. In the design of the experiments, primary human hepatocytes from a single donor were used to establish the MLHC with PBMCs from one to three different donors. Each PBMC donor was used for all experimental groups, resulting in 14 separate MLHC experiments.

Flow cytometry

For analysis of proliferative alloresponses, the PBMCs stained with PKH26 were analyzed on day 10 by flow cytometry. Additional staining for CD4 and CD8 was performed to distinguish T cell subpopulations, as previously described [20]. Flow cytometry measurements were performed using a BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and the results were analyzed using FACSDiva software (BD Biosciences, Franklin Lakes, NJ, USA).

Cytokine analysis

Luminex-based multiplex technology with the Bio-Plex Pro Human Th17 Cytokine Panel (Bio-Rad, Hercules, CA, USA) was used to generate cytokine profiles of culture supernatants, as previously described [20]. Bio-Plex Manager software version 6.0 (Bio-Rad, Hercules, CA, USA) was used to calculate standard curves and cytokine concentrations. The detection limit of all proteins was 1–10 pg/ml.

MTT assay

Primary human hepatocytes were investigated for metabolic activity of NAD(P)-H-dependent cellular oxidoreductase enzymes on day 10 of culture. The CellTiter 96® Aqueous One Solution Cell Proliferation Assay colorimetric method (Promega, Madison, WI, USA) was used as previously described [24].

Albumin synthesis

Albumin synthesis by primary human hepatocytes was measured using the Human Albumin enzyme-linked immunosorbent assay (ELISA) Quantitation Set (Bethyl Laboratories, Montgomery, Texas, USA), according to the manufacturer's instructions and previously reported [18].

Measurement of aspartate-aminotransferase (AST) activity and urea production

Quantification of aspartate-aminotransferase (AST) and urea, representing the degree of cell damage and the ability of ammonia detoxification of cultured hepatocytes, respectively, was performed from supernatants of cell cultures by standardized procedures at central laboratory of Hanover Medical School (Roche Molecular Diagnostics), as previously described [18].

DNA quantification

The DNA content of primary human hepatocytes was monitored in culture. Briefly, hepatocytes were harvested from 6-well plates and centrifuged at $10,847 \times g$ for 5 minutes. The supernatant was then decanted and the sediment dissolved in 40 µl proteinase K in 200 µl of binding buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris HCl, 20% Triton X-100, pH 4.4). After 10 minutes of incubation at 70°C , 100 µl of isopropanol was added, and the solution was centrifuged through a filter tube containing glass fibers for 1 minute at $8000 \times g$. The filter tube was subsequently centrifuged with 500 µl of inhibitor removal buffer (5 M guanidine-HCl, 20 mM Tris-HCl, 45% ethanol, pH 6.6) for 1 minute at $8000 \times g$, and washed three times with washing buffer (20 mM NaCl, 2 mM Tris-HCl, 80% ethanol, pH 7.5) for 1 minute at $8000 \times g$. The DNA on the glass fibers was then eluted in 50 µl of elution buffer by centrifuging

