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# Urine Stem Cells are Equipped to Provide B cell Survival Signals

**Running Title:** USCs Immune Regulation

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Urine Stem Cells, B cells, T cells, Mesenchymal Stem Cells, Cytokines, BAFF.

**Abstract:**

The interplay between mesenchymal stem cells (MSCs) and immune cells has been studied for MSCs isolated from different tissues. However, the immunomodulatory capacity of urine stem cells (USCs) has not been adequately researched. The present study reports on the effect of USCs on peripheral blood lymphocytes. USCs were isolated and characterized before co-culture with resting and with anti-CD3/CD28 bead stimulated lymphocytes. Similarly to bone marrow mesenchymal stem cells (BM-MSCs), USCs inhibited the proliferation of activated T lymphocytes and induced their apoptosis. However, they also induced strong activation, proliferation, and cytokine and antibody production by B lymphocytes. Molecular phenotype and supernatant analysis revealed that USCs secrete a range of cytokines and effector molecules, known to play a central role in B cell biology. These included B cell-activating factor (BAFF), interleukin 6 (IL-6) and CD40L. These findings raise the possibility of an unrecognised active role for kidney stem cells in modulating local immune cells.

**Significance statement:**

The active role of stem cells in maintaining tissue homeostasis has been well documented, particularly their ability to modulate the local immune response. Our study on the immunomodulatory properties of USCs shows that, like MSCs, they are capable of modulating immune cells. However, we uncovered an unknown capacity of USCs, residents of the kidney, to promote B lymphocyte functions, which could potentially change our understanding of the kidneys' normal immune environment and immune-mediated nephropathy. Further, our findings reveal a new possible therapeutic use of USCs as an immune adjuvant with clinical implications.

## 1. Introduction

Urine cytology has remained one of the most commonly used diagnostic tools in nephrology since it was first described almost 100 years ago [1]. The ability of some urine cells to grow in culture has been known since the 1970s [2]. However, the isolation and culture of undifferentiated cells from the urine with the capacity to expand, express mesenchymal stem cell (MSC) markers and differentiate into different lineages, has transformed this diagnostic tool into an important source of MSCs [3,4].

MSCs were first isolated from the bone marrow by Friedenstein *et al.* as fibroblast-like cells capable of differentiating into adipocytes, chondrocytes and osteoblasts [5,6]. Since then, similar cells have been found in small numbers in most adult tissues. MSCs strategically reside in perivascular spaces, from where they mobilize in response to local or distant tissue damage to promote tissue repair and healing [7]. Besides their regenerative capacity, MSCs have an immune-regulatory function [8,9], inhibiting most immune cells, including T cells [10], natural killer cells [11], monocytes and dendritic cells [12].

MSCs have been successfully cultured from different tissues such as umbilical cord, adipose tissue, endometrium, placenta, dental pulp and recently, urine (USCs) [7,13-15]. The renal origin of USCs was first discovered when the male karyotype of USCs was isolated from a female recipient of a male kidney donor. This was further confirmed by the expression of renal parietal cell markers and the close correlation between renal progenitor cells' transcriptome and USCs' transcriptome [16,17].

The non-invasive way of isolating USCs has quickly drawn attention to urine as a source of MSCs with a higher proliferation rate and higher myogenic, neurogenic and endogenic differentiation potential compared to other types [18-20].

USC characterization studies have shown unique podocyte markers expression as well as the secretion of growth factors and cytokines, such as VEGF, HGF, IGF-1, IL-6 and IL-8 [21] , all of which can modulate the immune cells [22-26]. These studies suggest that USCs could have

immunomodulatory properties. Moreover, recent research on the potential use of USCs for inflammatory bowel disease (IBD) has shown that USCs negatively modulate T cell functions [27]. However, compared to BM-MSCs, the immunomodulatory properties of USCs remain to be properly addressed.

In this study, we report on the effect of USCs on human peripheral blood lymphocytes in resting and activated culture conditions. BM-MSCs were used as a control in the proliferation study. We found that USCs modulate the function of both T and B lymphocytes. The effect of USC co-culture on T cells is similar to that of MSCs; USCs suppressed *in vitro* stimulated T cell proliferation and induced their apoptosis.

