Human Umbilical Cord Mesenchymal Stem Cells Inhibit the Function of Allogeneic Activated Vγ9Vδ2 T Lymphocytes In Vitro

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Background. Human umbilical cord mesenchymal stem cells (UC-MSCs) can regulate the function of immune cells. However, whether and how UC-MSCs can modulate the function of Vγ9Vδ2 T cells has not been fully understood. Methods. The PBMCs or Vγ9Vδ2 T cells were activated and expanded with pamidronate (PAM) and interleukin-2 (IL-2) with or without the presence UC-MSCs. The effects of UC-MSCs on the proliferation, cytokine expression, and cytotoxicity of Vγ9Vδ2 T cells were determined by flow cytometry. The effects of UC-MSCs on Fas-L, TRAIL-expressing Vγ9Vδ2 T cells, and Vγ9Vδ2 T cell apoptosis were determined by flow cytometry. Results. UC-MSCs inhibited Vγ9Vδ2 T cell proliferation in a dose-dependent but cell-contact independent manner. Coculture with UC-MSCs reduced the frequency of IFNγ+ but increased granzyme B+ Vγ9Vδ2 T cells. UC-MSCs inhibited the cytotoxicity of Vγ9Vδ2 T cells against influenza virus H1N1 infected A549 cells and also reduced the frequency of Fas-L+, TRAIL+ Vγ9Vδ2 T cells but failed to modulate the apoptosis of Vγ9Vδ2 T cells. Conclusions. These results indicated that UC-MSCs efficiently suppressed the proliferation and cytotoxicity of Vγ9Vδ2 T cells and modulated their cytokine production. Fas-L and TRAIL were involved in the regulation. Cell contact and apoptosis of Vγ9Vδ2 T cells were not necessary for the inhibition.

1. Introduction

Mesenchymal stem cells (MSCs) can spontaneously proliferate and differentiate varieties of cell types, including osteoblasts, chondrocytes, adipoblasts, skeletal myocytes, and tenocyte [1, 2]. Furthermore, previous studies have shown that MSCs have unique immunomodulatory properties and potent immunosuppressive activities because they inhibit alloreactive T cell responses [2–4]. Instead, MSCs have been demonstrated to inhibit the function of immune cells, such as αβ T cells [3, 5, 6], NK cells [3, 7–9], macrophage [3], and dendritic cells (DCs) [2, 3, 10]. In addition, MSCs can enhance regulatory T cell responses and help tissue repairs [11]. Because of unique features, they have been widely investigated as a new therapy for graft versus host disease (GvHD) [12, 13], myocardial infarction, stroke, lupus, arthritis, Crohn's disease, acute lung injury, chronic obstructive pulmonary disease (COPD), cirrhosis, multiple sclerosis, amyotrophic lateral sclerosis (ALS), and diabetes [13]. Among all sources of MSCs [2], umbilical cord derived MSCs (UC-MSCs) offer other feasible candidates for MSC-based therapies because of their abundant resources, noninvasive acquiring, low immunogenicity, and great capacity of ex vivo expansion [2, 14].

γδ T cells have unique innate and adaptive immunity features and account for approximately 1%–5% of circulating T cells [15, 16]. γδ T cells can respond to exotic factors [17, 18] and periphery blood Vγ9Vδ2 T cells, and the largest subset of γδ T cells can be activated by small nonpeptide phosphoantigens such as isopentenyl pyrophosphate (IPP)
and pamidronate (PAM) [19, 20] in an HLA-unrestricted manner [16, 21]. Functionally, \( \gamma \delta \) T cells play an important role in host defense, especially in homeostasis and surveillance [21]. Previous studies have shown that \( \gamma \delta \) T cells regulate the process of innate immunity [22], immune responses against infection [17, 23], cancer [15, 22, 24], and autoimmune disease [25]. Both \( \gamma \delta \) T cells play regulating roles in GVHD and some autoimmune diseases [13, 25, 26]; however, how \( \gamma \delta \) T cells respond to allogeneic UC-MSCs has not been clarified.