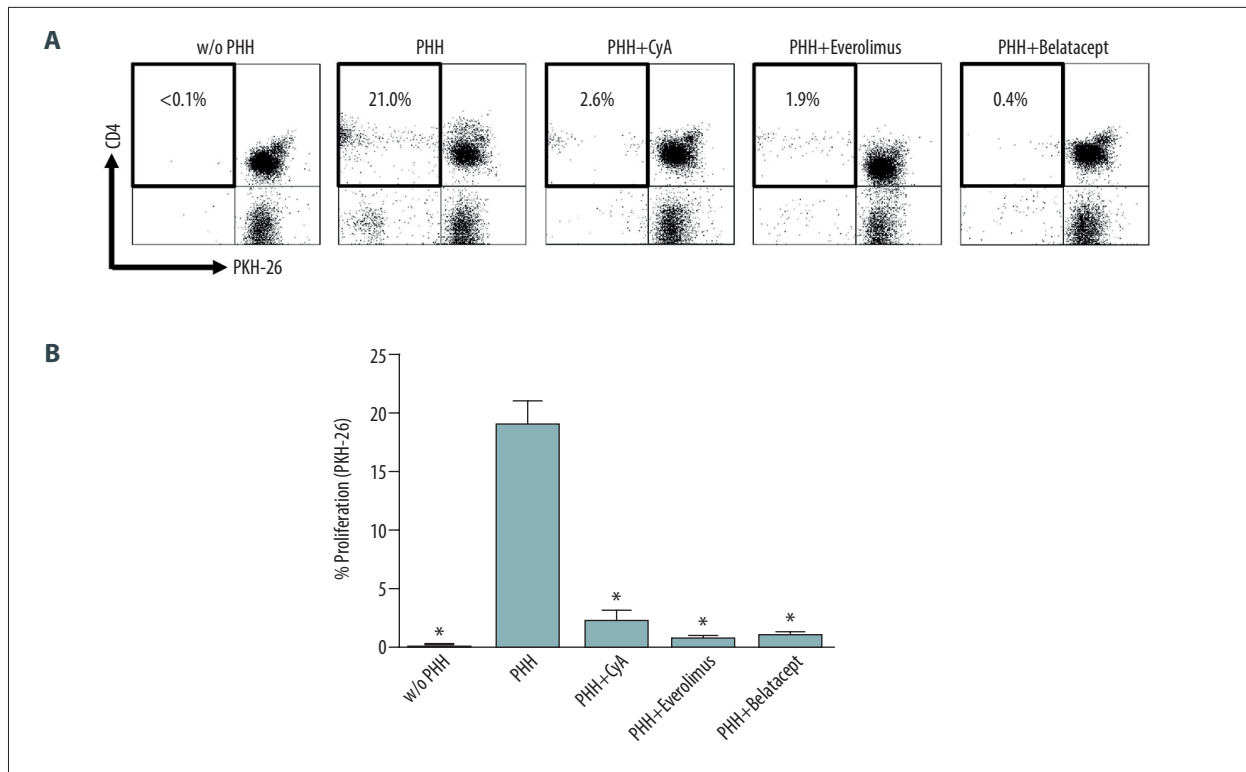


Figure 1. Flow cytometry characterized hepatocyte-induced T cell alloresponses in a mixed lymphocyte-hepatocyte culture (MLHC) in the presence or absence of Cyclosporine, Everolimus, and Belatacept. (A) Representative dot-plots of flow cytometry analysis shows the proliferative alloresponses on day 10 of the mixed lymphocyte-hepatocyte culture (MLHC) for different experimental groups, as indicated. w/o – without; CyA – Cyclosporine. (B) Bar charts summarizing the proliferative responses of peripheral blood mononuclear cells (PBMCs) cultured alone or in co-culture with primary human hepatocytes (PHHs) in the absence or presence of different immunosuppressive agents. The results are presented as the mean \pm SEM (n=14). * $p<0.0001$ compared with the PBMC+PHH group (referred to as PHH).

for 1 minute at 8000 x g and concentration and purity were measured using a NanoDrop® spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

Morphology of primary human hepatocytes *in vitro*

The morphology of primary human hepatocytes attached to the collagen-coated culture plates that were treated with and without immunosuppressants was assessed daily during the whole culture period using phase-contrast microscopy.

Statistical analysis

Statistical analysis was performed using SPSS statistical software, version 25.0 (IBM Corp, Armonk, NY, USA). The Mann-Whitney U test and the Wilcoxon signed-rank test were used, as appropriate. The results were expressed as the mean \pm standard error from the mean (SEM), unless indicated otherwise. A P-value <0.05 was considered to be statistically significant.

Results

Suppression of proliferative alloresponses in a mixed lymphocyte-hepatocyte culture (MLHC) with primary human hepatocytes (PHHs) by Cyclosporine, Everolimus, and Belatacept

The novel co-culture system of a mixed lymphocyte-hepatocyte culture (MLHC) was used to characterize the immune responses against allogeneic primary human hepatocytes (PHHs) and the immunosuppressive potential of Cyclosporine, Everolimus, and Belatacept. As previously described [20], the immune response induced by allogeneic primary human hepatocytes *in vitro* was predominantly CD4⁺ T cell-driven, and only limited proliferation was observed for CD8⁺ T cells (data not shown). This proliferative response was significantly reduced by all three immunosuppressive agents as determined on day 10 of culture (PHH: $19.1 \pm 1.9\%$; PHH+CyA: $2.3 \pm 0.9\%$ ($p<0.0001$), PHH+Everolimus: $0.8 \pm 0.2\%$ ($p=0.0001$); PHH+Belatacept: $1.1 \pm 0.2\%$ ($p=0.0001$) compared with PHH, respectively (Figure 1).