Surprisingly, USCs had a profound yet opposite effect on resting B cells; they stimulated B cell activation, proliferation across B cell sub-populations, including transitional, naïve and memory cells and induced antibody production. The stimulatory effect on B cells was mediated by both cell-cell contact and the secreted factors. USCs secrete a range of B cell immunostimulatory molecules such as soluble CD40L, BAFF and IL-6, known to provide survival signals to B cells.

We believe that this previously undescribed effect of urine-derived kidney stem cells on B cells is of particular importance given the increasing evidence of stem cells' contribution to tissue homeostasis and the central role that the immune system plays in the kidney.

## 2. Material and Methods

### 2.1. Human USCs isolation and expansion

Urine samples were collected from 6 healthy volunteers, aged between 22 and 45 years with no history of chronic illness or urinary tract infection symptoms during the previous 3 months, UniSA Human Ethics Committee approval no. 35945. USCs were isolated following the previously described protocol [28]. Samples were centrifuged (400g for 10 minutes), followed

by two washes of the pellets with phosphate-buffered saline (PBS) by centrifugation as above. The cell pellet was then resuspended in 2 ml of Renal Epithelial Cell Growth Medium BulletKit™ (REGM™; Lonza, Switzerland), 10% foetal bovine serum (FBS) (Gibco, USA) containing 100 μg/ml penicillin and streptomycin (P/S), seeded on tissue culture 4-well plates (Thermo-fisher, USA), coated with tissue culture certified gelatine (SCT, USA), and cultured at 37°C in a 5% $CO_2$. After 48 hours of culture, 50% of the media was replaced with new media and cultured for another 24 hours. Then, the culture media was gradually replaced by expansion media, composed of a 1:1 DMEM/F-12 and REGM™, 10% FBS, 100 μg/ml P/S, 1% NEAA (Gibco, USA), 5 ng/mL of bFGF, PDGF-BB and EGF (Peprotech, USA). During the culture, expansion media was replaced daily with a 50% fresh expansion medium. Confluent (80–90%) cells were detached with TryplE (Gibco, USA) and re-plated at 3000 cell/$cm^2$ in expansion media.

### 2.2. BM-MSCs culture and expansion

Human BM-MSCs were purchased from Lonza (Cat. No. PT-2501), cultured as described by the manufacturer using Mesenchymal Stem Cell Growth Medium BulletKit™ (Lonza, Switzerland) and expanded similar to USCs.

The absence of Mycoplasma or Alcoplasma contamination was tested by analysis of the USCs and BM-MSCs culture supernatant by PCR [29].

### 2.3. USCs characterization

USCs at passage 3-4 were collected, washed once with PBS and stained for CD73, CD105, CD14, CD34, CD45, HLA-DR, HLA-ABC, CD178, CD276, CD29, CD54, CD106 and isotype control antibodies **(Supplementary Table S1)** for 30 minutes at 4ºC. Then, the USCs were washed with PBS and analysed by FACScanto flow cytometry (BD, USA).

### 2.4. Confocal microscopy

BM-MSCs and USCs at passage three were cultured over coverslips, washed twice before fixing with 4% paraformaldehyde (30 mins). Cells were washed (X3) and blocked with bovine serum albumin (BSA) (10 mg/mL) and glycine (22.52 mg/mL) (30 mins) at room temperature. Cells were incubated with mAb to CD54 and isotype control diluted in PBS containing BSA (10 µg/mL), overnight in humidified chamber at 4 °C. Cells were washed and incubated with anti–mouse Alexa flour 647 according to manufacture instructions for 1 h at room temperature. Cells were washed and mounted using DAPI mounting media (Sigma-Aldrich, USA) for confocal microscope visualization (Olympus FV3000 Confocal Microscope, Japan).

### 2.5. USCs differentiation potential

USCs were collected from passage 4 and differentiated to either adipocytes, osteoblasts or chondrocytes for 21 days, followed by testing their differentiation using specific stains i.e. Oil red O for adipocytes, Alizarin Red for osteoblasts and Toluidine Blue for chondrocytes, all following a previously described protocol [30].