In this study, we isolated and expanded UC-MSCs as well as peripheral blood mononuclear cells (PBMCs) from healthy donors and examined the regulatory effect of allogeneic UC-MSCs on the proliferation, cytokine production, and cytotoxicity of \( \gamma \delta \) T cells in vitro. Our findings indicated that UC-MSCs inhibited the proliferation, cytokine production, and cytotoxicity of \( \gamma \delta \) T cells in vitro. Cell contact and apoptosis of \( \gamma \delta \) T cells were not necessary for the inhibition. Fas-L and TRAIL were involved in the regulation. We discussed the implications of our findings.

## 2. Materials and Methods

### 2.1. Preparation of UC-MSCs

The experimental protocol was approved by the Institutional Review Board of Sichuan University. UC-MSCs were isolated from human umbilical cords, as described previously [27]. UC-MSCs were cultured in a 75-cm\(^2\) flask in α-MEM medium (HyClone, Chengdu, China) containing 15% heat inactivated fetal bovine serum (FBS, BioWest, South America), 100 units/mL of penicillin, and 100 μg/mL of streptomycin at 37°C in 5% CO\(_2\) incubator. When the cells reached 80% confluency, UC-MSCs were digested with 0.25% trypsin and harvested for subculture or irradiation process. The expanded UC-MSCs were characterized for their abilities to self-renew and differentiate multiple lineages of cells (data not shown). UC-MSCs were irradiated by X-ray (30 Gy) on Precision X-ray, (X-RAD 320), and cocultured with PBMCs or \( \gamma \delta \) T cells.

### 2.2. Preparation of PBMCs and \( \gamma \delta \) T Cells

Human peripheral blood samples were obtained from healthy donors and PBMCs were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ, USA) density gradient centrifugation, as previously described [23]. The isolated PBMCs were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 2 μg/mL of pamidronate disodium (PAM), and 100 IU/mL of human recombinant IL-2 (Invitrogen, Carlsbad, CA, USA). The cells were exposed to freshly prepared medium supplemented with, or without, allogeneic irradiated UC-MSCs at cell ratios of 5:1 or 80:1 for 48 hours. The exposed cells were washed, stained with AP-cy7-anti-CD3 and APC-anti-TCRy, fixed, permeabilized, and intracellularly stained with FITC-anti-IFNγ, PE-anti-TNFα, PE-anti-IL-10, PE-anti-IFNγ, FITC-anti-granzyme B (BD Biosciences) to examine the frequency of cytokine-expressing \( \gamma \delta \) T cells, respectively.

### 2.3. PBMCs/\( \gamma \delta \) T Cells Cocultured with UC-MSCs

PBMCs were cocultured with UC-MSCs in 10% FBS RPMI 1640 medium containing PAM and IL-2 with or without transwell system. PBMCs (1.5 ∗ 10\(^6\)/well) were cocultured with 30 Gy-irradiated UC-MSCs at cell ratios of 5:1, 20:1, 80:1, or 320:1 for 48 hours to 14 days. In transwell system, PBMCs/\( \gamma \delta \) T cells were cultured at the bottom chamber of 24-well transwell plates (0.4 mm pore; Millipore, Bedford, MA, USA) and 30 Gy-irradiated UC-MSCs were cultured at the top chamber of the plates.

### 2.4. Proliferation Assay

The isolated PBMCs (1.0 ∗ 10\(^6\)/mL) were labeled with 1 μM 5,6-carboxylfluorescein diacetate succinimidyl ester (CFSE, Dojindo, Kumamoto, Japan) for 15 minutes. After being washed, the labeled PBMCs were cocultured with UC-MSCs in triplicate at the cell ratios of 5:1, 20:1, 80:1, and 320:1 for 14 days with PAM and IL-2. The cells were exposed to freshly prepared medium every three days. The cells were stained PE-anti-TCRy and APC-cy7-anti-CD3 (BD Biosciences, San Jose, USA) and the frequency of \( \gamma \delta \) T cell proliferation was determined by flow cytometry on a BECKMAN, FC 500, followed by analyzing the data with FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Furthermore, the CFSE-labeled PBMCs (1.5 ∗ 10\(^6\)/well) at the top chamber of 24-well transwell plates (0.4 mm pore; Millipore, Bedford, MA, USA) were cocultured with 30 Gy-irradiated UC-MSCs at the bottom chamber of the plates at the cell ratios of 5:1, 20:1, 80:1 for 48 days with PAM and IL-2. PBMCs alone at the top chamber served as controls. The PBMCs were stained with PE-anti-TCRy and APC-cy7-anti-CD3 to determine the proliferation of \( \gamma \delta \) T cells.

