Identification of Potential Immunogenic Molecules During the Allogeneic Transplantation of Human Adipose-derived Mesenchymal Stem Cells

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Research

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Abstract

Background: Given their low immunogenicity and multiple differentiation capacities, mesenchymal stem cells (MSCs) have the potential to be used for “off-the-shelf” cell therapy. However, MSC allorejection indicates that they are not fully immune privileged. In this study, we investigated the immunogenicity of human adipose-derived MSCs (Ad-MSCs) and identified potential immunogenic molecules.

Methods: To evaluate the immunogenicity of human Ad-MSCs *in vivo*, cells were transplanted into humanized mice (hu-mice), and T cell infiltration and clearance of human Ad-MSCs were observed by immunofluorescence and bioluminescence imaging. One-way mixed lymphocyte reaction (MLR) and flow cytometry were performed to evaluate the immunogenicity of human Ad-MSCs *in vitro*. High-throughput TCR repertoire sequencing and mass spectrometry were applied to identify potential immunogenic molecules.

Results: Allogeneic human Ad-MSCs recruited T cells during transplantation and caused faster clearance in hu-mice than NOD/ShiLtJGpt-*Prkdc*^{em26Cd52}*Il2rg*^{em26Cd22}/Gpt (NSG) mice. The proliferation and activation of T cells was significantly enhanced by human Ad-MSCs, and the expression level of HLA-II on human Ad-MSCs was dramatically increased after coculture with human peripheral blood mononuclear cells (PBMCs) *in vitro*. In addition, upregulated expression of alpha-enolase (ENO1) on the surface of human Ad-MSCs increased their immunogenicity, and ENO1 inhibitor treatment decreased the human Ad-MSC triggered proliferation of T cells *in vitro*.

Conclusions: We further confirmed the immunogenicity of human Ad-MSCs during allogeneic transplantation and provided a potential target, ENO1, for the safe clinical application of allogeneic human Ad-MSC therapy.

Background

Mesenchymal stem cells (MSCs) are progenitor cells that originate from the mesoderm. They were first discovered in bone marrow by Alexander Friedenstein in the late 1970s [1, 2]. Although they were found in bone marrow, MSCs can be isolated from numerous tissues, such as adipose tissue [3], the placenta [4], or the umbilical cord [5]. MSCs from different tissues share common characteristics and still exhibit heterogeneity [6, 7]. MSCs can differentiate into different cell types of the mesodermal lineage, such as adipocytes, chondroblasts and osteoblasts [8].

Since being discovered, MSCs have been widely investigated, and their potential has led to high expectations for clinical therapies. Due to their differentiation capacity, MSCs are of great value for supporting tissue regeneration and are used to treat a broad panel of diseases ranging from acute injuries (e.g., bone fractures, muscle trauma, and myocardial infarction) to chronic ischemic tissue injuries (e.g., critical limb ischemia) [9–12]. In addition, their immunoregulatory properties [13] enable MSCs to be applied for the treatment of graft-versus-host disease (GvHD) and autoimmune diseases [14–16]. Notably, MSCs were once conceived as “immune privileged,” and both autologous and

allogeneic major histocompatibility complex (MHC)-unmatched MSCs have been commonly used for clinical trials [17]. Klyushnenkova proposed that MSCs could be isolated from healthy individuals and expanded on a large scale *in vitro* to make MSCs an “off-the-shelf” product [18]. However, the expansion of clinical trials and research has produced successive studies showing that autologous and allogeneic MSC transplantation have different levels of efficacy [19], and allogeneic MSC therapy produces common side effects, such as pain and swelling [20, 21]. Thus, the immunogenicity of MSCs should not be ignored [22]. Allogeneic MSC transplantation induces both innate and adaptive immune responses, which ultimately lead to the immune rejection of MSCs and a reduction in the therapeutic effect [23–25]. However, it currently remains unknown how allogeneic MSCs provoke these immune responses since they lack MHC class II and costimulatory molecules, such as CD40, CD80 and CD86 [26].

As key cellular effectors of allograft rejection, T cells are armed with specialized T cell receptors (TCRs) to recognize and eliminate allografts. The diversity of the TCR repertoire allows T cells to recognize extensive collections of antigens. Specificity for the recognition of peptide-MHC complexes is provided by 3 complementarity-determining regions (CDRs) of the TCR: CDR1, CDR2, and CDR3 [27]. CDR3 is a highly polymorphic principal recognition site that largely engages the solvent-exposed chains of MHC-bound peptides [28]. High-throughput sequencing of TCR repertoires is a robust tool for deep sequencing the repertoire of TCRs and has been used in the development of tumor biomarkers and cancer immunotherapies [29]. In this study, for the first time, we used this technique to identify potential immunogenic molecules in human Ad-MSC during allogeneic transplantation.

Methods

Mice

Six- to eight-week-old male NSG mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and raised in our specific pathogen-free animal facilities under appropriate conditions, as described previously. All animal experiments were performed according to the guidelines of the Animal Care and Use Committee at the Institute of Basic Medical Sciences Chinese Academy of Medical Sciences.

Cell lines

Human Ad-MSCs were generously given by Professor Robert Chunhua Zhao. Human placenta-derived MSCs expressing GFP were a gift from Professor Hongcui Cao. MSCs were grown using a MesenCult Proliferation Kit (human) (STEMCELL Technologies, Canada) and used for studies before passage 3.

The HEK-293T cell line was obtained from the Cell Center at Peking Union Medical College, cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Thermo Fisher Scientific, USA) and used for lentiviral packaging.

The cells mentioned above were incubated in 5% CO_2 at 37 °C.

Modified one-way MLR

Modified one-way MLRs were performed to assess the ability of human Ad-MSCs to stimulate an immune response [30]. Briefly, mitomycin-treated (10 μg/ml at 37 °C for 2 h) human Ad-MSCs were prepared as stimulatory cells. Freshly isolated human PBMCs (MHC-mismatched with Ad-MSCs) were prepared as responder cells. Stimulator and responder cells were mixed in 96-well plates in triplicate at a 1 to 10 ratio and cultured in 200 μl of RPMI 1640 medium supplemented with 10% (v/v) FBS, 50 μM β-mercaptoethanol (2-ME) and 2 mM L-glutamine. Meanwhile, two other groups, PBMCs cultured alone and PBMCs stimulated with concanavalin A (ConA) (10 μg/ml; Sigma-Aldrich, USA), were set as negative and positive controls, respectively. Half the medium was replaced every 3 days, and the coculture was continued for a total of 12 days.