### 2.6. Peripheral Blood Mononuclear Cells (PBMC) isolation

Fresh blood samples were collected from 6 healthy donors after informed consent (Human ethics approval no. 35760). PBMC were isolated by the Ficoll method [31] and washed three times with PBS. The cell pellet was resuspended in complete media (CM) composed of IMDM (Gibco, USA) media, supplemented with 10% FBS, 2 mM L-glutamine and 100 µg/ml P/S. For B cell isolation, EasySep™ Human B Cell Enrichment Kit was used following the manufacturer's protocol (Stem Cell, USA), achieving a purity >97%. To study the B cell sub-populations, B lymphocytes were sorted out according to the expression of CD27, CD24 and CD38 into 3 sub-populations: transitional cells ($CD27^{-}CD38^{++}CD24^{++}$), naïve cells ($CD27^{-}CD38^{+}CD24^{+}$) and memory cells ($CD27^{+}CD38^{+}CD24^{++}$), using a FACSAria™ cell sorter (BD Bioscience, USA), achieving >98% purity, as described [32-34].

### 2.7. Co-culture studies

To test the effects of USCs on the immune cells, mycoplasma free USCs *vs* BM-MSCs, were co-cultured with PBMC. USCs or BM-MSCs were plated at 2.6 X $10^4$ cells/$cm^2$ on gelatine-coated 24-well plates. After 24 hours, cells were treated with Mitomycin C 10 µg/ml (Sigma-Aldrich, Germany) for 2 hours then washed with PBS twice. Cells grown in control wells were detached and counted prior to PBMCs co-culture. CFSE labelled PBMC in CM were added to the wells at a final ratio of 1:5. To study the effect of USCs in the immune stimulated PBMC condition, similar experiments were performed in the presence of anti-CD3/CD28 beads (Dynabeads™ Human T-Activator CD3/CD28, Thermo-Fisher, USA) at 1:4 beads/T cells ratio. Trans-well studies were carried out similarly using Trans-well inserts of 0.4 µm pore size (Corning, USA) [35]. USCs effect on B cells was also tested using USCs and purified B cells or B cell sorted sub-populations co-cultures at the same ratio.

### 2.8. Proliferation study

Proliferation was assessed by labelling the lymphocytes with CFSE (10 µM final concentration) using CellTrace™ (Thermo-Fisher, USA), following manufacturer instructions, before co-culture with USCs or BM-MSCs. Cells were co-cultured for 5 days and the percentage of divided cells was measured as previously described [36]. Cell viability was determined by Propidium Iodide (PI) staining or with FITC conjugated Annexin V (BD, USA) [37]. PBMC samples experimenting basal proliferation above 10% were excluded.

### 2.9. Activation marker expression

Lymphocyte activation markers (CD69 and CD40) were measured following PBMC co-culture with USCs for 24 hours. CpG-B ODN 2006 (3.2 µg/ml, Geneworks, Australia) was used as a positive control for B cell CD69 activation [38].

### 2.10. Antibody and cytokine quantification

Supernatant cytokine and Immunoglobulin isotypes concentrations were measured after 48 hours and five days respectively, by flow cytometry-based multiplex using a B cell panel 13 multi-analyte array (Cat.No.740527) and a human immunoglobulin isotyping panel (Cat.No.740637), following the manufacturer's protocol (BioLegend, USA).

### 2.11. RNA sequencing

USCs were processed for RNA sequencing using RNeasy Mini Kit (Qiagen, Germany) followed by DNase treatment. cDNA and gene libraries were generated using an Illumina MiSeq paired-end 300 bp protocol (Illumina, USA) performed at BIOZERON Biotechnology Co. (Shanghai, China). Overexpressed immune-related genes were queried using the online database, DAVID ontology [39], followed by gene ontology analysis for immune process using "clusterProfiler" R package [40]. Bar plots were generated using ggplot2 R package [41].

### 2.12. Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (GraphPad, CA, USA). The data were presented as Mean± SEM. Assuming a normal distribution of the data (using the Shapiro–Wilk normality test), the Paired Student t-test analysis was used to compare the mean of marker expression, cytokine concentration and FlowJo-calculated means of percentage of proliferating cells between two groups. Repeated Measurement One-way ANOVA followed by multiple comparisons using the Tukey test were used to test the significance between different groups. Geisser-Green house correction was employed whenever there was unequal variance, where Tukey test computed individual variance for each comparison. To normalize the distribution, the cytokine results underwent prior logarithmic transformation. The result was considered only significant if p-value $<0.05$. The experiments were repeated at least three times.