### 2.5. Cytokine Assay

Freshly isolated PBMCs were stimulated with PAM and IL-2 for 12 days and the cells were cocultured with, or without, allogeneic irradiated UC-MSCs at cell ratios of 5:1 or 80:1 for 48 hours. During the last 4-hour culture, the cells were treated with Brefeldin A. Subsequently, the cells were harvested, stained with APC-cy7-anti-CD3 and APC-anti-TCRy, fixed, permeabilized, and intracellularly stained with FITC-anti-IFNγ, PE-anti-TNFα, PE-anti-IL-10, PE-anti-perforin, or FITC-anti-granzyme B (BD Biosciences) to examine the frequency of cytokine-expressing \( \gamma \delta \) T cells, respectively.

### 2.6. Cytotoxicity Assay

Human adenocarcinomic alveolar basal epithelial A549 cells provided by the laboratory were cultured in 10% FBS RPMI 1640 medium. Furthermore, A549 cells were cultured overnight and, after being washed with phosphate-buffered saline (PBS), the cells were infected with influenza virus H1N1 (A/PR/8/34) [27] at a multiplicity of infection (MOI) of 2 for 1 hour, followed by washing out free virus with PBS. Freshly isolated PBMCs were stimulated with PAM and IL-2 for 12 days and were cocultured with allogeneic irradiated UC-MSCs at cell ratios of 5:1 or 80:1 for 60 hours. The contained \( \gamma \delta \) T cells were purified by negative selection, as described above. The purified \( \gamma \delta \) T cells (effector) were cocultured in triplicate with influenza virus- (H1N1-)
infected A549 cells (target) at an effector-to-target (E/T) ratio of 10:1 for 5 hours. Subsequently, the cells were stained with APC-cy7-anti-CD3 and 7AAD (BD Biosciences) and the percentages of dead A549 cells in total CD3-A549 cells were determined by flow cytometry.

2.7. Apoptosis Assay. freshly isolated PBMCs were stimulated with PAM and IL-2 in the presence or absence of irradiated UC-MSCs at the cell ratio of 5:1 or 80:1 for 14 days. The cells were harvested and stained with APC-cy7-anti-CD3, APC-anti-TCRVγ9Vδ2, and PE-anti-Fas-L or PE-anti-TRAIL. The frequency of Fas-L+ or TRAIL+ Vγ9Vδ2 T cells was determined by flow cytometry. In addition, some cells were stained with APC-cy7-anti-CD3, APC-anti-TCRVδ, FITC-Annexin-V, and 7-AAD to determine the frequency of apoptotic Vγ9Vδ2 T cells.

2.8. Statistical Analysis. Data are representative FACs charts or histograms or expressed as the mean ± SEM. The difference among groups was analyzed by ANOVA and post hoc t-test using GraphPad Prism software (version 5). A P value of <0.05 was considered statistically significant.

3. Results

3.1. UC-MSCs Inhibit the Proliferation of Allogeneic Vγ9Vδ2 T Cells. We characterized the frequency of peripheral blood Vγ9Vδ2 T cells by flow cytometry analysis and found that peripheral blood Vγ9Vδ2 T cells accounted for 3%–8% of T cells (data not shown). Following stimulation with PAM and IL-2 for 14 days, the percentages of Vγ9Vδ2 T cells reached 40%–90% of cultured T cells. To determine the effect of UC-MSCs on the expression of cytokines and functional enzymes, the isolated PBMCs were stimulated with PAM and IL-2 for 12 days and the cells were cocultured with, or without, irradiated UC-MSCs in triplicate at cell ratios of 5:1 or 80:1 for 48 hours (with Brefeldin A for the last 4 hours). Subsequently, the cells were harvested, stained with APC-cy7-anti-CD3 and APC-anti-TCRVδ, fixed, permeabilized, and intracellularly stained with FITC-anti-IFNγ, PE-anti-TNFα, PE-anti-IL-10, PE-anti-perforin, or FITC-anti-granzyme B. The percentages of TNFα+, IFNγ+, perforin+, granzyme B+, or IL-10+ Vγ9Vδ2 T cells were determined by flow cytometry.