To assess whether human Ad-MSCs stimulate the proliferation of T cells, PBMCs were labeled with 5 μM carboxyfluorescein succinimidyl ester (CFSE; 37 °C for 30 min), mixed with Ad-MSCs and then collected on days 3, 6, 9 and 12. PBMCs were then stained with APC-conjugated anti-human CD3 (Biolegend, USA).

To examine the activation of T cells by Ad-MSCs, PBMCs were collected on days 3, 6, 9 and 12 and stained with FITC-conjugated anti-human CD69, PE-conjugated anti-human CD25 and APC-conjugated anti-human CD3 (Biolegend, USA).

To examine the immunogenic molecules, Ad-MSCs were collected on the 7th day and subjected to 2-step staining with anti-human ENO1 (Santa Cruz, USA) and APC-conjugated anti-mouse IgG (Biolegend, USA) with 2 washes of PBS in the middle.

Cells were stained for 30 min at 4 °C and washed twice with phosphate-buffered saline (PBS) before analysis on a FACSort flow cytometer (BD Biosciences, USA).

Reconstitution of humanized mice

Hu-mice were generated as previously described [31]. Briefly, human PBMCs were freshly isolated from peripheral blood with Ficoll (TBD, China) and suspended in RPMI 1640. The concentration was adjusted to 1×10^8 cells/ml before use. Each NSG mouse was intravenously transfused with a 200 μl cell suspension (twenty million cells) via the tail vein.

Flow cytometry analysis of splenocytes in hu-mice

Splenocytes from reconstituted hu-mice and NSG mice were isolated 3 weeks after reconstitution and analyzed by flow cytometry. Briefly, cells were incubated with APC anti-human CD45 (clone HI30), PerCP anti-mouse CD45 (clone 30-F11), Alexa Fluor® 488 anti-human CD19 and PE anti-human CD3 (clone UCHT1) to confirm the presence of human T or B cells in hu-mice. For further analysis of T cells, the isolated cells were stained with FITC anti-human CD8 (clone HIT8a) and PE anti-human CD4 (clone RPA-T4). All staining was performed at 4 °C for 30 min, and the stained cells were washed before being analyzed by flow cytometry.

Luciferase lentiviral packaging

Lentivirus was generated in HEK-293T cells using a second-generation packaging system. Briefly, HEK-293T cells were plated in 10 cm^2 dishes and transfected at approximately 80% confluence with jetPRIME Reagent (Polyplus Transfection, France). Five micrograms/dish pCDH-luciferase plasmid vector, 3.33 μg/dish psPAX2 plasmid and 1.67 μg/dish pMD2G plasmid were mixed with 20 μl/dish jetPRIME Reagent for 10 min and then added to HEK-293T cells. At 8 h post transfection, the medium was completely changed. Cell supernatants were collected twice at 48 h and 72 h, filtered through a 0.45-μm pore size filter and then concentrated by centrifugation (25000 rpm for 2 h) at 4 °C in an ultracentrifuge (Beckman, USA). The concentrated lentivirus was titrated by infecting HEK-293T cells with serially diluted lentivirus.

Lentiviral transduction of Ad-MSCs

Human Ad-MSCs were transduced with lentivirus to express firefly luciferase. Briefly, 1×10^4 cells were grown in 12-well plates and incubated for 4 h with complete culture medium containing luciferase lentivirus (MOI = 30) and protaminesulfate (100 μg/ml). Then, the medium was completely changed. The luciferase activity in the transduced MSCs was measured after 48 h with an *In Vivo* Imaging System (IVIS) spectrum (Xenogen, USA) by adding D-luciferin (150 μg/ml). After lentiviral transduction, luciferase-MSCs were used for further study.

Allotransplantation of MSCs into hu-mice

After 2 weeks of human lymphocyte implantation, the reconstituted hu-mice were used in experiments. First, hu-mice were anesthetized by intraperitoneal injection of 2% sodium pentobarbital. For intramuscular transplantation, 1×10^6 human placenta-derived GFP-MSCs (in 200 μl of PBS) were injected into the left thigh of hu-mice at 5 different positions. Concomitantly, the right thigh was injected with 200 μl of PBS as a control. For subcutaneous transplantation, 1×10^7 human Ad-MSCs (in 200 μl of PBS) were mixed with Matrigel and subcutaneously embedded into the dorsal surface of hu-mice on the left side. Meanwhile, the right side dorsal surface of the mice was injected with 200 μl of PBS and Matrigel as a control. Seven days later, hu-mice were sacrificed for subsequent studies.

Bioluminescence imaging

Bioluminescence images were captured and analyzed by the IVIS Spectrum. Twenty minutes before imaging, hu-mice were intraperitoneally injected with D-luciferin (150 mg/kg; PerkinElmer, USA). Anesthesia was provided as inhaled isoflurane, and images were taken at 30 min and 7 days after allotransplantation of the luciferase-MSCs. Following the initialization and self-examination of the IVIS Spectrum, the mice were positioned in the cabinet, and images were captured. Imaging parameters were adjusted to field of view “D,” binning 8, and autoexposure time. The luciferase activity (total flux) represents the survival of human Ad-MSCs. The total flux of independent experiments was normalized and subjected to statistical analysis.

Immunofluorescence staining

The hu-mice that were transplanted with human placenta-derived MSCs were cardioperfused with 4% paraformaldehyde. The thighs were isolated, postfixed with 4% paraformaldehyde, embedded in optimal cutting temperature (OCT) compound and cut into slices (15 μm). The slices of thighs were blocked with 5% goat serum for 2 h at room temperature and incubated with primary antibodies overnight at 4 °C. After being washed 5 times with TBST, the sections were incubated with a secondary antibody and DAPI for 40 min at room temperature. The images were captured using a Zeiss LSM 780 laser scanning microscope (Zeiss, Germany).

TRA/TRB repertoire sequencing and data analysis

1. Sample collection

Hu-mice were reconstituted with human PBMCs. PBMCs from the same donor were collected in TRIzol (5×10^6 cells/ml) and stored at -80 °C as a control (PBMC group). Two weeks later, one hu-mouse was subcutaneously administered a mixture of human Ad-MSCs (1×10^7 cells per mouse) or PBS with Matrigel (BD, USA) separately on the dorsal surface of the right and left sides; a total of 10 hu-mice were subjected to the same treatment. Seven days later, all 10 hu-mice were sacrificed, and the administered cells were isolated. Tissues containing MSCs or PBS were gathered separately in TRIzol as MSC or PBS groups, frozen in liquid nitrogen and stored at -80 °C before TCR repertoire sequencing.

2. TRA/TRB repertoire sequencing of infiltrating T cells

Total RNA was extracted using a PE150 Kit (Illumina, USA). The TRA/TRB repertoire was amplified and sequenced using Illumina MiSeq by GeneWiz Inc. (Suzhou, China). Raw data were analyzed on the iRepertoire website (http://www.irepertoire.com).

