Peer Review File

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Reviewer A

Comments 1- Material and Methods line 206- NK cytotoxicity assay

The experiment, as performed, only reflect a decreased susceptibility of USCs to be lysed by NK cells compared to the other cells, rather than inhibition in NK cell activity.

To measure the inhibitory function on NK function would require assessing the effect of USCs on the ability of NK cells to lysing known target cells such as K562.

Reply 1: Thank you very much for your valuable comments and advice. In this study, We found the phenomenon that USCs had less cell lysis when mixed with nature killer cells comparing with SMCs and BMSCs. The CytoTox 96 Non-Radioactive Cytotoxicity Assay is a colorimetric alternative to radioactive cytotoxicity assays. The assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a 30-minute coupled enzymatic assay that results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Visible wavelength absorbance data are collected using a standard 96-well plate reader. The assay can be used to measure membrane integrity for cell-mediated cytotoxicity assays in which a target cell is lysed by an effector cell. It was used to analysis NK cytotoxicity had been published in literatures (Natural Killer Cell (NK-92MI)-Based Therapy for Pulmonary Metastasis of Anaplastic Thyroid Cancer in a Nude Mouse Model, Front Immunol. 2017 Jul 21;8:816.), it is the alternative method rather than K562 cell line to evaluate NK cells lysing effect, which might help us to understand the phenomenon of USCs inhibiting the NK cells lysing effects.

Comments 2- Discussion line 356:

The authors make an unstained claim that the possible association of the release of the proinflammatory cytokines and chemokines such as IL6, IL8, RANTES with the inhibitory

effect on lymphocyte proliferation. However, fail to measure molecules know to mediates the effect, such as IL-10 and PDL1.

Reply 2: The cytokines and chemokines released by USCs to the supernatants when cultured in absence of PBMNCs and in direct or indirect contact with PBMNCs were evaluated using the Human Cytokine Array Panel A kit (R&D Systems Inc., MN, USA). In this study, all the samples did not show significantly positive in IL-10， and PDL1 did not included in the Human Cytokine Array Panel A kit, we mostly focused on IL-6, IL-8 , MCP-1, RANTES and other cytokines and chemokines like GROα and GM-CSF might also play a role in immunomodulatory effects of USCs were added and mentioned in the discussion section line 354~358.

Changes in the text: RANTES and other cytokines and chemokines like GROα and GM-CSF might also play a role in immunomodulatory effects of USCs were added and mentioned in the discussion section line 354~358.

Comments 3- At basal level, it seems that USC secrete more cytokines/chemokines than BMSC, specially GROα than BMSC, not discussed in the paper.

Reply 3: GROα(Growth Regulatory Oncogene α), also known as CXCL1, is a chemokine thought to have mitogenic properties and chemoattract neutrophils. GROα was initially discovered as a growth-regulated gene that is overexpressed constitutively in tumorigenic cells and transcribed in normal cells only during growth stimulation. GROα is a potent chemoattractant for human neutrophils and stimulates neutrophil degranulation and enzyme release from cytochalasin B-treated human neutrophils. However, the immuno-regulatory mechanism of GROα was not totally clear, so we just mentioned it in the discussion section, not discussed in detail.

Changes in the text: Other cytokines and chemokines like GROα and GM-CSF might also play a role in immunomodulatory effects of USCs were added and mentioned in the discussion section line 354~358.

Comments 4- The number of sample studied is very limited to reach a reliable conclusion.

Reply 4: We recruited volunteers in different age providing urine samples for cells isolating

and further experiment in this study, we expanded cells clones and repeated the experiment triplicated for all procedure to make the work consistently.

Comments 5- Finally, recent papers have shown that USCs have strong B cell stimulatory properties, questioning their possible use to treat autoimmune disorders. The authors should address such concerns.

Reply 5: We researched with human peripheral blood mononuclear cells in this study, we do not study it with B cell separately, we could not gave the comments about USCs effects to B cells in this study.

Changes in the text: We added and mentioned the limitations of this study in discussion section line 371~380 to concern the more research work should carry out in the following study.

Reviewer B

Wu and co-authors' intent was to profile and test immune-modulatory capacity constitutively expressed by urine-isolated (stem) cells. The cells collected and purified in adult urine have been shown some characteristics important for cell-based treatments. Urine-derived cells, most likely resulted from exfoliating process in the interface of renal glomerulus, have been collected and proved MSC-like properties. MSC term stands for mesenchymal stromal cells, frequently miscalled as mesenchymal stem cells. Stem cells definition is quite broad range, and cells benefit from such definition should satisfy all the stemness requirements. Thus, the real question is: do the USC satisfy all the stem cell requirement?

Several issue and/or concerns need to be addressed before considering the manuscript for publication:

Comments 1: Multipotency: MSC (and maybe USC) have been shown multipotency capacity (if limited to mesoderm lineage or including also the other 2 germ layer is still under debate)

and anti-inflammatory and immune-modulatory properties.

The authors stated multipotency capacity in USC (page 6, line 105: “USCs can be efficiently induced into ectodermal, mesodermal, and endodermal lineages”), however such affirmation needs to be sustained and supported by reference(s).

