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TISSUE-SPECIFIC STEM CELLS

STEM CELLS

Urine stem cells are equipped to provide B cell survival signals

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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 coculture 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 unrecognized active role for kidney stem cells in modulating local immune cells.

KEYWORDS
B cells, BAFF, cytokines, mesenchymal stem cells, T cells, urine stem cells

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 noninvasive 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 coculture 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 subpopulations, 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.

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. The present study on the immunomodulatory properties of urine stem cells (USCs) shows that, like mesenchymal stem cells, they are capable of modulating immune cells. However, the authors 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. Furthermore, the authors' findings reveal a new possible therapeutic use of USCs as an immune adjuvant with clinical implications.

2 | MATERIALS AND METHODS

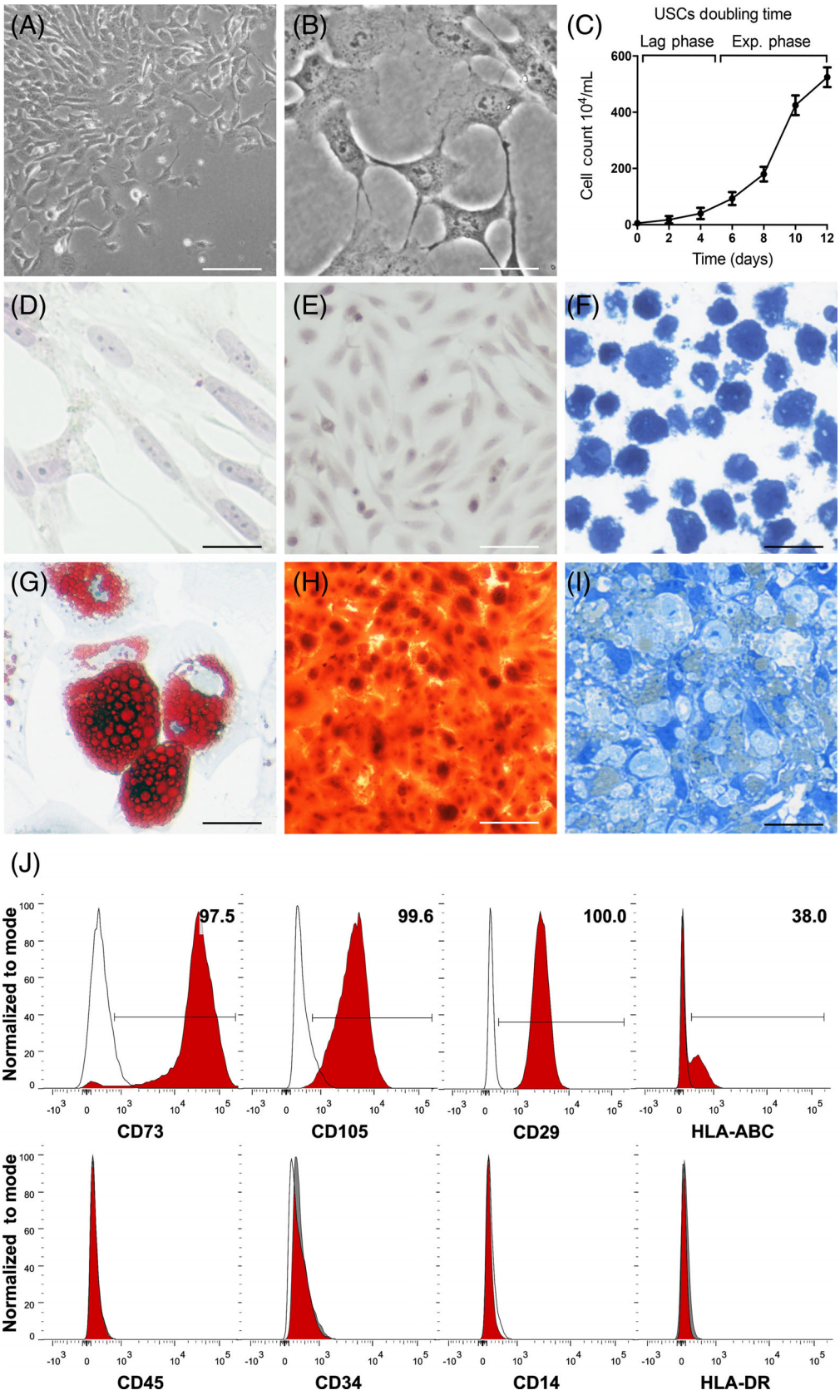
2.1 | Human USCs isolation and expansion

Urine samples were collected from six 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 (400*g* 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% fetal bovine serum (FBS) (Gibco) containing 100 μg/mL penicillin and streptomycin (P/S), seeded on tissue culture 4-well plates (Thermo Fisher Scientific), coated with tissue culture certified gelatine (SCT), 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), 5 ng/mL of bFGF, PDGF-BB, and EGF (Peprotech). During the culture, expansion media was replaced daily with a 50% fresh expansion medium. Confluent (80%-90%) cells were detached with TryplE (Gibco) 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]



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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 (Supporting Information Table S1) for 30 minutes at 4◦C. Then, the USCs were washed with PBS and analyzed by FACScanto flow cytometry (BD).