3. Data analysis

To comprehensively analyze the TRA/TRB differences among the MSC, PBS and PBMC groups, we used the unique CDR3/total CDR3 sequences and the frequency of dominant sequences to assess CDR3 diversity as described in ref.39. We also analyzed the CDR3 length and VJ usage frequency of the unique CDR3 sequences. Finally, we selected 5 CDR3 peptides from the MSC group for synthesis with a biotin tag to search for potential immunogenic molecules in human Ad-MSCs.

Biotinylated CDR3 peptide pulldown assay

To identify potential human Ad-MSC immunogenic molecules during allotransplantation, we used biotinylated CDR3 peptides to capture proteins in human Ad-MSC cell lysates. The proteins were then pulled down by streptavidin beads for subsequent identification. Briefly, 1.5×10^7 human Ad- MSCs were washed twice with cold PBS and harvested in 1 ml of immunoprecipitation (IP) lysis buffer (25 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1% Triton X-100, 0.5% deoxycholate) containing protease and phosphatase inhibitors. The cells were incubated for 30 min on ice, subjected to ultrasonication (100 V

for 3 s × 10 times) and centrifuged at 15,000 × g for 30 min. The cell supernatants were precleared with 150 μl of uncoupled Dynabeads magnetic beads streptavidin C1 beads (Thermo Fisher Scientific, USA) for 2 h and incubated with 150 μl biotinylated irrelevant peptide (biotin-SPGH)-coupled streptavidin C1 beads for 8 h. The precleared samples were divided into 5 groups and incubated with 5 biotinylated CDR3 peptides coupled to streptavidin C1 beads overnight at 4 °C. All pulldown complexes were washed 3 times with IP lysis buffer, denatured by the addition of 5 × SDS loading buffer containing β-mercaptoethanol, and boiled at 100 °C for 10 min. After the pulldown complexes were centrifuged at 15,000 × *g* for 2 min, the supernatants were collected for further SDS-PAGE and silver staining.

SDS-PAGE and silver staining

The pulldown complexes (15 ~ 20 μl) were loaded on 10% SDS-PAGE gels for electrophoresis. Silver staining was performed using Pierce Silver Stain for Mass Spectrometry according to the manufacturer's instructions (Thermo Fisher Scientific, USA). Briefly, after SDS-PAGE, the gel was washed twice with ultrapure water for 5 min and fixed twice with a solution containing 30% ethanol and 10% acetic acid for 15 min at room temperature. Then, the gel was washed with ethanol (10%) and ultrapure water wash and incubated in sensitizer working solution for 1 min and stain enhancer for 5 min in succession with the indicated washes. Finally, the developer working solution was added. When the desired staining intensity was reached, Stop Solution was used to stop the reaction. The gel was immediately subjected to protein band excision for peptide identification by mass spectrometry.

Mass spectrometry identification

Mass spectrometry identification was conducted by the Centralab Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). Briefly, the silver-stained protein bands were excised and sliced into 0.5-1 mm^3 pieces. Then, the gel pieces were subjected to destaining, protein alkylation reduction, enzymatic hydrolysis and polypeptide desalination. Shotgun proteomics analyses were performed using an EASY-nLCTM 1200 UHPLC system (Thermo Fisher Scientific, USA) coupled with an Orbitrap Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, USA) operating in the data-dependent acquisition (DDA) mode. The resulting spectra from each fraction were searched separately against the UniProt database. For protein identification, proteins with at least 2 unique peptides were identified at the peptide and protein levels at a false-discovery rate (FDR) of less than 5.0%. Proteins containing similar peptides and those that could not be distinguished based on MS/MS analysis were grouped separately as protein groups.

Data analysis

Two-tailed Student's *t*-test (paired or unpaired) was applied to analyze the significant difference between two groups with a normal distribution; otherwise, the Mann-Whitney test was applied. The comparison of more than two groups was analyzed by ANOVA with Bonferroni's post hoc test. All data are displayed as the mean ± standard deviation (SD), except for specific indications. $P \leq 0.05$ was considered statistically significant.

Results

Most human Ad-MSCs were rejected after being transplanted into hu-mice

To investigate the immunogenicity of human MSCs during transplantation, we prepared hu-mice by transfusion of freshly isolated human PBMCs into NSG mice to temporarily reconstitute the human immune system [31, 32]. Human immune cells can last for approximately 4 weeks in NSG mice [31]. After 3 weeks, splenic lymphocytes from reconstituted hu-mice were analyzed by flow cytometry. The results showed that both human and mouse $CD45^+$ immune cells were present in hu-mice (Fig. 1A and 1B). Further analysis of the human $CD45^+$ population revealed that there were human $CD3^+$ T cells at an appropriate CD4/CD8 ratio (Fig. 1B). However, human $CD19^+$ B cells were not detectable (Fig. 1A), which was not described and explained in previous studies (ref 31 and 32). Thus, in this study, we mainly focused on investigating the interaction between allogeneic transplanted human MSCs and T cells in this hu-mice model.

Next, we genetically modified human Ad-MSCs to express luciferase (luciferase-MSCs) by lentiviral transduction to monitor the survival of human Ad-MSCs in the hu-mice (Fig. 1C). Luciferase-MSCs were mixed with Matrigel and subcutaneously embedded in the backs of hu-mice or NSG control mice. The luciferase activity indirectly representing the surviving cells was examined at 30 min and 7 days post inoculation. The results showed that significantly fewer luciferase-MSCs survived in hu-mice than NSG mice after 7 days (Fig. 1D and E), suggesting that the presence of human T cells in hu-mice accelerates the clearance of allotransplanted luciferase-MSCs.

We also intramuscularly transplanted human placenta-derived MSCs expressing GFP (GFP-MSCs) into hu-mice and found that there were infiltrating human $CD3^+$ T cells around the GFP-MSCs but no human $CD19^+$ B cells (Fig. 1F and G), which might be attributed to the lack of detectable B cells in the reconstituted hu-mice, as mentioned above. To exclude the immunogenicity of GFP, we subcutaneously transplanted human Ad-MSCs into the backs of hu-mice. Compared to that in the PBS control group, the percentage of human $CD3^+$ T cells around Ad-MSCs was significantly higher, and the percentage of human $CD19^+$ B cells was negligible and not significantly different between the two groups (Fig. 1H and I). Together, these results indicate that the allotransplanted human MSCs (adipose- or placenta-derived) were subject to immune rejection in hu-mice, which further confirms the immunogenicity of human MSCs.