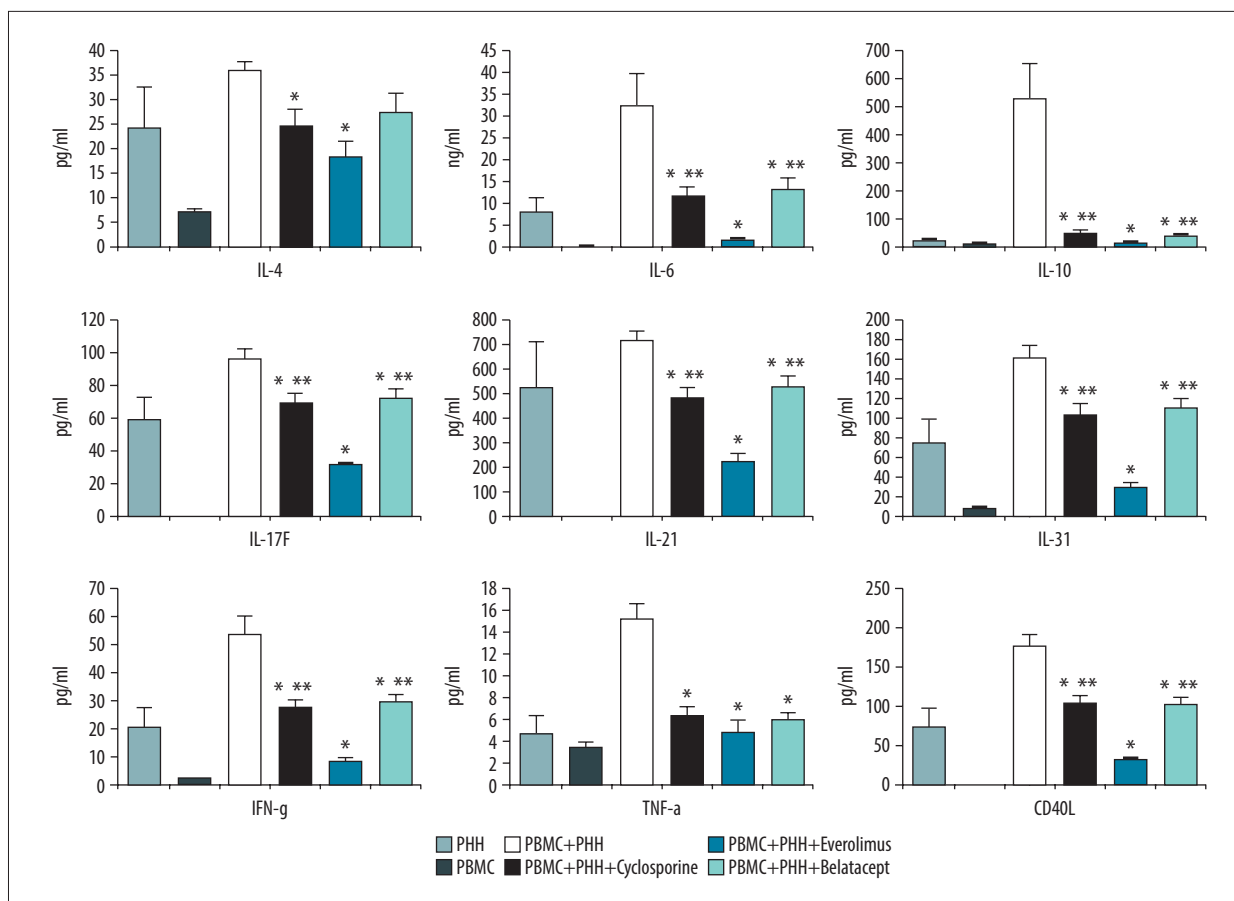


Figure 2. Hepatocyte-induced cytokine responses and characterization of the suppressive effects of Cyclosporine, Everolimus, and Belatacept. Bar charts summarize the results of cytokine analysis of the culture supernatants on day 10 of the mixed lymphocyte-hepatocyte culture (MLHC) and controls. Expression levels of selected cytokines for respective experimental groups are represented as the mean \pm SEM for primary human hepatocytes (PHH) (n=3), peripheral blood mononuclear cells (PBMCs) (n=7), PBMC+PHH (n=7), PBMC+PHH+Cyclosporine (n=7), PBMC+PHH+Everolimus (n=7) and PBMC+PHH+Belatacept (n=7), respectively. * p<0.05 compared with PBMC+PHH. ** p<0.05 compared with PBMC+PHH+Everolimus.