2.4 | Confocal microscopy

BM-MSCs and USCs at passage three were cultured over coverslips, washed twice before fixing with 4% paraformaldehyde (30 minutes). Cells were washed (X3) and blocked with bovine serum albumin (BSA) (10 mg/mL) and glycine (22.52 mg/mL) (30 minutes) 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 hour at room temperature. Cells were washed and mounted using DAPI mounting media (Sigma-Aldrich) 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 that is, 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 isolation

Fresh blood samples were collected from six healthy donors after informed consent (Human ethics approval no. 35760). Peripheral blood mononuclear cells (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) 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), achieving a purity >97%. To study the B cell subpopulations, B lymphocytes were sorted out according to the expression of CD27, CD24 and CD38 into three subpopulations: transitional cells ($CD27^{-}CD38^{++}CD24^{++}$), naïve cells ($CD27^{-}CD38^{+}CD24^{+}$) and memory cells ($CD27^{+}CD38^{+}CD24^{++}$), using a FACSAria cell sorter (BD Bioscience), achieving >98% purity, as described.[32-34]

2.7 | Coculture studies

To test the effects of USCs on the immune cells, mycoplasma free USCs vs BM-MSCs, were cocultured with PBMC. USCs or BM-MSCs were plated at 2.6×10^4 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 coculture. CFSE labeled 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 Scientific) 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).[35] USCs effect on B cells was also tested using USCs and purified B cells or B cell sorted subpopulations cocultures at the same ratio.

2.8 | Proliferation study

Proliferation was assessed by labeling the lymphocytes with CFSE (10 μM final concentration) using CellTrace (Thermo Fisher Scientific), following manufacturer instructions, before coculture with USCs or BM-MSCs. Cells were cocultured for 5 days and the percentage of divided cells was measured as previously

FIGURE 1 Characterization of USCs showing mesenchymal stem cell nature. USCs were isolated from healthy volunteers and their MSC characteristics were confirmed by their ability to differentiate into adipocytes, osteoblasts and chondrocytes, together MSC expression markers. A,B, Inverted phase light micrograph shows morphological appearance of the USCs where (A) USCs (P0) show stellate shaped morphology (×100, scale bar = 100 μm). B, USCs at P1 showing multiple projections reaching to nearby cells (×400, scale bar = 20 μm). C, USCs growth curve showing initial lag phase (4-6 days) followed by exponential growth with 24-30 hours doubling time (n = 3). D, Red O staining counterstained by Mayer's Hematoxylin of the undifferentiated USC (×400, scale bar = 20 μm). E, Alizarin Red staining of undifferentiated USC (×100, scale bar = 100 μm). F, Toluidine Blue staining of sectioned undifferentiated USC pellet showing clear intercellular spaces (×400, scale bar = 20 μm). G, Oil Red O staining of differentiated adipocytes, counterstained by Mayer's hematoxylin, showing red staining of intracellular lipid vacuoles (×200, scale bar = 50 μm). H, Alizarin Red staining of induced osteogenic cells showing orange discoloration of the mineralized nodules (×200, scale bar = 50 μm). I, Toluidine Blue staining of sectioned induced chondrogenic pellet showing pale blue staining of extracellular matrix (×400, scale bar = 20 μm). J, Representative sample of immune phenotype characterization of 10^5 USCs by flow cytometry showing positive expression of MSC markers CD73, CD29, CD105, and negative expression for the hematological markers CD14, CD34, CD45, and HLA-DR (MHC class II). The cells showed low expression of HLA-ABC (MHC class I)