## 3. Results

### 3.1. USCs characterization

USCs were initially cultured in 4-well plates where cell clones started to appear between Day 7 and Day 13 of culture, with predominantly stellate shaped morphology and intercellular projections **(Fig 1A, B)**. The growth rate of the cells was proportional to their density, with an initial lag phase leading into a stage of exponential growth, doubling after two to three days up to passages seven or eight **(Fig 1C)**.

The USCs mesenchymal nature was confirmed by testing their differentiation potential into adipocytes, osteoblasts and chondrocytes where the successful induction, unlike the untreated cells (**Fig. 1D-1F**), demonstrated by the red staining of the intracellular vacuoles with Oil Red O stain of the induced adipocytes **(Fig. 1G)**, the orange staining of the calcified osteogenic nodules with Alizarin Red stain in the induced osteoblasts **(Fig. 1H)** and the pale blue staining of the extracellular matrix with Toluidine Blue stain in the induced chondrocytes **(Fig. 1I)**.

For further characterization, phenotypic analysis was performed at passage 3-4, showing high expression of MSC markers CD29, CD73 and CD105 up to 97%±2% and negative for hematopoietic markers CD45, CD14, CD34 and HLA-DR with low expression of HLA-ABC **(Fig. 1J)**, as expected [42,43].

### 3.2. USCs' immune-modulatory effect on PBMC

#### 3.2.1. Inverted phase-contrast light microscope of USCs and PBMC co-culture

Monitoring of the PBMC culture wells showed that in the resting conditions compared to the PBMC cultured alone **(Fig. 2A.I),** USCs seemed in causing the potential recruitment of the PBMC **(Fig. 2A.II)**. The addition of anti-CD3/CD28 beads to the PBMC caused the formation of proliferation aggregates **(Fig. 2A.III),** consequent with their stimulation. However, in the

USCs' co-culture, PBMC seemed to be forming discrete aggregates with the USCs **(Fig. 2A.IV)**. This cell association was also observed in syngeneic cultures (data not shown).

#### 3.2.2. Similar to BM-MSCs, USCs inhibit T lymphocyte proliferation in the activated condition

The co-culture of USCs or BM-MSCs without any additional stimulation showed that neither USCs nor BM-MSCs caused significant T cell proliferation **(Fig. 2B)**. The presence of anti-CD3/CD28 beads resulted in a significant increase in T cell proliferation, which significantly decreased in the presence of both BM-MSCs and USCs **(Fig. 2B),** which is in line with immune suppressive capacity of MSCs extensively reported [44].

#### 3.2.3. Unlike BM-MSCs, USCs significantly stimulate B cell proliferation in the resting condition

On the other hand, the study of B cells in the same co-culture conditions showed a peculiar result. While the presence of BM-MSCs had a modest effect on B cell proliferation, USCs induced a significant proliferation of these B cells **(Fig. 2C).** USCs induced proliferation seemed to be limited to the B cells population of the PBMC **(Supplementary Fig. 1).** Similar to $CD3^+$ T cells, the presence of anti-CD3/CD28 beads resulted in an increase of $CD19^+$ B cells, probably as consequence of T cell activation with the resulting CD40L expression and cytokine release by activated T cells [45]. Notably, the presence of either USCs or BM-MSCs significantly supressed B cell proliferation in this condition, possibly reflecting the supressing effect on T cells and a decrease in the T cell dependent B cell stimulation **(Fig. 2C)**.

### 3.3. USCs induced apoptosis of T cells in the activated condition and decreased inflammatory cytokine production

To elucidate the mechanism behind the USCs' immunosuppression, T cells' viability under activated and resting conditions was analysed as a possible mechanism of MSC-induced immune suppression [46-48]. The PI viability studies revealed a decrease in the percentage of

the viable T cells co-cultured with USCs, which reached statistical significance in the presence of anti-CD3/CD28 beads **(Fig. 3A).** Further analysis of T cells showed a significant increase in the percentage of apoptotic T cells under the same conditions, measured by Annexin V staining **(Fig. 3B)**. These results were confirmed by PI and Annexin V double staining (**Fig. 3C**). These results indicate that USCs not only inhibit the proliferation of T cells cultured under activating conditions, but they also induce their apoptosis, as described for other MSCs [49,50].