Reply 1: The multiportency of USCs have been well addressed in our previous study and publicshed (Bharadwaj S, Liu G, Shi Y, et al. Multipotential differentiation of human urine-derived stem cells: potential for therapeutic applications in urology. Stem Cells 2013;31:1840–56.).

Changes in the text: The reference have been added at the reference section, as shown in the16th reference.

Comments 2: At page 14; line 253, the authors stated, “Typical embryonic markers”. What do they actually refer to? None of the classic embryonic markers (i.e., SSEA-3 or SSEA-4, TRA-1-60 or TRA-1-81) have been tested. Neither Oct4, nanog, sox-2 genes. Please comment.

Identity: MSC have been previously described as cells (Dominici et al., Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006):

1. MSC must be plastic-adherent when maintained in standard culture conditions;
2. must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules
3. MSC must differentiate to osteoblasts, adipocytes and chondroblasts in vitro

In the current manuscript few of such critical assessment have been shown. Notably, endoglin (CD105) has not been analyzed. Please motivate and/or edit it.

Reply 2: The cell surface markers of USCs were screened in this study, including CD29, CD44, CD54, CD73, CD90, CD146, CD166 were positive expressed in USCs, otherwise, there were negative expressed of CD11, CD14, CD19,CD31,CD34, CD45, CD79 in USCs samples, we try to address that USCs have some characteristics and cell surface markers similar to bone marrow mesenchymal stem cells but not totally same as it, that suggested that USCs might share some characteristics as mesenchymal stem cells but not the MSC we

already known about, USCs might be a novel MSCs like cells. The expression of SSEA-4, CD105, Oct4, sox-2 had been researched in our group's previous publication (Bharadwaj S, Liu G, Shi Y, et al. Multipotential differentiation of human urine-derived stem cells: potential for therapeutic applications in urology. Stem Cells 2013;31:1840–56.) we did not repeat it in this study.

Comments 3: Telomerase activity appears to be extremely variable between clones. (page 14, lines 266-268: "specific clones were selected from 3 donors. Each donor provided two USCs clones, one with high telomerase activity (USC-TA+) and another one with low telomerase activity (USC-TA-"). Please comment. Why such clones have been selected? Further analysis is probably required to draw any conclusion and correlate such activity with cell quality. Why authors hypothesised that differences in telomerase activity would reflect on immune-modulatory effects is not clear. Please motivate. Lines 272-281 are quite redundant and repetitive. Please revise

Reply 3: For the study of different clones with high and low telomerase activity, in the current study, the MLR and NK cytotoxicity assays indicated the absence of a significant difference between the inhibitory effects of USCs-TA+ and USCs-TA−, suggesting that the telomerase activity seemed to have little or no effect on the immunomodulatory properties of USCs, even though the USC-TA+ cells were more easily expanded to a suitable quantity for clinical usage. However, it is worth to further address in vivo whether USC-TA+ can maintain their immunomodulatory properties for longer periods than USC-TA− cells.

Changes in the text: We carefully revised the manuscript with the comments including lines 272~281.

Comments 4: Immune effects on PBMNC or NK should probably be investigated in the lights of specific mechanism and molecules (as previously described on other immunomodulatory cells)

The authors claimed (page 17, line 306) "This study demonstrated that human USCs express immunological markers and possess immunomodulatory properties in similar levels to MSC". Such affirmation is quite ambitious. No specific immunomodulatory pathways nor specific

membrane-bound immunomodulatory proteins has been detected (the authors mostly identified classical markers and ECM adhesion subunits).

Results are extremely limited, lacking biomolecular evaluations and specific inhibitory effects. Any particular immune-modulatory pathways have been depicted nor characterized. The sole immune-modulatory molecule actually tested is non-polymorphic HLA-G, proving lack of expression for such important immunomodulatory molecules. The additional immune-effective marker currently analysed is CD73. Any other ecto-nucleotidases has been analysed or measured (neither in classical ATP nor alternative GADPH pathway)

The study is not actually analysing and profiling immune-modulatory properties but immunosuppressive potentials towards Mixed lymphocyte (PBMNC aka peripheral blood mononuclear cells).

Reply 4: Thank you for your reliable comments very much, in this study, we presented the phenomenon of immunosuppressive effects of USCs, we screened the cell surface markers of USCs, tested the mixed lymphocyte reaction, NK cytotoxicity assay, and analyzed cytokine and chemokines released by USCs, compared with bone marrow mesenchymal stem cells and smooth muscle cells, we also found USCs did not express HLA-D and HLA-G, try to address the characteristics of USCs in the immune system recognizing and the potential for immunosuppressive and immunomodulatory properties of USCs. We did not perform the further biomolicular evaluations and specific inhibitory effects, we revised the manuscript followed your reliable comments and added the limitations of this study in discussion section line 371~380 and reveal to complete the research in the following study.

Changes in the text: We revised the manuscript followed your reliable comments and added the limitations of this study in discussion section line 371~380 and reveal to complete the research in the following study.