Human Ad-MSCs stimulate the proliferation and activation of T cells in vitro

MSCs have been used to treat GvHD because of their immunomodulatory properties. MSCs can inhibit lymphocyte proliferation when added into a mixed lymphocyte reaction (MLR) [33]. However, whether MSCs directly elicit a proliferative response is unknown [30, 34, 35]. In this study, we performed a modified one-way MLR (detailed in the Methods section) and found that human Ad-MSCs stimulated the proliferation of $CD3^+$ T cells on days 9 and 12 after coculture with PBMCs. The proportions were approximately 7.5% and 32.5%, respectively. When PBMCs were cultured alone, the proportion of $CD3^+$ T

cells was approximately 1.9% and 6.8%, respectively (Fig. 2A). The statistical analysis showed significant differences between these two groups (Fig. 2B).

We also examined the activation markers of T cells during one-way MLR and found that the expression of CD69 and CD25 on the surface of $CD3^+$ T cells was significantly upregulated by coculture with human Ad-MSCs for 6 days compared to no coculturing (Fig. 2C and D). The expression of these two markers continuously increased during coculture, but the expression of only CD25 was significantly different from that in the PBMC-only group on days 9 and 12. The possible reason for this result might be that CD69 is an early T cell activation marker, and the change is most obvious in the early stage [36].

These results demonstrate that human Ad-MSCs stimulate the proliferation and activation of $CD3^+$ T cells *in vitro*, and they are not fully immune privileged.

From the perspective of human Ad-MSCs during one-way MLR, we analyzed the expression level of immunogenic molecules, including CD40, CD80, HLA-ABC and HLA-DR, and found that human Ad-MSCs did not express CD40, CD80 or HLA-DR before coculture with PBMCs (MSC day 0). However, when human Ad-MSCs were cocultured with PBMCs for 7 days, the expression levels of CD40 and HLA-DR were increased dramatically compared to Ad-MSCs cultured alone. CD80 expression was also slightly upregulated even though no significant difference was observed between the groups (Fig. 2E). These results suggest that the immunogenicity of human Ad-MSCs increases when they are exposed to PBMCs.

Specific T cells were selectively recruited by human Ad-MSCs after transplantation

Comprehensive analysis of the TCR or immunoglobulin repertoire by high-throughput sequencing can be used to assess the enrichment of antigen-specific T cell or B cell clones in many diseases or the immunogenicity of specific antigens [29, 37]. It may also provide clues for elucidating the underlying mechanisms of antitumor immunity in specific tumors [38]. In this study, we analyzed the TCR repertoire of human Ad-MSC-recruited T cells with the aim of finding dominant TCR clones that respond to human Ad-MSCs. The samples were collected as shown in Fig. 3A and detailed in the Methods section. Briefly, hu-mice were reconstituted with human PBMCs, and PBMCs were collected as a control (PBMC group). After two weeks, one hu-mouse (a total of 10) was subcutaneously administered a mixture of human Ad-MSCs (MSC group) or PBS (PBS group) with Matrigel separately on the dorsal surface of its right and left sides. Seven days later, tissues from the MSC or PBS group were isolated individually and then mixed together within each group as one sample. All 3 samples were subjected to TCR repertoire sequencing and data analysis. First, we evaluated the diversity of the T cell receptor α-chain (TRA) and T cell receptor β-chain (TRB) repertoires within MSC, PBS and PBMC groups by using the frequency of the dominant sequence and the unique CDR3/total CDR3 sequences, as described previously [39].

The results showed that the dominant CDR3 sequence frequency (first, top 5, and top 10 frequencies) of TRA and TRB was higher in the MSC group than in the PBMC control group (original data were shown in the additional files), and the ratio of unique CDR3 to total CDR3 sequences in the MSC group was markedly reduced (Table 1). These results suggest that the diversity of the TCR repertoire is significantly

reduced in the MSC group because human Ad-MSCs selectively, not randomly, recruit T cells. This result further proves the immunogenicity of human Ad-MSCs. To exclude the influences of Matrigel, we established a PBS group and found that the TCR diversity in the MSC group was generally greater than that in the PBS group. These results indicate that some of the TCR clones of the MSC group were human Ad-MSC-specific.

The CDR3 length is also a characteristic of the TCR repertoire. As shown in Fig. 3B, the CDR3 lengths of TRA and TRB in the 3 groups were mainly between 10 and 20 amino acids (aa) and showed no difference among the groups. The VJ gene usage of TRA and TRB was significantly different among the three groups (Fig. 3C). T cells in the MSC and PBS groups selectively adopted certain VJ genes with a high frequency.

Based on the TCR repertoire analysis, we conclude that human Ad-MSCs selectively recruit T cells, perhaps owing to their specific immunogenic molecules.

Table 1
The diversity of the TRA and TRB repertoires

Features	Top 1 frequency	Top 5 frequency	First 10 frequency	Unique CDR3/ Total CDR3
MSC α chain	0.07	0.25	0.42	0.00016
PBS α chain	0.11	0.40	0.64	0.00018
PBMC α chain	0.06	0.14	0.16	0.05970
MSC β chain	0.06	0.18	0.29	0.00098
PBS β chain	0.08	0.36	0.62	0.00034
PBMC β chain	0.05	0.11	0.13	0.09456

ENO1 binds to the CDR3 peptide of the predominant TCR of human Ad-MSC-recruited T cells

The diversity of TCRs depends on the rearrangement of V (D) J genes, which is ultimately reflected by the CDR3 peptides [40]. In this study, we selected dominant CDR3 peptides from the TCR repertoire of human Ad-MSC-recruited T cells (MSC group) that might respond to human Ad-MSCs, according to the strategies shown in Fig. 3D. Based on the frequency, the top 30 CDR3 peptides in the MSC group were compared with all CDR3 peptides in the PBS group to exclude Matrigel-specific CDR3 as much as possible. In the TRA analysis, 26 CDR3 peptides overlapped, and 4 were unique in the MSC group. In the TRB analysis, all of the top 30 CDR3 peptides were present in the PBS group. We selected these 4 unique CDR3 peptides (clones 11, 19, 25, and 30) plus clone 2 of TRA in the MSC group (clone 2 ranked no. 2 in the MSC group and no. 34 in the PBS group, indicating that it might also be a specific TCR) and artificially synthesized them with a biotin tag for subsequent experiments (Table 2).