To further understand the suppressive effects of USCs on T cells under activated conditions, we proceeded to measure cytokine concentration in the supernatants of these co-cultures. The addition of anti-CD3/CD28 beads increased the measured cytokine concentrations, including IFNɣ, which exceeded the upper limit of detection (>10 ng/ml). However, the presence of USCs significantly decreased the concentrations of the inflammatory cytokines TNFα (1571± 124.4 *vs* 160.6±15.51 pg/ml), TNFβ (3176.7±116.5 *vs* 183.1±20.4 pg/ml) and IL13 (688.5±192 *vs* 135.6±12.7 pg/ml). Surprisingly, the presence of USCs increased IL-6 (2680±611.4 *vs* 42081±7028 pg/ml), BAFF (30.42±8.302 *vs* 2630.4±342.6 pg/ml), APRIL (9.163± 3.749 *vs* 279.4±54.14 pg/ml), and CD40L (162.4±18.83 *vs* 261.3±21.84 pg/ml) concentrations, although the latter did not reach statistical significance (Mean±SEM, n=3) **(Fig. 3D)**.

Combining this cytokine increase with the observed USCs' induced B cell stimulation observed before, we proceeded to analyse the USCs-B cell stimulatory effect further.

### 3.4. USCs cause activation, proliferation and antibody production of enriched B cells

In order to exclude the possible contribution of HLA-mismatch to the observed proliferative USCs effect on B cells, we proceeded to include syngeneic USCs. While USCs induced 28.2% of the matched B cell proliferation, they induced 30% of the mis-matched B cell proliferation compared to control **(Fig. 4A)**.

The study of the USCs effect on B cell activation measure by CD69 expression showed that USCs co-culturing with B cells caused a significant increase in the expression of CD69, similar

to the CpGB stimulation, used as positive control **(Fig. 4B)**. In addition, USCs caused increased expression in the co-stimulatory molecule CD40 compared to untreated (1735±461 *vs* 2695±205 MFI, n=3), **(Supplementary Figure 2).**

To further study the effect of USCs on B cells, excluding any effect of T cells, and to determine whether the effect of USCs was towards a particular B cell sub-population, we co-cultured USCs with highly $CD19^+$ purified B cells. Again, USCs induced purified B cell proliferation **(Fig. 4C)**. To test for the selective stimulation on B cell sub-populations, we proceeded to sort the B cells into transitional ($CD19^+CD27^-CD38^{++}CD24^{++}$), naïve ($CD19^+CD27^-CD38^+CD24^+$) and memory B cells ($CD19^+CD27^+$) **(Fig. 4D)**. USCs co-culture with the sorted B sub-populations showed enhanced proliferation in all sub-populations studied, compared to cells cultured alone **(Fig. 4E)**.

Given that the main function of B cells is antibody production, we proceeded to measure the USCs effect on antibody production in supernatants collected from the co-culture of USCs with purified B cells, under resting conditions, and in the presence of the Toll-like receptor 9 (TLR9) ligand, CpGB known to induce antibody production [38]. We found that the presence of USCs induced a significant increase in antibody secretion, namely, IgM, IgG1, IgG2, IgG3, IgG4 and IgA **(Fig. 4F)**. The addition of CpGB, as expected, significantly increased the production of all antibodies. However, the presence of USCs further significantly enhanced the CpGB-mediated antibody production for IgM, IgG1, IgG2, IgG3, IgG4, IgA and IgE **(Fig. 4G)**, **(Supplementary Table S2.A)**, further confirming the USCs' B cell stimulatory properties.

### 3.5. USCs effects on B cells are mediated by both cell-cell contact and secreted factors

To identify the mechanism behind B cell stimulation, we performed Trans-well experiments were PBMC and USCs were co-cultured in close cell-cell contact or physically separated. We also included BM-MSCs as a control to rule out the culture conditions as a contributing factor. Again, direct cell-cell contact induced a significant B cell proliferation. However, it also

induced, although to a lesser extent, a significant proliferation of $CD19^+$ B cells in Trans-well. These results suggested that USCs' effect on B cells was not dependant on cell-cell-contact **(Fig. 5A)**. No proliferation was observed with BM-MSCs Trans-well co-culture.