To further characterize the immune reaction between PBMCs and primary human hepatocytes with and without the immunosuppressive agents, Cyclosporine, Everolimus, and Belatacept, measurement of cytokine levels on day 10 in supernatants of the MLHC was performed (Figure 2). All cytokine levels were highest in the control group (PHH+PBMC), which showed that an inflammatory milieu with a significant reduction of cytokine secretion occurred following treatment with Cyclosporine, Everolimus, or Belatacept. Cyclosporine, Everolimus, and Belatacept significantly reduced the levels of the proinflammatory cytokines IL-6 and CD40 ligand as well as T helper (Th)₂-associated cytokines IL-10, IL-21, and IL-31. Everolimus showed the most effective suppression of proinflammatory and Th₂-associated cytokines, which was significantly greater than the effect of Cyclosporine and Belatacept for most of the cytokines studied. However, in the case of tumor necrosis-alpha (TNF-alpha), which is likely to play a major role in liver inflammation after hepatocyte

transplantation (HCTx) and activation of neutrophils and Kupffer cells [25], the effect of Everolimus was not significant, in part due to low secretion even without immunosuppressive drugs. The peak level of the anti-inflammatory cytokine IL-10 in the control group was interpreted in terms of counter-regulation and part of the tolerogenic potential of primary human hepatocytes. However, IL-10 secretion might have been suppressed by interfering with nuclear factor of activated T cells (NFAT), mTOR, and even by the blockade of co-stimulatory signals.

Effect of Cyclosporine, Everolimus, and Belatacept on the metabolic activity of primary human hepatocytes

The MTT assay for cell viability showed significant reduction of metabolic activity of NAD(P)-H-dependent cellular oxidoreductase enzymes on day 10 of culture, following treatment with Everolimus when compared with the control group

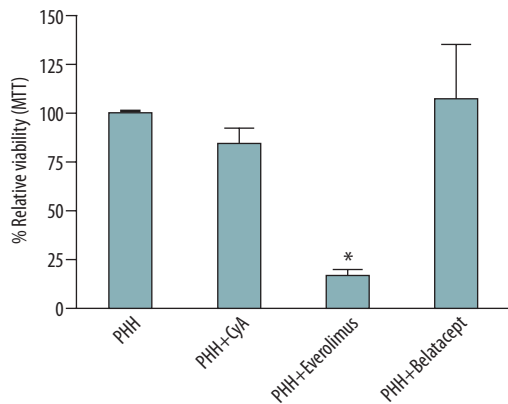


Figure 3. The effect of immunosuppression on the metabolic activity of primary human hepatocytes (PHHs) treated with Cyclosporine, Everolimus, and Belatacept. Bar charts summarize the results of the metabolic activity of NAD(P)-H-dependent cellular oxidoreductase enzymes on day 10 of culture expressed as relative viability in treated (Cyclosporine, Everolimus, and Belatacept) compared with untreated primary human hepatocytes (PHHs). The percentage of relative viability for respective experimental groups is presented as the mean \pm SEM (n=7). * $p < 0.05$ for comparison of PHH+Everolimus with all other groups.

(PHH+PBMC; $p=0.0223$) as well as to the other immunosuppressant agents ($p=0.0156$, respectively) (Figure 3). Also, treatment with Cyclosporine showed only a slight reduction of enzyme activity, and treatment with Belatacept showed a minor increase when compared with the control group, but these findings did not reach statistical significance. This finding was not unexpected for Belatacept, as primary human hepatocytes do not express the ligands for CTLA-4, CD80, and CD86, under non-inflammatory conditions.

In contrast to these findings, there were no differences in the morphology of cultured hepatocytes between the experimental groups. On phase-contrast microscopy, cells in all study groups showed the typical morphological appearance of primary human hepatocytes. Hepatocytes had a regular polygonal shape and were either mononuclear or were multinucleated (data not shown).