Table 2
Synthesized MSC-CDR3 peptides of the TRA

Clone ID	Count	Fraction	AA SEq. of the CDR3 α chain
2	63737	0.0598	CAMSAVTSGSRLTF
11	31447	0.0295	CADNSGYALNF
19	21886	0.0205	CAATIGGADGLTF
25	15911	0.0149	CAVRPSWTSGSRLTF
30	12810	0.0120	CAMSAGGGSYIPTF

First, the binding of the 5 CDR3 peptides to human Ad-MSCs was tested by flow cytometry. Clones 2 and 25 of the CDR3 peptides bound directly to Ad-MSC surface molecules (Fig. 4A), and all 5 CDR3 peptides bound to intracellular Ad-MSC molecules (Fig. 4B). Then, we performed biotinylated CDR3 peptide pull-down assays, SDS-PAGE and silver staining (detailed in the Methods section) to identify proteins recognized by the CDR3 peptides. Silver staining revealed a unique protein band at 45 ~ 55 kDa, which was captured by the clone 2 CDR3 peptide (Fig. 4C). The band was excised and subjected to mass spectrometry analysis and database searching. A total of 10 proteins with at least 2 unique peptides were identified for the protein band, among which ENO1 and AMBP proteins showed highly matching unique peptide counts (Fig. 4D).

Whether these 2 proteins are associated with the immunogenicity of human Ad-MSCs has not been reported before. Notably, ENO1 can be used as a diagnostic marker for various tumors, and its expression is positively correlated with the malignancy of tumors, such as liver, lung and pancreatic cancers [41, 42]. Researchers have also detected anti-ENO1 antibodies in patients with unexplained habitual abortion [43]. Thus, we mainly focused on investigating the connection between ENO1 and the immunogenicity of human Ad-MSCs.

Verification of ENO1 as a potential immunogenic molecule in human Ad-MSCs

We examined the expression level of ENO1 on the human Ad-MSC surface using flow cytometry. As shown in Fig. 5A, approximately 1.4% of human Ad-MSCs expressed ENO1 under normal culture conditions (MSC-0 day), whereas under one-way MLR conditions, the proportion of ENO1-expressing human Ad-MSCs increased significantly after 7 days (MSC + PBMC-7 day). We also observed that when treated with mitomycin and cultured alone for 7 days (MSC-7 day), the expression of ENO1 on the surface of human Ad-MSCs was upregulated; however, the overall level was approximately 20% lower than that in the cells cocultured with PBMCs (Fig. 5B).

Having confirmed that human Ad-MSCs stimulate the proliferation of $CD3^+$ T cells on the 12th day *in vitro* (Fig. 2B), we wondered whether the extent of proliferation would be altered by the addition of the ENO1 inhibitor ENOblock (10 μM) to the medium. We found that ENOblock treatment significantly decreased the proliferation of $CD3^+$ T cells stimulated with human Ad-MSCs (from 15.5–2.6%) and exerted no effects

on the proliferation of the positive control group (PBMC + ConA; Fig. 5C and D). Our results indicate that ENO1 is a potential immunogenic molecule in human Ad-MSCs.

Discussion

MSCs bear unique immunoregulatory properties and have long been considered immune privileged. The allogeneic transplantation of MSCs can be used to treat a wide range of diseases. There are currently a large number of clinical trials on allogeneic MSC therapy registered at *ClinicalTrials.gov.* Reports also show that allogeneic MSCs are not completely immune privileged; they cause adaptive and innate immune responses and are finally rejected [23]. Most of these studies were performed using animal models, such as rhesus monkeys [44], pigs [45] and rodents [46], which may not well simulate the allorejection process of human MSCs. Therefore, in this study, we used hu-mice model and assessed the immunogenicity of human Ad-MSCs *in vivo*. The hu-mice we reconstituted harbored mature and functional T cells; however, no B cells were observed, which was unfortunate because humoral immunity also plays a role in the allorejection process of MSCs [47]. Hence, additional studies using refined humanized mouse models, for instance, those established by transplanting a human fetal thymus, liver, and $CD34^+$ cells into NSG mice [48], are still needed to better understand the immune rejection process of human Ad-MSCs.

We also found that allogeneic human Ad-MSCs stimulated the proliferation and activation of T cells in modified one-way MLR, and the expression of HLA-II and CD40 was increased on the surface of human Ad-MSCs exposed to human PBMCs, thus increasing their immunogenicity and recognition by immune cells (Fig. 2E). This result is consistent with previous findings published by Prasanna and Raicevic [49, 50]. The TCR repertoire of human Ad-MSC-recruited T cells was analyzed for the first time in our study. We found that the diversity of both TRA and TRB was significantly reduced, indicating that the allotransplanted human Ad-MSCs selectively recruit T cells that harbor specific TCR clones. Using the CDR3 peptide of these specific TCR clones, we identified some possible immunogenic candidates, including ENO1.

The expression level of ENO1 on the surface of freshly isolated human Ad-MSCs was low but significantly increased by coculture with PBMCs. When an ENO1 inhibitor was added to the *in vitro* human Ad-MSC and PBMC coculture system, the Ad-MSC-induced proliferation of T cells was decreased. Therefore, we believe that the upregulated expression of ENO1 on the surface of human Ad-MSCs increases their immunogenicity.

ENO1 is a 48 kDa metabolic enzyme involved in the glycolytic pathway that catalyzes 2-phosphoglyceric acid to phosphoenolpyruvic acid [51]. It also exhibits other activities in addition to its catalytic function depending on its cellular and extracellular localization [52]. When expressed on the cell surface, ENO1 serves as a receptor and activator of plasminogen that mediates the activation of plasmin and extracellular matrix degradation, thus enabling the cell migration process [53]. ENO1 overexpression and posttranslational modifications may be important in many biological processes, such as cellular stress,

bacterial and fungal infections, autoantigen activity, and the occurrence and metastasis of cancer [54]. ENO1 has previously been reported to act as an autoantigen in patients with rheumatoid arthritis. After the arginine in its epitope is citrullinated, ENO1 can be presented via HLA-DRB1*04:01 to stimulate T cell responses directly [55]. In this study, for the first time, we connected ENO1 to the immunogenicity of human Ad-MSCs during allotransplantation. However, the mechanisms still need further investigation.

Conclusion

Our study further confirms the immunogenicity of allogeneic human Ad-MSCs both *in vitro* and *in vivo* and identify a potential immunogenic molecule, ENO1. Our findings provide a theoretical basis for improving the efficiency and safety of allogeneic human Ad-MSC therapies.

Abbreviations

Ad-MSCs
adipose-derived mesenchymal stem cells
Hu-mice
humanized mice
MLR
mixed lymphocyte reaction
TCR
T cell receptor
NSG
NOD/ShiLtJGpt-*Prkdc*^{em26Cd52}*Il2rg*^{em26Cd22}/Gpt
ENO1
alpha-enolase
MHC
major histocompatibility complex
CDR
complementarity determining region
PBMCs
peripheral blood mononuclear cells
GFP
green fluorescent protein
GvHD
graft-versus-host disease
ConA
concanavalin A
CFSE
carboxyfluorescein succinimidyl ester

Declarations

Acknowledgments

All authors are acknowledged for their contribution to the study.