Therefore, we proceeded to identify the production of known B cell related cytokines by USCs. We found that in the USCs-PBMC co-culture supernatant, there was a significantly higher concentration of BAFF (1111±189 *vs* 3722±119pg/ml) and APRIL (17±4 *vs* 56±22pg/ml) compared to BM-MSCs-PBMC co-culture (**Fig. 5B**), **(Supplementary Table S2.B)**.

Further analysis was conducted on the USCs co-culture supernatant for more B cell-related cytokines. Analysis of the co-culture supernatant revealed a significant increase, compared with that of PBMC alone, in CD40L (40.7±10.9 *vs* 294.7±29.8pg/ml), IL-6 (1600±575 *vs* 37018±769.7 pg/ml), IL10 (7.5±3.4 *vs* 233.5±73pg/ml), BAFF (28.9±7.3 *vs* 1256.1±84.5pg/ml) and APRIL (27.7±9 *vs* 60.6±10pg/ml) (Mean±SEM, n=3) **(Fig. 5C,D)**.

The measurement of these cytokines in of 48 hours culture supernatant of USCs ($10^5$ cell/1.5ml) showed the presence of BAFF (467±20 pg/ml), CD40L (120±6 pg/ml) and IL-6 (8319.6±688.8 pg/ml) (Mean±SEM, n=3) **(Fig. 5E)**, demonstrating the USCs origin of the cytokines. While MSCs supernatant analysis of similar cell number showed presence of CD40L (59.7±6.2 pg/ml) and IL-6 (2060.4±83.1 pg/ml) with no detectable levels of BAFF or APRIL in the supernatant **(Fig. 5F).** Levels of IL2, IL4, IL12p70, IL13, IL17A and TNFβ were below the range of detection.

### 3.6. Surface expression of immunomodulatory factors by USCs

To further study the expression of immunomodulatory molecules that could better explain the above observations, including the PBMC recruitment pattern described in Fig 2A II and IV, we first performed USCs RNA-seq and identified over expressed immune-related genes. The gene ontology analysis revealed the expression of many genes involved in T, B cells immune functions and cytokines signalling **(Supplementary Figure 3A, B, C)** and **(Supplementary Table S3)**.

Using flowcytometry to confirm expression of relevant molecules, we found that USCs highly express the adhesion molecules CD29 or β1 integrin (3275±179 MFI) **(Fig. 1G)**, CD54 or ICAM-1(1553±30 MFI), and CD106 or VCAM-1(1325±77 MFI) **(Fig. 6A)**, which might correlate with the cell behaviour in the *in vitro* co-culture mentioned above. With regard to the T immunosuppressive function, we found that USCs highly expressed CD276 (2137±184 MFI) (**Fig. 6A**), known to play a role in downregulating T cell functions [51,52], while the stem cells were negative for CD178. Although both, BM-MSCs and USCs expressed CD276 (>98%), BM-MSCs expressed lower level of CD106 (53.3±5.5% *vs* 74.9±6.5%) and CD54 (7.9±2.6% *vs* 91.38±3.5%) than USCs (**Fig. 6C**). Confocal imaging of the USCs and BM-MSCs stained with CD54 confirmed these results, while USCs showed strong staining, BM-MSCs were negative (**Fig. 6B, D**).

## 4. Discussion

USCs' simple isolation and rapid expansion *in vitro* make them an attractive source of MSCs for therapeutic use, potentially for the treatment of nephropathies and other tissue regeneration [3,19,53]. Moreover, USCs offer a non-invasive way of studying the biological functions of kidney stem cells shed in urine [16,54].

Here, we investigated USCs' immunomodulatory properties, which have been extensively studied for other MSCs, especially BM-MSCs [46,55-57]. To test the effect of the USCs under immune-stimulated conditions, anti-CD3CD28 beads were used as these mimics the activation state generated by antigen presentation with T cell receptor (TCR) signalling [58].