Functional analysis of primary human hepatocytes treated with Cyclosporine, Everolimus, and Belatacept

To further assess the potential impact of immunosuppression on the functional capacity of primary human hepatocytes and to further evaluate the findings of the MTT assay, subsequent longitudinal studies on cultured primary human hepatocytes

were performed (Figure 4). As an indicator of cell damage, the levels of aspartate transaminase (AST) in the culture supernatants were assayed, but did not show significant differences between the control and different treatment groups: AST levels were seen to increase until day 5 of culture with stable enzyme secretion from then on until the end of the experiment on day 10 (Figure 4A). Regarding the synthesis of albumin, which was specific for hepatocyte function, and the production of urea, there were no significant differences between the experimental groups. The levels of albumin and urea peaked on day 3 of culture and remained stable until day 5, before slowly declining until day 10 in all groups (Figure 4B, 4C). Consistent with these results, the DNA content, measured by NanoDrop® spectrophotometry, also increased until day 5 of culture, and then significantly decreased towards the end of the experiment (Figure 4D).

Discussion

The optimal immunosuppression for hepatocyte transplantation (HCTx) is currently unknown, and most centers use protocols used from orthotopic liver transplantation even though hepatocytes are highly immunogenic compared with whole organ liver transplants [6]. In the present study, HCTx was simulated *in vitro* by the establishment of primary human hepatocytes in a mixed lymphocyte-hepatocyte culture (MLHC). Primary human hepatocytes acted as donor cells, and human peripheral blood mononuclear cells (PBMCs) represented the recipient's immune system. The three immunosuppressive agents, Cyclosporine, Everolimus, and Belatacept, commonly used for solid organ transplantation, were studied.

The findings from the present study indicated all three immunosuppressant agents studied, Cyclosporine, Everolimus, and Belatacept, effectively reduced the allospecific proliferative T cell response of CD4⁺ T cells towards primary human hepatocytes *in vitro*. We recently have shown that allogeneic primary human hepatocytes induce a primarily CD4⁺ T cell-driven immune response *in vitro* with concomitant upregulation of MHC class II on hepatocytes [20]. In our previous study, CD8⁺ T cells did not show comparable proliferative responses, despite increased expression of the early activation marker CD69 [20]. Therefore, in the present study, we focused on the CD4⁺ T cell response to further characterize the immune response towards primary human hepatocytes [20].

There have been few previous studies on the effects of immunosuppressive agents on primary human hepatocytes, and the monitoring of rejection in HCTx remains a challenge [26]. Therefore, this study further aimed to investigate the impact of Cyclosporine, Everolimus, and Belatacept in terms of cytokine secretion, metabolic activity, albumin synthesis, urea