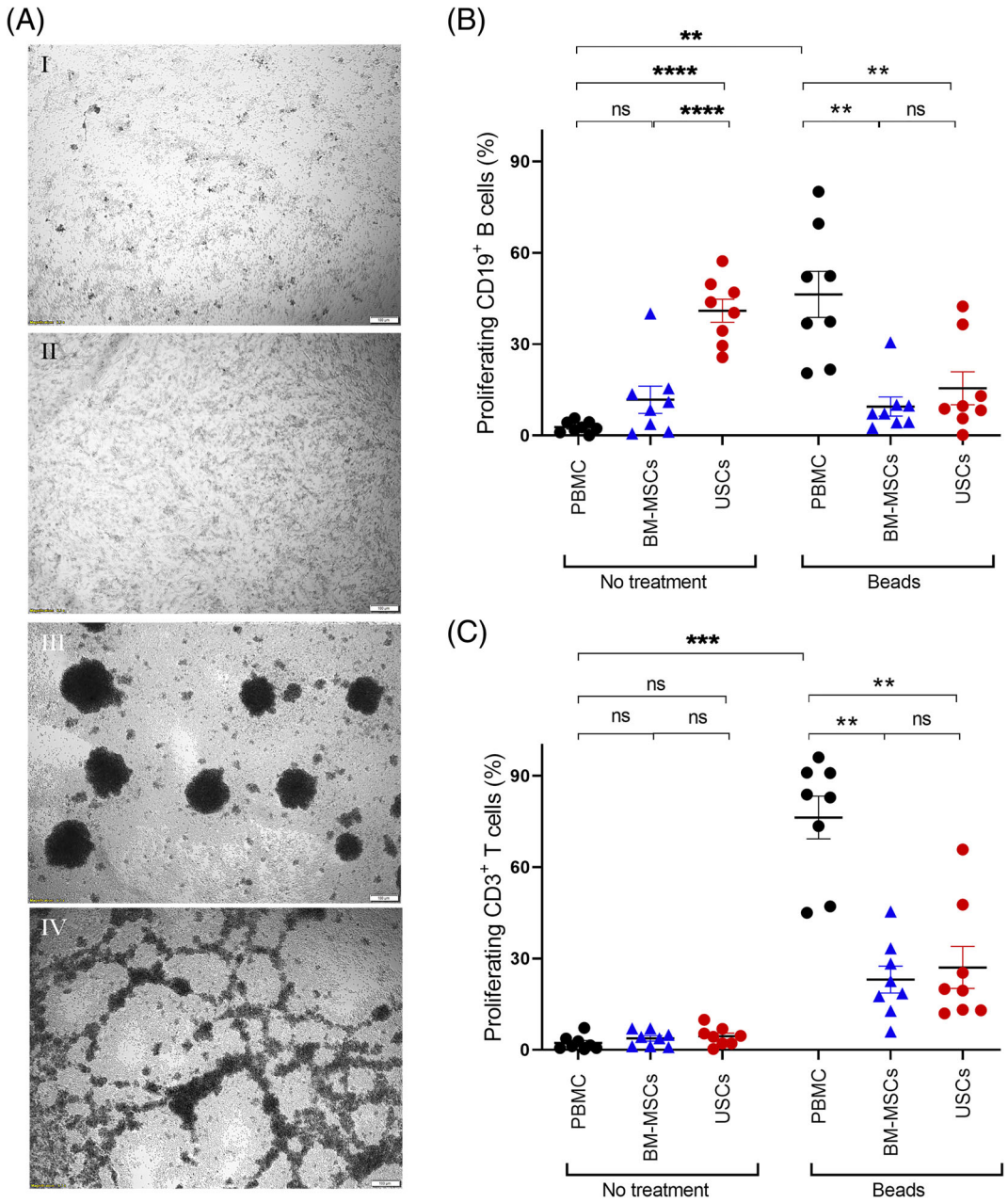
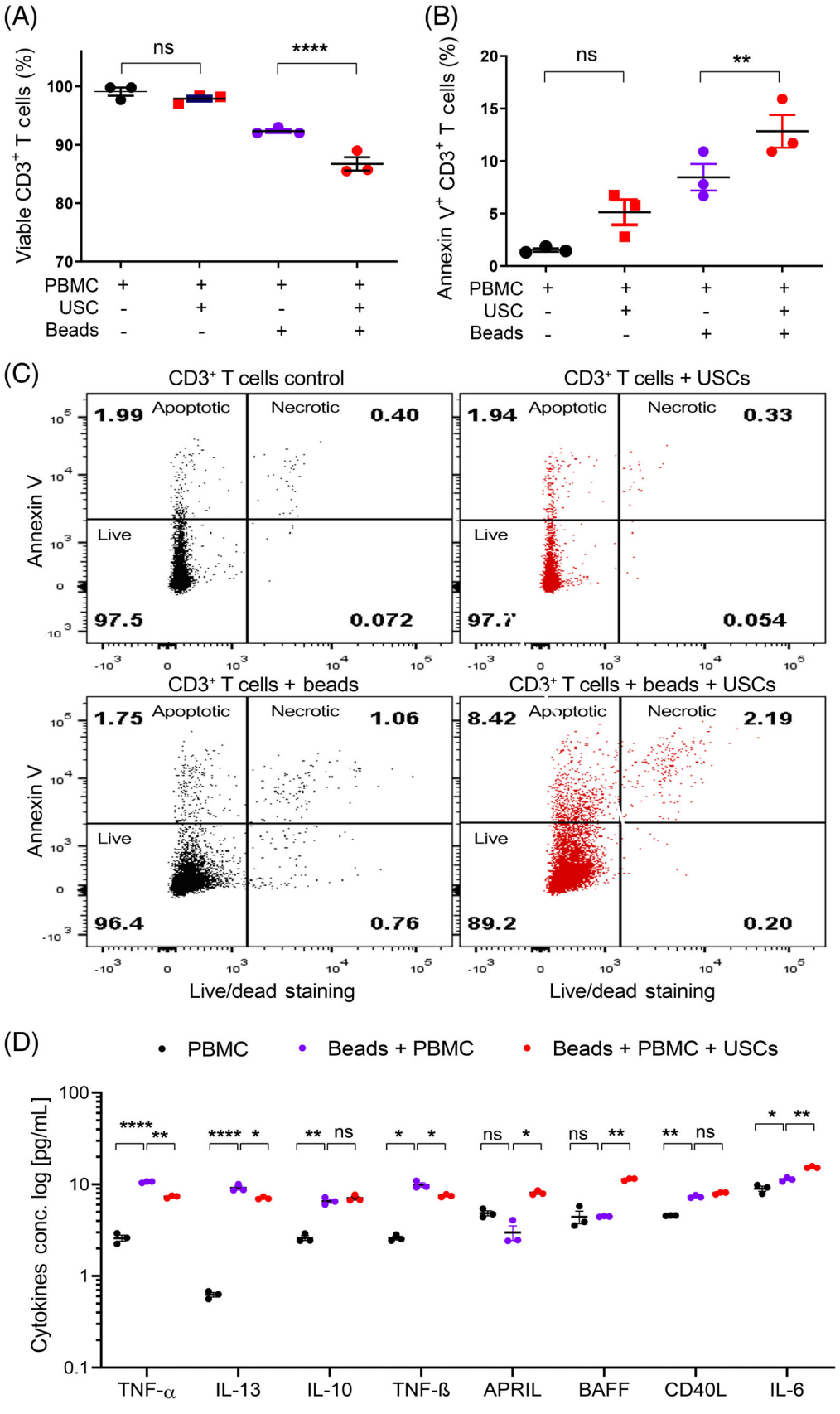