Following slow growth during the first four days of culture, USCs expanded exponentially, doubling every 24-48 hours, obtaining up to $1.6x10^7$ USCs from 100 ml of voided urine reaching up to passage 6-7. Phenotypic characterization showed that more than 97% of the USCs were positive for the MSC markers CD73, CD29 and CD105 [42], and negative for hematopoietic markers such as CD45, CD19, CD34, CD14 and MHC class II [43], with low MHC class I expression [59,60]. Additionally, using standard differentiation protocols [30], we successfully differentiated the USCs to adipocytes, osteoblasts and chondrocytes, further confirming their MSC properties.

Our study revealed that USCs have the capacity to interact and modulate T and B cell functions, both in resting and activating culture conditions. We found that USCs express adhesion molecules, known to play a role in cell-cell contact during the immune response. These molecules, which included CD54 and CD106, mediate the adhesion of immune cells to endothelial cells, particularly following immune cell activation [61,62]. The high expression of these molecules in USCs, in particular CD54 (ICAM-1), compared to BM-MSCs may account for the consistent USCs-PBMC aggregates observed when co-cultured in the presence

of anti-CD3/CD28 beads **(Fig. 2A.IV)** [63]. Furthermore, these molecules have shown correlation to the immunosuppressive capacity of the MSCs [64].

Regarding the effect of USCs on T lymphocytes, we found that neither the BM-MSCs nor the USCs co-culture with PBMC resulted in T cell proliferation. This result suggests that USCs exert poor allogenic stimulation, which could be explained by the lack of expression of MHC class II and low expression of MHC class I.

The MSCs' T cell immunosuppressive capacity, particularly under activating conditions, has been extensively demonstrated [46-50], and was also observed in our study. We found that both BM-MSCs and USCs significantly inhibited T cell proliferation in the presence of anti-CD3/CD28 stimulatory beads. In addition, we observed that USCs decreased the production of inflammatory cytokines (TNFα and TNFβ) and induced T cell death by apoptosis when cultured under stimulatory conditions, as other MSCs [46,47].

Interestingly, during the USCs phenotype, we found high expression of CD276 - one of the suggested molecular players of the MSC immunosuppression [52], which was similarly expressed by BM-MSCs.

However, on B cells, the effect of USCs was surprisingly different. USCs induced a significant proliferation of $CD19^+$ B cells when co-cultured with PBMC. This effect was reproducible when cultured with highly purified B cells (>97%). This indicates that the effect was cell-specific and not dependent on T cells, ruling out the possible contribution of HLA mismatch. This was further confirmed by the observation of B cell proliferation of syngeneic USCs and PBMC co-culture. We also observed that USCs induced the expression of co-stimulatory and activation markers CD40 and CD69 respectively, and significantly induced the secretion of IgM, IgGs and IgA antibodies. Of note, we found that the level of B cell activation by USCs was comparable to the stimulation resulting from CpG-B, which is known to induce strong B cell activation [38]. Additionally, the presence of USCs synergized with CpGB, significantly

enhancing CpG-mediated B cell antibody production. We therefore propose that the production of CD40L by USCs and the known effect of CpGB enhancing CD40 expression on B cells [65], may contribute to this synergism, previously described in other cellular systems [66].

Given that B cells are a heterogenous cell population, we sought to establish whether USCs had the same proliferation-inducing effect on different B cell sub-populations. We found that USCs significantly induced proliferation in all three major B cell sub-populations (transitional, naïve and memory) that were studied. This again confirmed that the activation of USCs was T cell-independent.

The analysis of possible contributors to USCs' B cell stimulation using Trans-well studies showed that although the stimulation was stronger when cultured in direct cell-cell contact, USCs significantly induced B cell proliferation when physically separated, suggesting the contribution of secreted factors, as soluble molecules or extracellular vesicles, currently under study.

We found that USCs indeed, constitutively secrete several molecules known to directly contribute to B cell survival, activation and differentiation, notably, CD40L, APRIL, BAFF, and as previously reported IL-6 [19,67]. These findings of USCs secreting CD40L and BAFF were surprising, given that their expression is mainly limited to immune cells. For example, CD40L is primarily expressed by activated T cells [68] and bind to CD40 molecules expressed on activated B cells leading to further B cell activation, proliferation, immunoglobulin class switching and antibody secretion [69]. This finding is highly relevant, considering that, as described earlier, USCs also induce the expression of CD40 on B cells. Furthermore, the high ICAM-1 expression in USCs could be contributing to the higher B cell stimulation observed during cell/cell contact conditions as it had been reported to lower B cell stimulatory threshold [70].