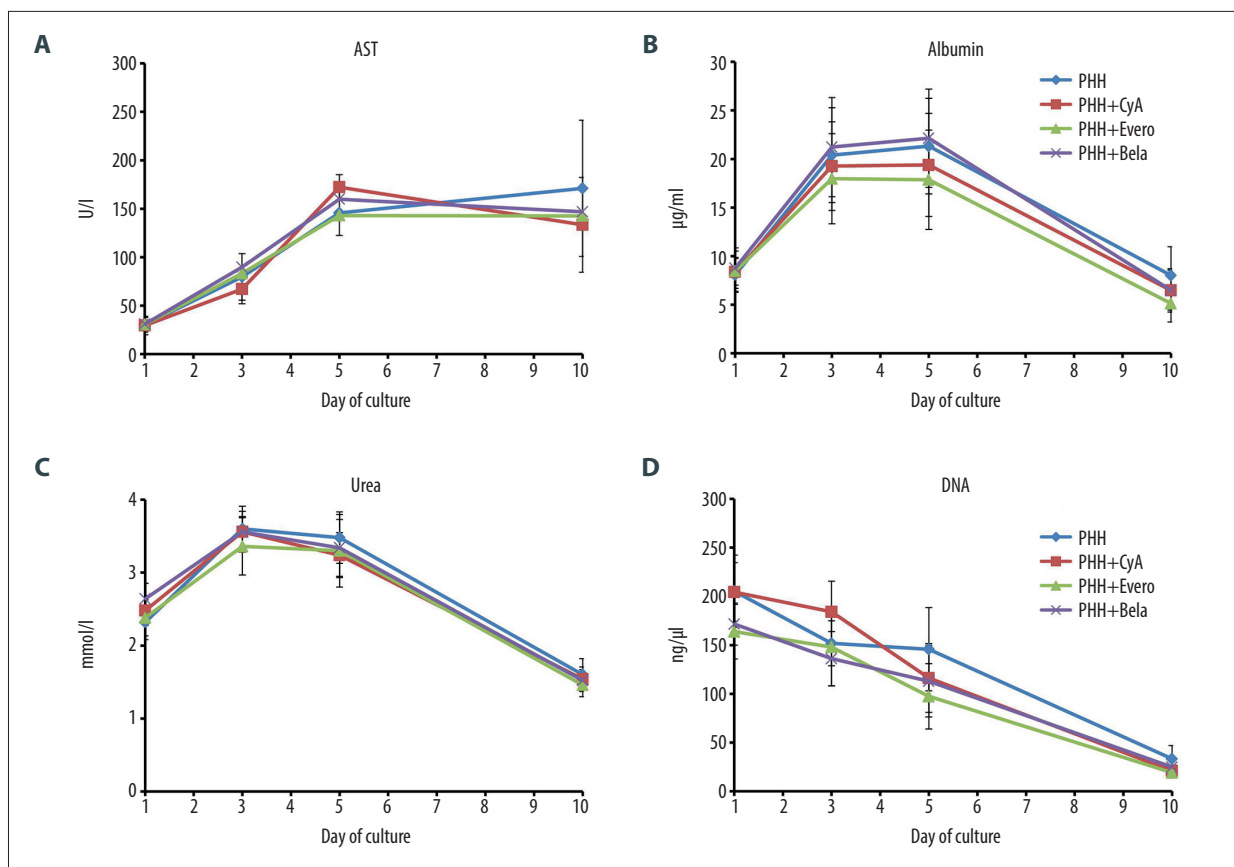


Figure 4. The effect of immunosuppression on the functional capacity of primary human hepatocytes (PHH) *in vitro* treated with Cyclosporine, Everolimus, and Belatacept. Diagrams show the detection of aspartate transaminase (AST) in the culture supernatant (A), albumin synthesis (B), urea production (C) and DNA content (D), determined on days 1, 3, 5 and 10 of hepatocyte culture in the absence of immunosuppression (blue line) or in the presence of Cyclosporine (CyA; red line), Everolimus (Evero; green line), and Belatacept (Bela; purple line). Data are presented as the mean \pm SEM (n=5).

production, aspartate transaminase (AST), and DNA content of primary human hepatocytes. The MTT assay, often used as a cell viability assay, showed that metabolic activity of NAD(P)-H-dependent cellular oxidoreductase enzymes were significantly reduced following treatment with Everolimus compared with the control, Cyclosporine, and Belatacept groups. This result raises the question of whether Everolimus significantly affects cell viability, which could be a disadvantage for its use in HCTx.

Inhibition of mTOR represents an important immunosuppressive strategy following transplantation. However, delayed liver regeneration has been reported in the initial phase after orthotopic liver transplantation [27–29]. Furthermore, several previously published studies have shown a significant decrease in proliferating hepatocytes after treatment with mTOR inhibitors, based on the function of the phosphoinositide 3-kinase (PI3K) signaling pathway on survival, autophagy, and proliferation [29–31]. Fouraschen et al. showed that the mTOR inhibitor, rapamycin, regulated not only cell proliferation but also increased hepatic autophagy during liver regeneration [32].

Previous studies have shown that mTOR inhibitors, including Everolimus, could potentially reduce cell proliferation and engraftment in HCTx. However, the clinical significance of this observation remains uncertain, as in patients treated *de novo* with rapamycin after living-donor liver transplantation, as well as in liver resection animal experiments, had no increase in mortality during rapamycin treatment, despite reduced hepatocyte proliferation [30,33]. Also, the metabolic activity detected by the MTT assay reflects viable cell metabolism and is not specific for cell proliferation [34]. Because markers of cell injury, such as AST, and markers of metabolism, including albumin synthesis, urea production, and DNA content, were comparable in all four groups in this study, the clinical impact of the reduction of cell viability in the MTT assay for Everolimus should be interpreted with caution. In this context, also the use of mycophenolic acid as an antiproliferative agent should be discussed, as previous reports have shown its successful use in experimental hepatocyte transplantation. Loukoupoulos et al. showed the effective use of mycophenolic acid and the mTOR inhibitor, sirolimus, as monotherapy or in

combination, respectively [35]. These results support the *in vitro* findings of the present study for Everolimus. However, it would still be interesting to know whether mycophenolic acid also inhibits metabolic activity in these non-proliferating human primary hepatocytes. Since regulatory T cells play an important role in transplant tolerance [36,37], we focused on Everolimus in this study due to its beneficial effect on this T cell subpopulation, as previously reported for liver transplantation [38].