Author Contributions

1. W. performed experiments, analyzed data and wrote the manuscript.
2. F. and C. L. performed animal culture and immunofluorescence staining.
3. F. performed human Ad-MSC isolation and characterization.
4. X., Y. H. and H. C. assisted flow cytometry and TCR-repertoire analyses.
5. C. provided human placenta-derived MSCs and valuable suggestions.
6. Z., W. H. and R C.Z. designed the scheme of the study and edited the manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the

corresponding author upon reasonable request.

Ethics approval and consent to participate

This animal study was approved and performed strictly according to the guidelines of the Animal Care and Use Committee at the Institute of Basic Medical Sciences Chinese Academy of Medical Sciences.

Consent for publication

Not applicable.

Competing interests

The authors state no conflicts of interest in this study.

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Figures

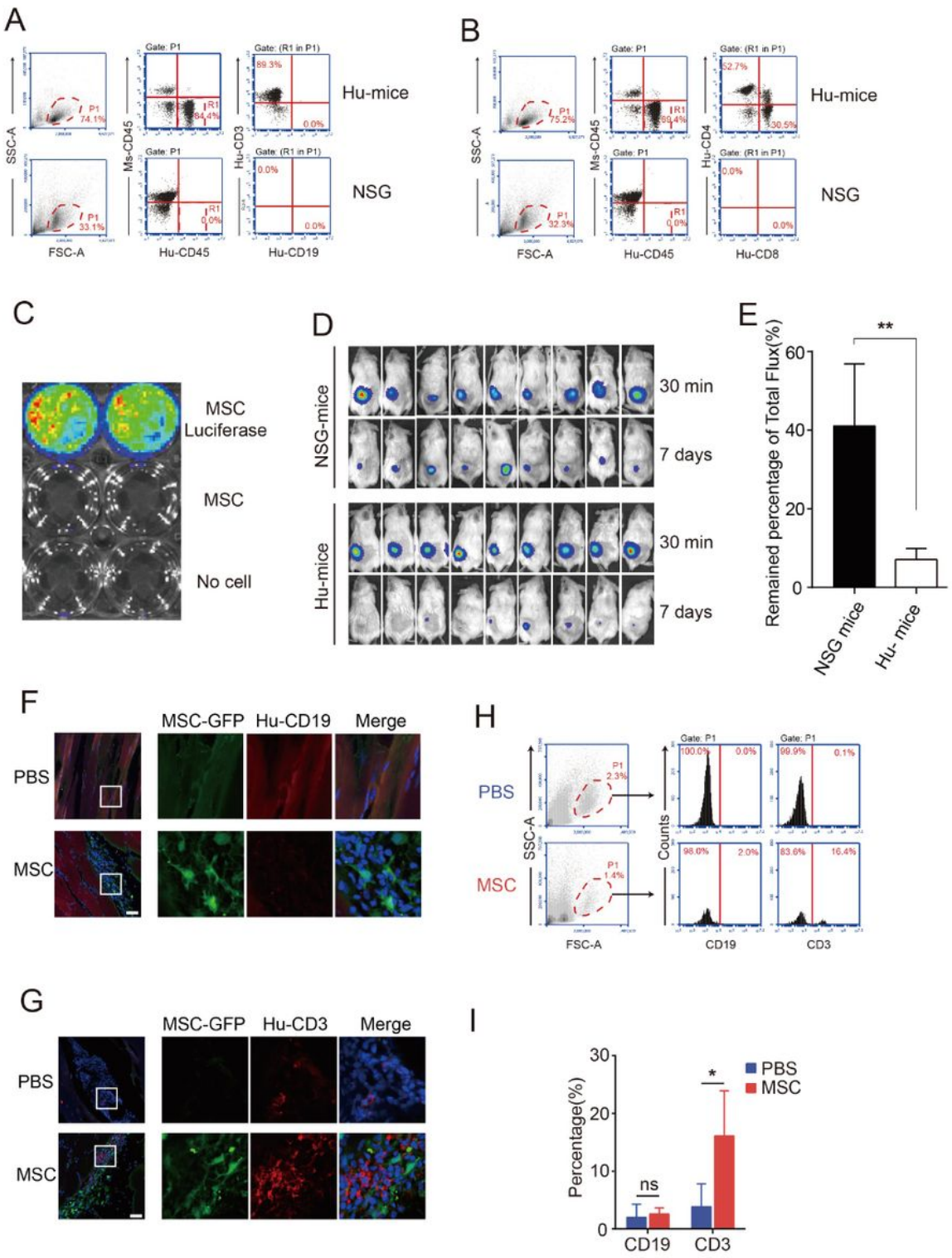


Figure 1

Immune rejection after allotransplantation of human MSCs in hu-mice A&B. Flow cytometry to analyze the splenic lymphocytes of reconstituted hu-mice (upper) and NSG mice (lower). C. Luciferase activity in Ad-MSCs after 48 h of lentivirus infection. D. Detection of luciferase-MSC activity (total flux) in Hu-mice and NSG mice by IVIS Spectrum at 30 min and 7 days after allotransplantation. All animals from 3 independent experiments are shown. E. Quantification of the results shown in panel (D). The initial (30

min) total flux of each mouse was normalized to 1. After normalization, the percentage of the remaining luciferase activity was calculated (n=9). The data are shown as the mean ± SD. **, P < 0.01, Mann-Whitney Test. F&G Immunofluorescence assays were performed to detect immune cell (human CD19+ B cells and CD3+ T cells) infiltration on the 7th day after human placenta-derived GFP-MSC transplantation. DAPI (blue), scale bar =50 μm, area of white rectangle=100×100 μm2. H. Flow cytometry to detect immune cell (human CD19+ B cells and CD3+ T cells) infiltration on the 7th day after human Ad-MSC transplantation. Representative data are shown. I. Quantification of the flow cytometry analysis shown in panel (i); n=6. Data are shown as the mean ± SD. *, P < 0.05 by paired Student's t test.