3.2. UC-MSCs Regulate Cytokine Production by Vγ9Vδ2 T Cells. Activated Vγ9Vδ2 T cells can express different cytokines and cytotoxic enzymes. To determine the effect of UC-MSCs on the expression of cytokines and functional enzymes, the isolated PBMCs were stimulated with PAM and IL-2 for 12 days and the cells were cocultured with, or without, irradiated UC-MSCs in triplicate at cell ratios of 5:1 or 80:1 for 48 hours (with Brefeldin A for the last 4 hours). Subsequently, the cells were harvested, stained with APC-cy7-anti-CD3 and APC-anti-TCRVδ, fixed, permeabilized, and intracellularly stained with FITC-anti-IFNγ, PE-anti-TNFα, PE-anti-IL-10, PE-anti-perforin, or FITC-anti-granzyme B. The percentages of TNFα+, IFNγ+, perforin+, or IL-10+ Vγ9Vδ2 T cells were determined by flow cytometry.

3.3. UC-MSCs Inhibit the Cytotoxicity of Vγ9Vδ2 T Cells against Influenza Virus-Infected A549 Cells. Activated Vγ9Vδ2 T cells have cytotoxicity against influenza virus-infected A549 cells [23]. To determine the impact of UC-MSCs on the cytotoxicity of Vγ9Vδ2 T cells, the isolated PBMCs were stimulated with PAM and IL-2 for 12 days and were cocultured with, or without, irradiated UC-MSCs in triplicate at cell ratios of 5:1 or 80:1 for 60 hours. After purification of Vγ9Vδ2 T cells, the purified Vγ9Vδ2 T cells (effector) were cocultured with influenza virus- (H1N1-) infected A549 cells (target) at an effector-to-target (E/T) ratio of 10:1 for 5 hours. Subsequently, the cells were stained with APC-cy7-anti-CD3 and 7AAD, and after being gated on CD3-A549 cells, the percentages of dead A549 cells in total A549 cells were determined by flow cytometry (Figure 4(a)).
Coculture of Vγ9Vδ2 T cells with UC-MSCs significantly reduced the percentages of dead A549 cells and the inhibitory effects of UC-MSCs on Vγ9Vδ2 T cell-mediated cytotoxicity against influenza virus-infected A549 cells tended to be dose-dependent (Figure 4(b)).

3.4 UC-MSCs Modulate the Fas-L and TRAIL Expression and Activated Vγ9Vδ2 T Cell Apoptosis. Finally, we investigated the impact of UC-MSCs on the apoptosis of activated Vγ9Vδ2 T cells. The isolated Vγ9Vδ2 T cells were stimulated with PAM and IL-2 in the presence or absence of irradiated UC-MSCs at a ratio of 80 : 1 or 5 : 1 for 14 days. Subsequently, the cells were harvested and stained with APC-cy7-anti-CD3, FITC-Annexin V, and 7-AAD and the percentages of apoptotic cells were determined by flow cytometry. There was no significant difference in the percentages of apoptotic cells among these groups of Vγ9Vδ2 T cells (Figure 5(a)). Furthermore, some cells were stained with APC-cy7-anti-CD3, APC-anti-TCRVγ9Vδ2, and PE-anti-Fas-L or PE-anti-TRAIL. The percentages of Fas-L+ or TRAIL+ Vγ9Vδ2 T cells were determined by flow cytometry. Quantitative analysis indicated that the percentages of Fas-L+ Vγ9Vδ2 T cells
4. Discussion