Despite the previously reported disappointing results for Belatacept in *de novo* liver transplant recipients from Klintmalm et al. [39], in our *in vitro* study, Belatacept effectively suppressed T cell responses without a negative impact on cell viability or metabolic competence. A previous *in vivo* study showed that the CD40L/CD40 system played an important part in T cell-mediated rejection of allogeneic hepatocytes, whereas the CD28 co-stimulatory pathway via CD80 and CD86 had a minor role [40]. The role of these co-stimulatory pathways in CD4 and CD8 knock-out mice was further studied by Gao et al., who showed that only CD4⁺ T cells were able to promote rejection of hepatocytes via the CD28, CD80, CD86 pathway [41]. Primary human hepatocytes do not usually express MHC class II or co-stimulatory molecules such as CD80 and CD86 under non-inflammatory conditions. However, we and others have demonstrated that during inflammation MHC class II, as well as CD80, can be upregulated on primary human hepatocytes by interferons [20,42]. With primary human hepatocytes acting as antigen-presenting cells under these conditions, the primarily CD4⁺ T cell-driven immune response observed in primary human hepatocytes in an MLHC were effectively suppressed by Belatacept. This finding thus is supported by the previously mentioned report by Gao et al., suggesting a role of the CD28, CD80, and CD86 pathway.

In the present study, Cyclosporine also suppressed T cell responses without a negative impact on cell viability or metabolic competence. This finding was supported by several previous studies that showed that calcineurin inhibitors improved hepatic regeneration and increased the mitotic index in regenerating liver cells *in vivo* [43–45]. Since calcineurin inhibitors act at the level of blocking the phosphatase calcineurin to prevent NFAT2 dephosphorylation nuclear translocation and activity as a transcription factor, this suppression of T cell proliferation in response to primary human hepatocytes was expected.

The main limitation of this study was that this was an *in vitro* model study of immunological processes involved in the interaction between hepatocytes and immune cells. The innate immune system was not represented in this model, but this has a critical role after HCTx since 70–80% of initially transplanted cells are destroyed by sinusoidal effects, oxidative stress, and cytokine-mediated toxicity [46]. Also, the number of immunosuppressive drugs analyzed was limited with limited availability

of primary human hepatocytes. However, currently, a tacrolimus-based immunosuppressive regimen is preferred to the use of Cyclosporine in the clinical setting. Also, Cyclosporine and tacrolimus have a similar mechanism of action, which is why Cyclosporine was used as the standard control in this study.

Clinically, it is important to have several immunosuppressive treatment options after HCTx, due to the variety of potential indications for the procedure, and due to patients with a variety of concomitant diseases. Cyclosporine has a broad spectrum of clinical side effects that include neurotoxicity, nephrotoxicity, and the risk of *de novo* malignancies [47,48]. Everolimus and Belatacept show less risk of nephrotoxicity and are good alternatives in patients with preexisting kidney injury. However, the use of Everolimus can be associated with severe side effects, such as hyperlipidemia and thrombocytopenia. Belatacept has only been approved for treatment in adults but has recently successfully been used in adolescents [49]. However, currently, there are no data on the use of Belatacept in pediatric patients in solid organ transplantation or in patients who are found to be Epstein-Barr virus (EBV) seropositive.

Conclusions

This study aimed to investigate the efficacy of the immunosuppressive agents, Cyclosporine, Everolimus, and Belatacept to suppress the alloresponse of primary human hepatocytes in a mixed lymphocyte-hepatocyte culture (MLHC) and their potential hepatotoxicity *in vitro*. Cyclosporine, Everolimus, and Belatacept suppressed the alloresponse of primary human hepatocytes in an MLHC without significant cytotoxicity or functional impairment of hepatocytes. There was some evidence that Everolimus could negatively influence cell viability since metabolic activity determined by the MTT assay was significantly decreased. However, this finding could not be confirmed by morphological appearance or further functional testing of treated hepatocytes. The findings regarding Belatacept were unexpected, as freshly isolated primary human hepatocytes do not express the CTLA-4 ligands, CD80, and CD86. However, within our 10-day co-culture period, a proinflammatory milieu was detected, which may be associated with induction of unusual cell expression patterns, including for CD80 and CD86.

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Conflict of interest

None.

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