Comments 5: The statement at page 17 (line 324-326) “the absence of co-stimulatory molecules and MHC-II markers on cultured USCs suggested that they possessed hypoimmunogenic properties, allowing them to escape their recognition by the recipient’s immune system” is actually over-stating and too much. Please rephrase or sustain such affirmation. Another interesting syllogism ”MSCs, even from different origins, possess

significant immunomodulatory potential. Since USCs may be an optimal source for stem cell therapy in cases of immune dysfunction". The authors should clearly state that they consider USC as alternative source for MSC, with related characteristic and properties.

Reply 5: We presented the phenomenon of immunosuppressive effects of USCs in this study, USCs shared similar stem cell markers with BMSCs, but also expressed different ones, the absence of co-stimulatory molecules and MHC-II markers on cultured USCs might help USCs to escape their recognition by the recipient's immune system. suggesting that USCs might be a new type of adult stem cells with hypoimmunogenic characteristics that require further characterization. The immunomodulatory mechanism of USCs is not clear yet even we have found some of immunosuppressive effects possessed by USCs, it still worth to study further in the future.

Changes in the text: We adjusted and revised the manuscript with your comments, especially in line 324-326 .

Reviewer C

Thank you for taking the time to investigate the characteristics of these novel class of cells. I am of the view that these urine-derived stem cells hold a lot of potential in promoting research and clinical use of cell therapy in the field of immunology, urology and beyond. With regards to the paper under review, the title and conclusions are appropriate and methods are clearly written. The functional characteristics and cytokine expression profile of the USCs found in this study enriches our knowledge and increases the therapeutic appeal of the USCs. However, I am of the view that for the graphical presentation of the cytokine profile on page 31, it would benefit readers more if the graph was divided into about 4 smaller graphs (say A, B, C, D). This would make it more eligible and well-organized. We look forward to reading the future work stated by the authors.

Reply: Thank you very much for your encouragement and advice for revising and improving our manuscript. We are quite appreciated for your earnest work. We adjusted the figures in the revised manuscript and try to make them much clearer.

Changes in the text: We adjusted the figures in the revised manuscript and try to make them

much clearer.

Reviewer D

Comments 1: Cells from the urine are cells expelled from the epithelial structure, e.g. the renal tubular epithelium and the bladder epithelium. They should therefore be fully characterized - also using epithelial markers. The characterization should be shown in full (as histograms) and not just mentioned in the text.

Reply 1: Thank you for your reliable comments and consideration very much, in this study, we tried to present the phenomenon of immunosuppressive effects of USCs, the origin of USCs is still controversial, some study suggest USCs come from renal tubular and other study suggest it might originate from parietal cells of nephron capsule epithelial cells, otherwise, our study show USCs have some characteristics of immunosuppressive or so call immuno-evacuation effects, that might help us to understanding the characteristics of USCs further more, and know about USCs being used in immunomodulate therapy potential. The epithelium markers expressed by USCs have been addressed detailed in our group's previous publication (Bharadwaj S, Liu G, Shi Y, et al. Multipotential differentiation of human urine-derived stem cells: potential for therapeutic applications in urology. Stem Cells 2013;31:1840–56.) we did not show the detail in this study.

Comments 2: Please show the morphology of the cultured cells.

Reply 2: Thanks for your comments and suggestion, we added the morphology of cultured cells in figure 2-1.

Changes in the text: We added the morphology of cultured cells in figure 2-1.

Comments 3: The conclusion is not supported by the results

line3: novel source for cell therapy? Really....please add references!

Reply 3: This study we investigated the USCs immunomodulatory effects. Our results showed that USCs expressed typical MSC-like surface cell markers and possessed good immunomodulatory properties, comparable to those of BMSCs. Stem cell therapy has been

demonstrated to possess the capacity to modulate the immune system and reduce the severity of the disease in animal models of autoimmune disorders, mesenchymal stem cells (MSCs) are commonly used for cell therapy, harvesting MSCs requires invasive medical procedures, which could cause potential complications and even risk to the donor's lives. USCs can be isolated from fresh voiding urine and propagated through a simple, noninvasive, and low-cost manipulation, making them novel and more attractive for tissue regeneration and stem cell therapy potential.

Changes in the text: The references have been added and adjusted as reference 11th, 15th, 16th.

Comments 4: line 52: emryonic markers? Please clarify.

Reply 4: what we mean is USCs exhibited cell surface expression of embryonic stem cell markers and mesenchymal stem cells markers CD29, CD44, CD54, CD73, CD90, CD146, and CD166.

Comments 5: The entire manuscript should be linguistically revised and contains some typing errors.

Please revise Fig 1 (histograms?)

Fig. 4 must be completely reworked and presented in a scientifically customary manner.

Reply 5: The manuscript has been carefully linguistically typing and grammar revised. Figure 1 is calculated and output by Flowjo software directly. Figure 4-1, Figure 4-2, Figure 4-3 have been reworked.

Changes in the text: Figure 4-1, Figure 4-2, Figure 4-3 have been reworked.