MSCs have potent immunoregulatory activities and have been tested in the clinical trials for intervention of different inflammatory diseases [13]. UC-MSCs have more advantages than bone marrow-derived ones because of their noninvasive nature and having less immunogenicity as well as powerful proliferative capacity [2, 14]. UC-MSCs have been demonstrated to inhibit the function of αβ T cells [3, 5, 6], NK cells [3, 7–9], macrophages [3], and DCs [2, 3, 10] but positively regulate Tregs [11]. In this study, we examined the effect of UC-MSCs on the proliferation and cytotoxicity of Vγ9Vδ2 T cells as well as their cytokine and effector expression in vitro. We found that UC-MSCs inhibited the proliferation of PAM/IL-2 stimulated Vγ9Vδ2 T cells in a dose-dependent and cell-cell contact-independent manner. Furthermore, we found that UC-MSCs inhibited the expression of IFNγ but enhanced granzyme B expression in activated Vγ9Vδ2 T cells. In addition, UC-MSCs inhibited the cytotoxicity of activated Vγ9Vδ2 T cells against influenza virus-infected A549 cells. The expression of Fas-L and TRAIL on Vγ9Vδ2 T cells were inhibited by UC-MSCs as well. However, UC-MSCs failed to modulate the spontaneous apoptosis of activated Vγ9Vδ2 T cells. Previous studies have shown that bone marrow-derived MSCs inhibit effector T cell proliferation and IFNγ and TNFα expression and cytotoxicity against cancer cells [28, 29]. Our data extended previous findings and suggest the notion that UC-MSCs are powerful inhibitors of T cell immunity. To the best of our knowledge, this was the first report on the regulatory effects of UC-MSCs on the activation and function of human Vγ9Vδ2 T cells. Our novel findings may provide new insights into understanding of the regulatory mechanisms underlying the action of UC-MSCs.

In this study, we found that UC-MSCs inhibit the proliferation of PAM/IL-2 stimulated Vγ9Vδ2 T cell proliferation in a cell-cell contact-independent manner. While inhibition of T cell proliferation is associated with inducing T cell apoptosis. However, we did not observe that UC-MSCs significantly modulated the apoptosis of activated Vγ9Vδ2 T cells in vitro, which may be associated with lower levels of Fas-L and TRAIL expression in Vγ9Vδ2 T cells with the presence of UC-MSCs. These findings suggest that UC-MSCs may secrete soluble factors that inhibit Vγ9Vδ2T cell proliferation. MSCs can secrete many factors, including transforming growth factor-β (TGF-β) [30], hepatic growth factors (HGF) [31], prostaglandin E2 (PGE2) [30], IL-10 [30], indolamine 2,3-dioxygenase (IDO) [32, 33], nitric oxide (NO) [30], heme oxygenase-1 (HO-1) [34], and human leukocyte antigen-G (HLA-G) [35], and TGF-β, IL-10, and PGE2 are potent inhibitors of T cell immunity [4, 36–38]. These factors together with other unknown factors may act to inhibit T cell proliferation directly and indirectly. We are interested in further investigating what factors positively regulate granzyme B expression in Vγ9Vδ2 T cells.

We speculate that the interaction of UC-MSCs with Vγ9Vδ2 T cells may be similar to that with NK cells [39]. It is possible that minor allogeneic antigens on UC-MSCs may trigger IFNγ Vγ9Vδ2 T cell responses and upregulate granzyme B expression, leading to the cytotoxicity against