FIGURE 2 USC effects on PBMC culture compared to BM-MSCs. To analyze the USCs immune-modulatory effect, USCs and BM-MSCs were cultured with PBMC for 5 days either with no treatment, or in presence of anti CD3/CD28 beads (Beads), mimicking antigen presentation signaling. Cultures were monitored using inverted phase light microscope (A) and proliferation of the T & B cells were analyzed by flow cytometry (B, C). (A.I) PBMC cultured alone, (A.II) PBMC and USCs (1:5 ratio) cocultured, (A.III) PBMC cultured in the presence of the beads showing characteristic proliferation aggregates, (A.IV) PBMCs and USCs cocultured in the presence of beads, showing discrete aggregates in association with USCs (scale bar = 100 μm, original magnification ×100). B, Proliferation analysis of CD19+ B cells from PBMC cultured under similar conditions, either alone or cocultured with either BM-MSCs or USCs (2.7 ± 0.6 vs 11.78 ± 4.48 vs 40.96 ± 3.7%), or in the presence of the beads (46.3 ± 7.5% vs 9.5 ± 3.15 vs 15.56 ± 5.4) (n = 8). USCs induced a significant B cell proliferation in resting condition. C, Proliferation analysis of CD3+ T cells from PBMCs cultured alone or cocultured with either BM-MSCs or USCs (2.3 ± 0.8 vs 3.7 ± 0.8 vs 4.5 ± 1.07%) or in the presence of the beads (76.2 ± 7 vs 23.06 ± 4.4 vs 27.06 ± 6.8%) (n = 8). Neither USC nor MSCs induced T cell proliferation, however, they both significantly inhibited beads induced proliferation. Values are presented as mean ± SEM. Statistical analysis was done by RM One-way ANOVA followed by Tukey multiple comparison test. $^{*}P < .05$ and $^{**}P < .01$

described.[36] Cell viability was determined by Propidium Iodide (PI) staining or with FITC conjugated Annexin V (BD).[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 coculture with USCs for 24 hours. CpG-B ODN 2006



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(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 5 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).

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) 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, California). 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 <.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 (Figure 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 2-3 days up to passages seven or eight (Figure 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 (Figure 1D-F), demonstrated by the red staining of the intracellular vacuoles with Oil Red O stain of the induced adipocytes (Figure 1G), the orange staining of the calcified osteogenic nodules with Alizarin Red stain in the induced osteoblasts (Figure 1H) and the pale blue staining of the extracellular matrix with Toluidine Blue stain in the induced chondrocytes (Figure 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 (Figure 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 coculture

Monitoring of the PBMC culture wells showed that in the resting conditions compared to the PBMC cultured alone (Figure 2A.I), USCs seemed in causing the potential recruitment of the PBMC (Figure 2A. II). The addition of anti-CD3/CD28 beads to the PBMC caused the

FIGURE 3 USCs induce T cell apoptosis in beads activated condition. The effect of USCs on cytokine production and T cell viability was measured after 48 hours and 5 days of coculture respectively. A, PI staining of CD3+ T cells population from PBMCs cultured alone vs cocultured with USCs (99.1 ± 0.7% vs 98.3 ± 0.6%), or in the presence of anti CD3/CD28 beads (93.5 ± 0.3% vs 82.5 ± 4.9%). Viability of beads-stimulated T cells significantly decreased in the presence of USCs. B, Annexin V staining for apoptotic CD3+ T cells of PBMC cultured alone vs in USCs coculture (1.5 ± 0.17 vs 5.1 ± 1.2%), or in the presence of anti CD3/CD28 beads (8.5 ± 1.3 vs 13.0 ± 1.5%), showing the increase of apoptotic cells cultured under activated condition, which significantly increases in the presence of USCs. C, Representative sample of Annexin/Live Dead double staining of CD3+ T cells population from PBMCs cultured either alone or with untreated USCs, or with anti CD3/CD28 beads. Double negative are live cells, Annexing V positive Life/Dead negative are apoptotic cells and double positive necrotic cells. D, The effect of USCs on PBMCs cytokine production in response to anti-CD3/CD28 beads measured by beads array shows that USCs significantly suppressed the production of the inflammatory cytokines, TNF-α, TNF-β and IL-13 by T cells in response to anti-CD3/CD28 stimulation, while significantly increasing Il-6, BAFF and APRIL. Values are presented as mean ± SEM (A, B, D; n = 3). *P* values were determined by RM One-way ANOVA, with Geisser–Greenhouse correction followed by Tukey multiple comparison. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001