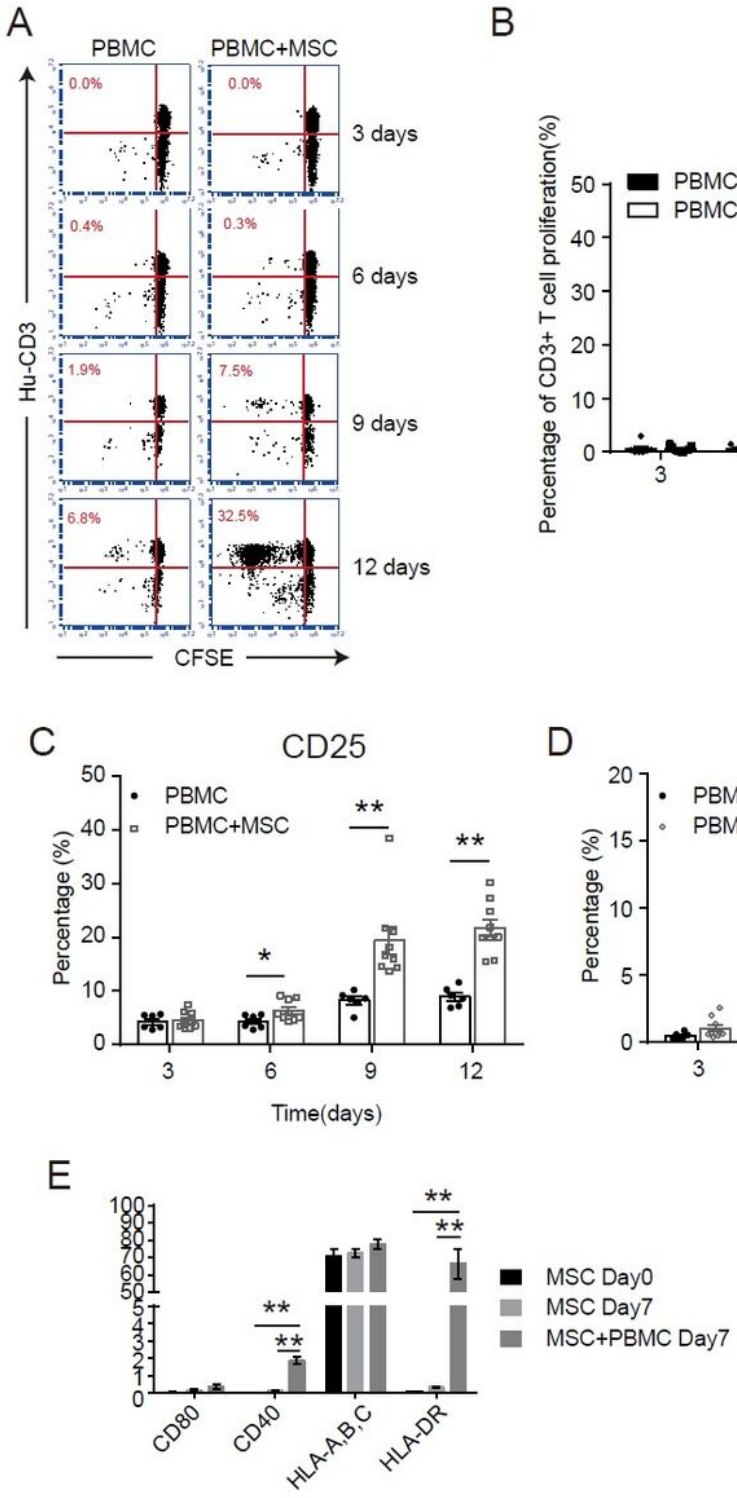


Figure 2

Human Ad-MSCs stimulate the proliferation and activation of T cells in vitro A. Flow cytometry and one-way MLR were used to evaluate the proliferation of CD3+ T cells in PBMCs stimulated with human Ad-MSCs in vitro. PBMCs were labeled with CFSE before being cocultured. CFSE dilution represents cell proliferation. PBMC: cultured alone; PBMC + MSC: coculture. B. Quantification of the proliferation of CD3+ T cells shown in panel (A). C&D Expression level of CD69 (C) or CD25 (D) on the surface of CD3+ T

cells during one-way MLR. E. Expression levels of CD40, CD80, HLA-A, B, C and HLA-DR on the surface of human Ad-MSCs before one-way MLR (day 0) and after 7 days (day 7). Statistics: For (B), (C) and (D), 3 independent experiments were conducted (n=9); for (E), n=5; mean ±SD, *, P < 0.05; **, P < 0.01 by Student's t test.