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**Figure 2:** UC-MSCs inhibit the proliferation of γδ T cells in a cell-cell contact-independent manner. PBMCs from three healthy donors were labeled with CSFE and cocultured with, or without, UC-MSCs at the different ratios in transwell or together in 6-well plates, followed by simulation with PAM and IL-2 for 14 days. Subsequently, the cells were stained with fluorescent antibodies, as described in the method section. The γδ T cells were gated on CD3+TCRγδ+ and the percentages of proliferative γδ T cells were determined by flow cytometry. Data are expressed as the mean percentages ± SEM of each group of cells from three separate experiments. (a) The percentages of proliferative γδ T cells following a separated coculture in transwell plates. (b) The percentages of proliferative γδ T cells following coculture in transwell or 24-well plates. *P < 0.01 versus the controls. UC-MSC: umbilical cord mesenchymal stem cell; PBMC: peripheral blood mononuclear cell.
Figure 3: UC-MSCs modulate the expression of cytokines and bioactive effectors in γδ T cells. PBMCs were isolated and stimulated with PAM and IL-12 for 12 days. The enriched γδ T cells were cocultured with UC-MSCs at the indicated ratios for 48 hours and the cells were stained with different fluorescent antibodies, as described in the method section. Subsequently, the percentages of IFNγ+, granzyme B+, TNFα+, perforin+, or IL-10+ γδ T cells were characterized by flow cytometry. Data are representative flow cytometry charts or expressed as the mean percentages ± SEM of each group of cells from nine subjects of nine separate experiments. (a–e) Quantitative analysis of the percentages of γδ T cells. * P < 0.05 versus the controls. UC-MSC: umbilical cord mesenchymal stem cell; PBMC: peripheral blood mononuclear cell; TNFα: tumor necrosis factor; IFNγ: interferon-γ; IL-10: interleukin-10.
UC-MSCs by the granzyme-NKG2D pathway. Simultaneously, inhibitory factors, such as PGE\textsubscript{2} [32, 33], TGF-\(\beta\) [3], IDO [32, 33], NO [33], and IL-10 [10, 40], secreted by UC-MSCs downregulate the function of V\(\gamma\)9V\(\delta\)2 T cells and the serine protease inhibitor 9 (SERPINB9) produced by UC-MSCs attenuates the enzyme granzyme B-mediated cytotoxicity. Subsequently, inhibitory factors secreted by UC-MSCs control the function of V\(\gamma\)9V\(\delta\)2 T cells by reducing IFN\(\gamma\), Fas-L, and TRAIL expression in V\(\gamma\)9V\(\delta\)2 T cells. Immunosuppressive functions of different sources of MSCs are varying and their functions are regulated by many other immunocompetent cells in vivo [41]. Therefore, we are interested in further investigating the precise mechanisms underlying the action of UC-MSCs in regulating the function and survival of V\(\gamma\)9V\(\delta\)2 T cells.

Even though the mechanisms underlying the cytotoxicity of V\(\gamma\)9V\(\delta\)2 T cells against virus infection are still incompletely understood, the protective role of V\(\gamma\)9V\(\delta\)2 T cells has been proved in acute and chronic virus infections. Following the infection with different strains of influenza viruses, V\(\gamma\)9V\(\delta\)2 T cells can secrete antiviral cytokines and directly kill virus-infected target cells [42–44], which can be enhanced by phosphoantigen stimulation [44, 45]. UC-MSCs can significantly inhibit the cytotoxicity of V\(\gamma\)9V\(\delta\)2 T cells against H1N1 influenza virus in vitro. This result indicates that maybe the viral infection, especially in H1N1 influenza virus infection, UC-MSCs suppress the antiviral protection of V\(\gamma\)9V\(\delta\)2 T cells. This needs to be proved in further studies.

In summary, our data indicated that human UC-MSCs inhibited the PAM/IL-2 stimulated V\(\gamma\)9V\(\delta\)2 T cell proliferation in a cell-to-cell contact-independent manner and modulated cytokine secretion by V\(\gamma\)9V\(\delta\)2 T cells. Furthermore, UC-MSCs inhibited the cytotoxicity of activated V\(\gamma\)9V\(\delta\)2 T cells against influenza virus-infected A549 cells. These regulations may be mediated by the inhibition of Fas-L and TRAIL expression by V\(\gamma\)9V\(\delta\)2 T cells but not by inducing the apoptosis of V\(\gamma\)9V\(\delta\)2 T cells. Our novel data support that UC-MSCs can exhibit immune regulatory capacity by inhibiting
UC-MSCs modulate the expression of Fas-L and TRAIL on γδ T cells but do not affect the spontaneous apoptosis of activated γδ T cells. PBMCs were cocultured with, or without, the different ratios of UC-MSC in the presence of PAM and IL-2 for 14 days. The cells were stained with fluorescent antibodies, as described in in the method section. The percentages of apoptotic γδ T cells and Fas-L+ or TRAIL+ γδ T cells were characterized by flow cytometry analysis. Data are representative flow cytometry analysis of the frequency of apoptotic γδ T cells or expressed as the mean percent ± SEM of each group of cells from 19 subjects. (a–c) The quantitative analysis of apoptosis, Fas-L, and TRAIL. *P < 0.05, **P < 0.05 versus the controls. UC-MSC: umbilical cord mesenchymal stem cell; PBMC: peripheral blood mononuclear cell; Fas-L: Fas ligand; Trail: tumor necrosis factor-related apoptosis-inducing ligand.

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