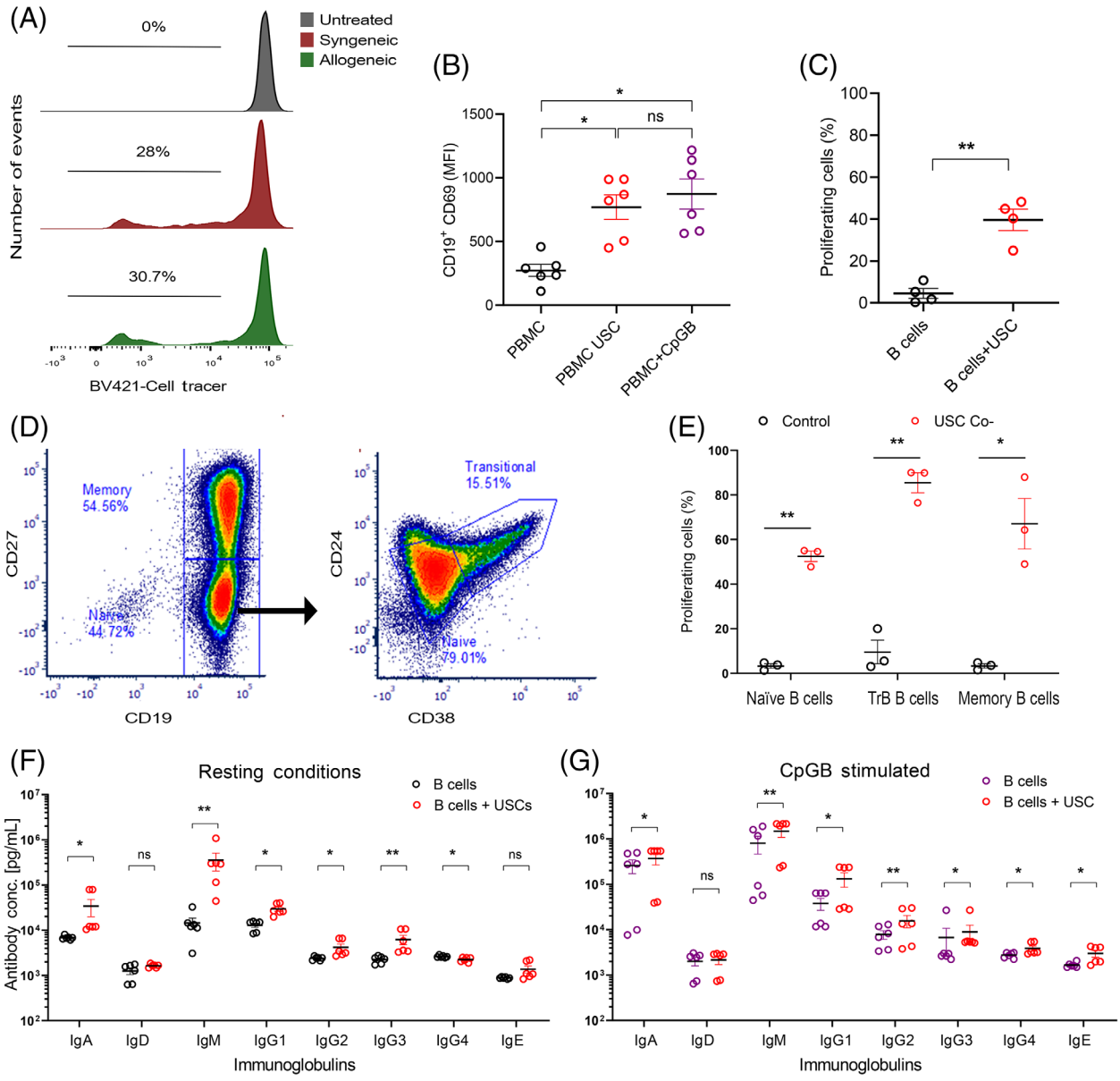
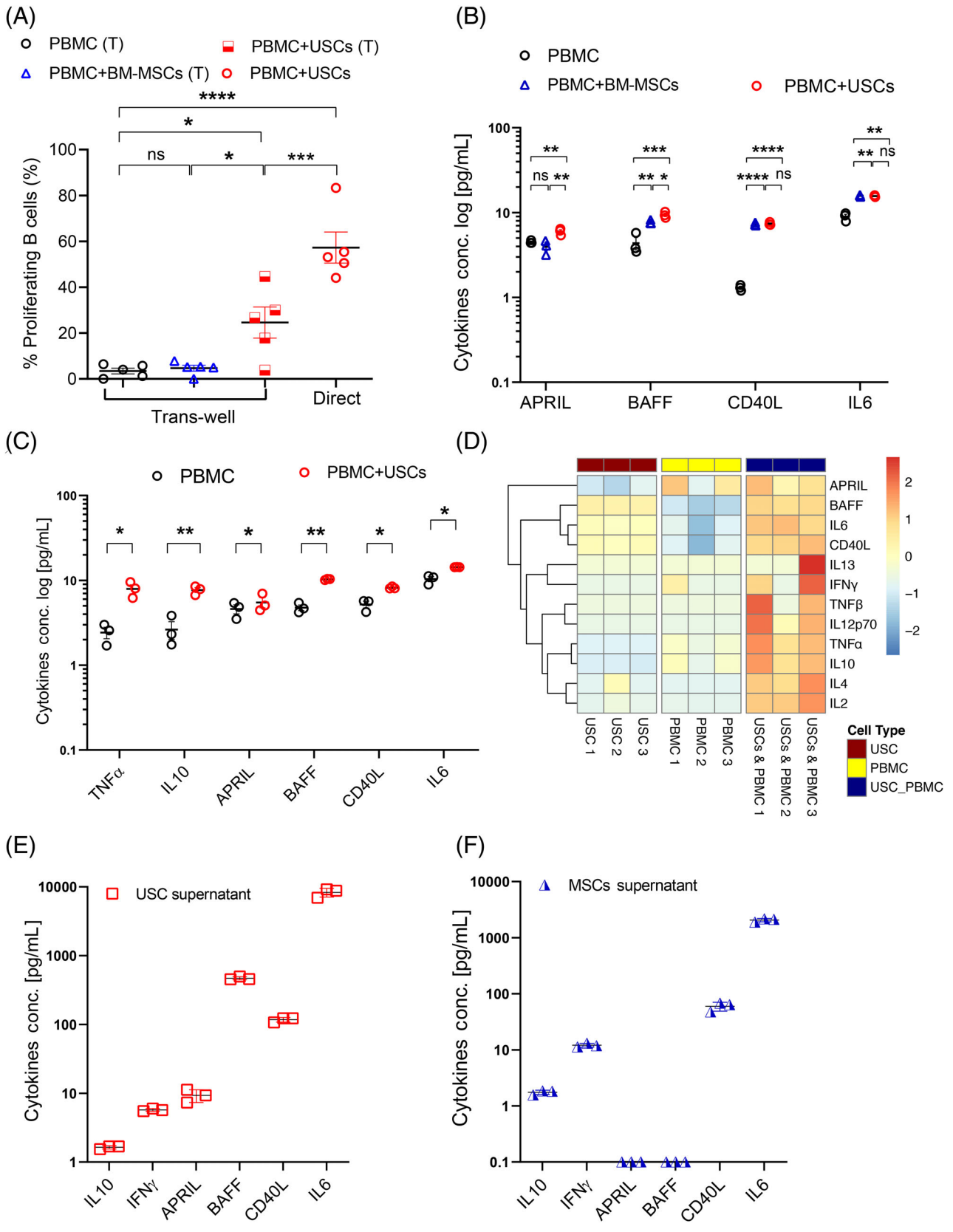