Similarly, BAFF is normally produced by lymphoid stromal cells, macrophages and dendritic cells in the germinal centre. It binds to three different receptors - BAFF-R, TACI/CAML and BCMA - which are constitutively expressed by B cells [71], leading to NF-kB activation in B cells and the transcription of anti-apoptotic genes [72]. These molecules are also important in maintaining long lived plasma cells and inducing B cell differentiation [73,74], and their dysregulation could result in the arrest of B cell differentiation and the development of primary immunodeficiencies [75,76] while their increase could result in the survival and expansion of autoreactive B cells and autoimmunity [77,78].

It worth noticing that in spite of the consistent BAFF expression at protein level, it didn't correlate its expression at gene level, which has previously been described for other cytokines [79].

BAFF expression by BM-MSCs has been previously reported in association to TLR-4 priming [80] and interestingly, high BAFF expression had been previously reported in BM-MSCs isolated from rheumatoid arthritis patients [81] but not from healthy individuals, confirmed in our study.

The effect of BM-MSCs on B cells has been previously studied, with conflicting results [82], [83-85] attributed to the difference in the starting population, the purity of isolation and culture conditions [86]. In our study, we found that BM-MSCs did not have a significant effect on B cell functions.

Although interesting, we believe that our study has several limitations that should be considered for future studies. For example, evaluating the effects of urine and the *in vitro* culture on the behaviour of USCs, the role of other cytokines and molecules not properly addressed in this study such as INFɣ, and to expand the effect of USCs to more immune cells subpopulations such as Monocytes/Macrophages, NK and dendritic cells. Also, although the B

cell stimulatory functions of BAFF and CD40L is well stablished, blocking their activity would validate their role in this system.

Given that MSCs play a central role in modulating the cell microenvironment, we theorise that the effect of USCs on T and B cells, as described in this study, could contribute to the homeostasis of the kidney immune microenvironment in health as well as in pathological conditions such as infections, autoimmunity, diabetic nephropathy and organ rejection. Interestingly, production of BAFF has also been recently reported by the renal tubular epithelium (REC), which correlated with the degree of kidney damage in SLE nephritis and is thought to provide survival signal to the long-lived intra-renal plasma cell niche [87,88]. The findings that USCs also secrete BAFF and are capable of modulating B cells functions suggest that these cells might also contribute to the renal immune pathologies, therefore, the study of USCs in these conditions could provide new insights on their pathogenesis, possible diagnostic and therapeutic targets.

## 5. Conclusion

In this study, we examined the effect of USCs on B and T cells in a PBMC microenvironment, both in a resting condition and under stimulation. Our study shows that similarly to MSCs, USCs exert a direct immunosuppressive effect on T cell functions under stimulatory conditions. However, in a resting condition, we found that USCs have a stimulatory effect on B cells, which is T cell-independent, and mediated by both cell-cell contact as well as soluble factors. USCs constitutively secrete CD40L, IL-6 and BAFF, while inducing the secretion of APRIL when cultured with PBMC. These molecules are known to play a central role in B cell biology and survival.

We believe that the study of USCs has the potential to provide unique information of different kidney pathologies [89] and although the physiological function of USCs within the kidney is not known, our results raise the possibility of USCs having an immune modulatory role in

kidney infection and pathologies, for example, in the formation of tertiary lymphoid tissue observed in kidney inflammatory conditions such as in organ rejection and Systemic Lupus erythematous [90-92], where coincidently, kidney derived BAFF has been reported to play a central role [93].

Finally, we believe that the intrinsic B cell stimulatory capacity of USCs opens up new possibilities for their therapeutic use in conditions where immune stimulation, in particular B cells, is required, for example, cancer immunotherapy, immunization and immunodeficiencies.

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**Conflicts of Interest**

All authors declared no potential conflicts of interest.

**Data Availability Statement**

The data that supports the findings of this study are available on request from the corresponding author.

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