FIGURE 4 USCs induce the activation, proliferation and antibody secretion of B cells. To study the effect of USCs on B cells, we included the study of syngeneic and allogenic B cells to exclude the role of HLA mismatch and T cell dependent stimulation. A, Representative sample of B cell proliferation from PBMC either alone (untreated, Black) or cocultured with USCs either matched to the donor's lymphocytes (Syngeneic, Red) or nonmatched (Allogenic, Green), showing the percentage of proliferating B cells. B, Early activation marker, CD69, MFI expression on B cells (CD19+) following 24 hours of USCs coculture compared to CpGB activation (273 ± 47 vs 770 ± 96.7 vs 874 ± 118 MFI) (n = 6). C, Proliferation analysis of enriched B cells (>97% purity) cocultured with USCs (4.5 ± 2.3 vs 39.6 ± 5.1%) (n = 4). D, B cell sorting strategy based on sorting out the CD19 + CD27+ (memory B cells) followed by separating the CD19+CD27-populations into CD38+CD24+ (naïve B cells) and CD38++CD24++ (transitional B cells, TrB) subpopulations. E, Proliferation analysis of the obtained B subpopulations cocultured with USCs, naïve (3.2 ± 0.9 vs 50.3 ± 2.3%), transitional B cells (9.5 ± 5.2 vs 85.4 ± 4.5%), or memory B cells (3.6 ± 0.9 vs 67.2 ± 11.3%) (n = 3). F, Immunoglobulins concentration analysis in enriched B cell culture supernatant compared to USC cocultured. G, Analysis of immunoglobulins concentrations in enriched B cells culture supernatants in presence of CpGB compared to USCs cocultured (n = 6). Values are presented as mean ± SEM. Statistical analysis was done by One-way ANOVA followed by Tukey multiple comparison test (B) or paired Student *t* test (C,E) or Wilcoxon matched-pairs test (F,G). **P* < .05, ***P* < .01, *** *P* < .001

formation of proliferation aggregates (Figure 2A.III), consequent with their stimulation. However, in the USCs' coculture, PBMC seemed to be forming discrete aggregates with the USCs (Figure 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 coculture of USCs or BM-MSCs without any additional stimulation showed that neither USCs nor BM-MSCs caused significant T cell



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proliferation (Figure 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 (Figure 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 coculture 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 (Figure 2C). USCs induced proliferation seemed to be limited to the B cells population of the PBMC (Supporting Information Figure S1). 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 suppressed B cell proliferation in this condition, possibly reflecting the suppressing effect on T cells and a decrease in the T cell dependent B cell stimulation (Figure 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 analyzed 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 cocultured with USCs, which reached statistical significance in the presence of anti-CD3/CD28 beads (Figure 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 (Figure 3B). These results were confirmed by PI and Annexin V double staining (Figure 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 cocultures. 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 42 081 ± 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) (Figure 3D).

Combining this cytokine increase with the observed USCs' induced B cell stimulation observed before, we proceeded to analyze 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 mismatched B cell proliferation compared to control (Figure 4A).

The study of the USCs effect on B cell activation measured by CD69 expression showed that USCs coculturing with B cells caused a significant increase in the expression of CD69, similar to the CpGB stimulation, used as positive control (Figure 4B). In addition, USCs caused increased expression in the co-stimulatory molecule CD40 compared to untreated (1735 ± 461 vs 2695 ± 205 MFI, n = 3), (Supporting Information Figure S2).

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 toward a particular B cell subpopulation, we cocultured USCs with highly $CD19^+$ purified B cells. Again, USCs induced purified B cell

FIGURE 5 USCs secrete cytokines known to modulate B cells. USCs and BM-MSCs were cultured physically separated from PBMC in Transwell and the B population was analyzed for its proliferation. To elucidate on B cell stimulatory secreted factors, PBMC, USCs coculture, BM-MSCs coculture and USCs supernatants were analyzed for B cell related cytokines. A, Proliferation analysis of the B cells from PBMC cultured in Trans-wells (T) either alone, or over either BM-MSCs (T) (3.45 ± 1.3 vs 4.7 ± 1.2%) or USCs (T), or in direct contact with USCs (24.6 ± 6.7 vs 57.2 ± 6.8%) (n = 5). B, Analysis of cytokines concentration in supernatants collected from PBMCs cultured either alone or with BM-MSCs or USCs for possible B cell stimulants showing significant higher BAFF and APRIL concentration in USCs coculture. C, Heat map presenting log 10 B cell related cytokines concentration (Z-score normalized by row) in supernatants collected from USCs, PBMCs and USCs + PBMC. D, Analysis of the B cell related cytokines concentration in supernatants collected from PBMC compared to USCs cocultured showing significant increase in TNFα, IL10, IL6, BAFF, APRIL, and CD40L in the presence of USCs. E, Cytokines concentration in supernatant collected from 10^5 USCs, cultured for 48 hours. F, Cytokines concentration in supernatant collected from 105 BM-MSCs, cultured for 48 hours (n = 3). Values are presented as mean ± SEM. B,D, Cytokines are presented after log treatment of the data. *P* values were determined by One-way ANOVA (A,B) or paired *t* test (D), $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

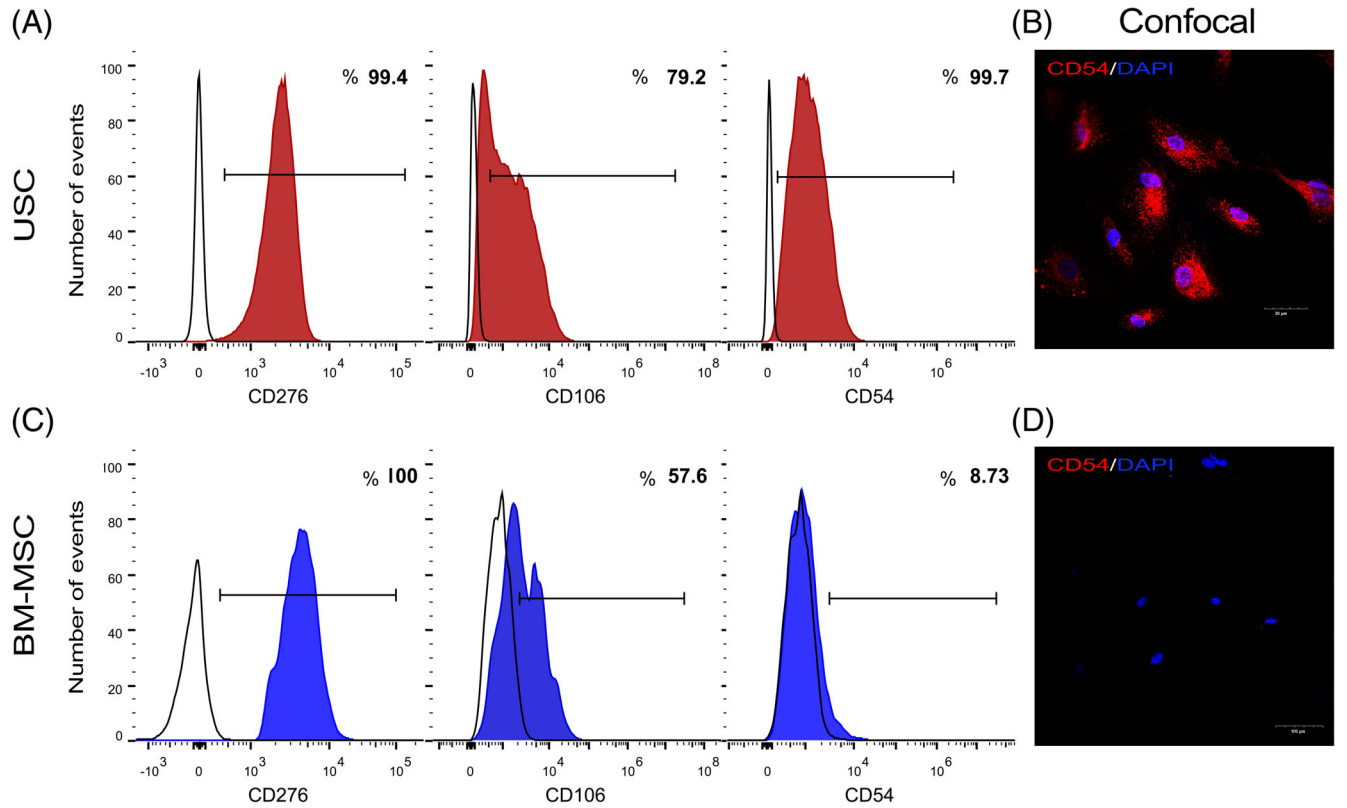

FIGURE 6 Study of surface markers expression by USCs and BM-MSCs (representative sample). A, Flow cytometry histograms of USCs (10^5) stained for the membrane expression of adhesion molecules CD54 (ICAM-1) and CD106 (VCAM-1) and the T cell function related molecules CD276 by USCs (shaded red histograms) compared to isotype control (non-shaded). B, Confocal image of DAPI (blue) stained USC after staining with CD54 mAb (red) (×600, scale bar = 30 μm). C, Flow cytometry histograms of BM-MSCs (10^5) stained for the membrane expression of adhesion molecules CD54 (ICAM-1) and CD106 (VCAM-1) and the T cell function related molecules CD276 (shaded blue histograms) compared to isotype control (non-shaded). D, Confocal image of DAPI (blue) stained BM-MSC after staining with CD54 mAb (red) (×600, scale bar = 100 μm). Three different cell lines were tested and the mean ± SEM was calculated for each antibody as following USCs vs BM-MSC CD276 (98.8 + 0.13 vs 98.1 + 0.8%), CD106 (74.9 ± 6.5 vs 53.3 ± 5.5%) and CD54 (98.8 ± 0.1 vs 7.9 ± 2.6%)

proliferation (Figure 4C). To test for the selective stimulation on B cell subpopulations, we proceeded to sort the B cells into transitional ($CD19^+CD27^-CD38^{++}CD24^{++}$), naïve ($CD19^+CD27^-CD38^+CD24^+$) and memory B cells ($CD19^+CD27^+$) (Figure 4D). USCs coculture with the sorted B subpopulations showed enhanced proliferation in all subpopulations studied, compared to cells cultured alone (Figure 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 coculture 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 (Figure 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 (Figure 4G), Supporting Information 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 where PBMC and USCs were cocultured 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 dependent on cell-cell-contact (Figure 5A). No proliferation was observed with BM-MSCs Trans-well coculture.

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

Further analysis was conducted on the USCs coculture supernatant for more B cell-related cytokines. Analysis of the coculture supernatant revealed a significant increase, compared with that of PBMC alone, in CD40L (40.7 ± 10.9 vs 294.7 ± 29.8 pg/mL), IL-6 (1600 ± 575 vs 37 018 ± 769.7 pg/mL), IL10 (7.5 ± 3.4 vs 233.5 ± 73 pg/mL), BAFF (28.9 ± 7.3 vs 1256.1 ± 84.5 pg/mL), and APRIL (27.7 ± 9 vs 60.6 ± 10 pg/mL) (Mean ± SEM, n = 3) (Figure 5C,D).

The measurement of these cytokines in 48 hours culture supernatant of USCs (10^5 cell/1.5 mL) 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) (Figure 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 (Figure 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 Figure 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 signaling (Supporting Information Figure S3A-C) and (Supporting Information 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) (Figure 1G), CD54 or ICAM-1(1553 ± 30 MFI), and CD106 or VCAM-1(1325 ± 77 MFI) (Figure 6A), which might correlate with the cell behavior in the in vitro coculture condition mentioned above. With regard to the T immunosuppressive function, we found that USCs highly expressed CD276 (2137 ± 184 MFI) (Figure 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 (Figure 6C). Confocal imaging of the USCs and BM-MSCs stained with CD54 confirmed these results, while USCs showed strong staining, BM-MSCs were negative (Figure 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 noninvasive 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) signaling.[58]

Following slow growth during the first 4 days of culture, USCs expanded exponentially, doubling every 24-48 hours, obtaining up to 1.6×10^7 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 cocultured in the presence of anti-CD3/CD28 beads (Figure 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 coculture 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 cocultured 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 coculture.

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 subpopulations. We found that USCs significantly induced proliferation in all three major B cell subpopulations (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 center. 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 antiapoptotic 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 did not 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,[8283-85] 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 behavior of USCs, the role of other cytokines and molecules not properly addressed in this study such as INFγ, 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 theorize 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 intrarenal 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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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

A.A.Z.: conception and design, data collection, data analysis and interpretation, manuscript writing and final approval; G.B.P.: conception and design, data collection, manuscript revision; M.A.-H: conception and design, data collection, manuscript revision; A.E.: data analysis; J.Y.: data collection; L.B., G.M.M.: manuscript revision; X.-F.Z.: conception and design and manuscript final approval; P.R.H.: conception and design, data analysis and interpretation, manuscript writing and final approval.

DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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