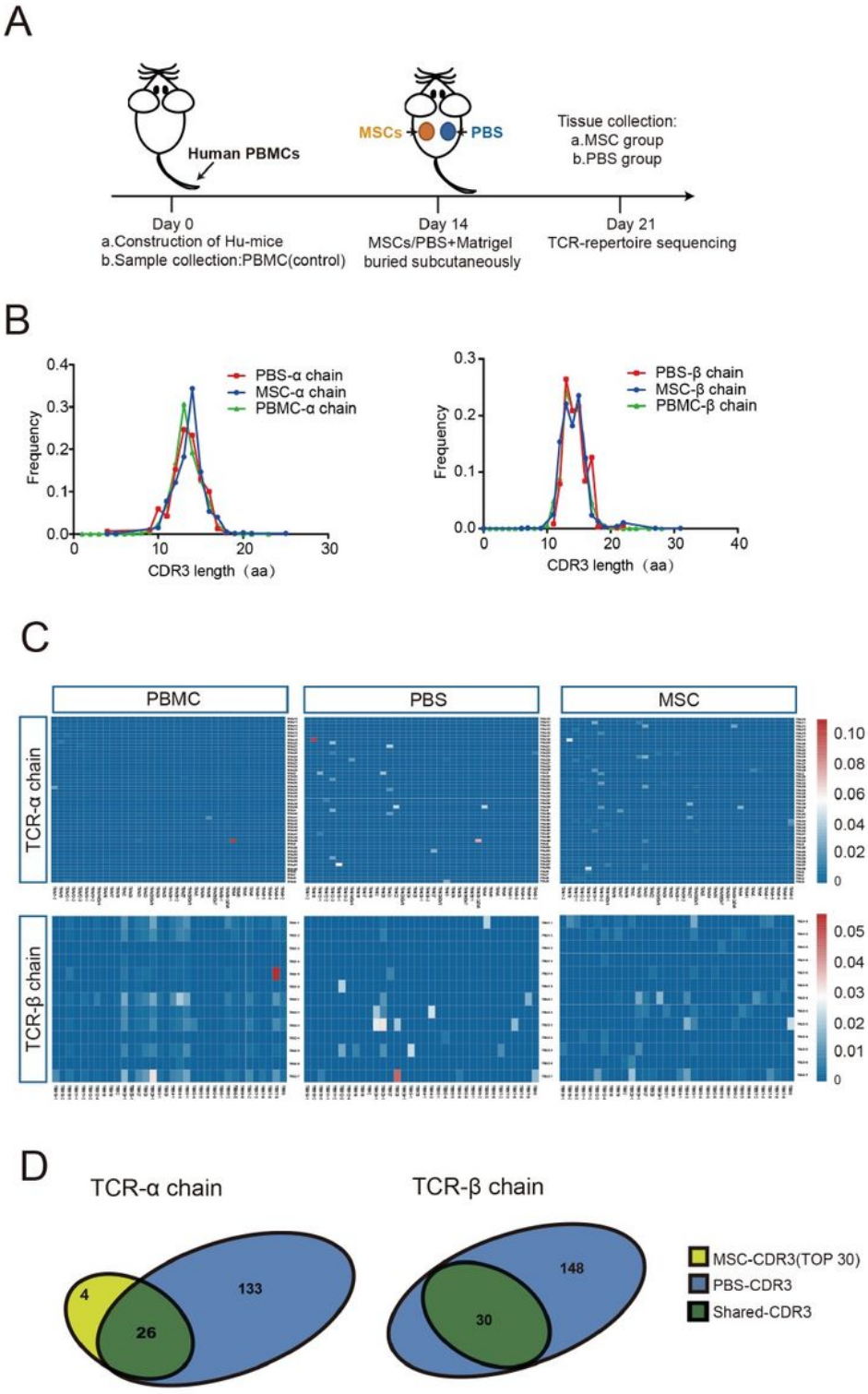
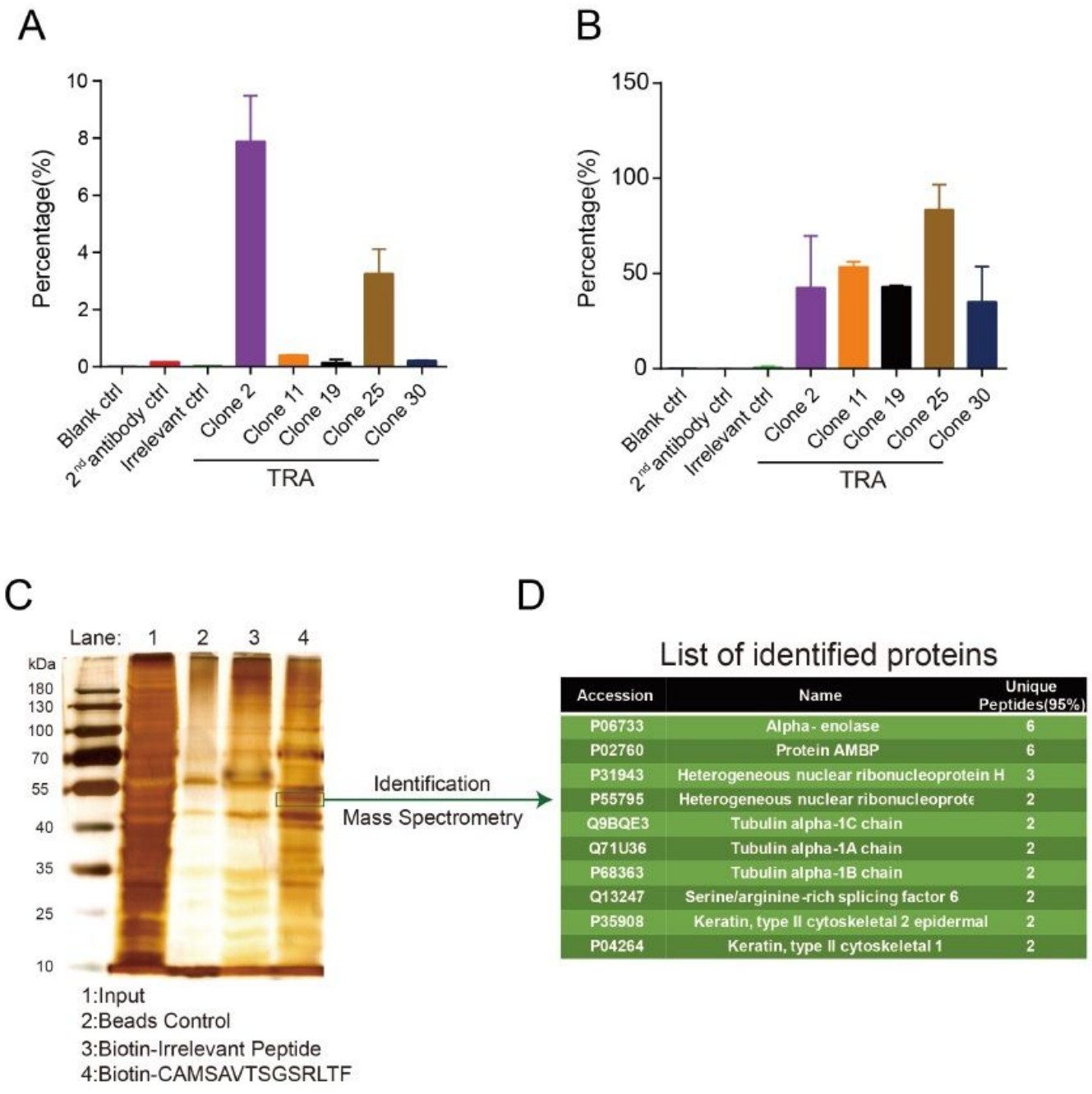


Figure 3

TRA/TRB repertoire analysis of infiltrating T cells A. Sample collection procedure for TCR repertoire sequencing. B. Comparison of unique CDR3 peptide length distribution among the 3 groups. C. Heatmaps of TRA/TRB chain VJ gene usage. The blue color indicates the lowest frequency, and the red color indicates the highest frequency. D. Venn diagram: The top 30 CDR3 peptides in the MSC group (MSC-CDR3 TOP30) were compared with all CDR3 peptides in the PBS group (PBS-CDR3). Shared CDR3 represents overlapping CDR3 peptides.



Accession	Name	Unique Peptides(95%)
P06733	Alpha - enolase	6
P02760	Protein AMBP	6
P31943	Heterogeneous nuclear ribonucleoprotein H	3
P55795	Heterogeneous nuclear ribonucleoprote	2
Q9BQE3	Tubulin alpha-1C chain	2
Q71U36	Tubulin alpha-1A chain	2
P68363	Tubulin alpha-1B chain	2
Q13247	Serine/arginine-rich splicing factor 6	2
P35908	Keratin, type II cytoskeletal 2 epidermal	2
P04264	Keratin, type II cytoskeletal 1	2

Figure 4

Identification of proteins pulled down by biotinylated CDR3 peptides A. Flow cytometry was used to examine the affinity of 5 selected CDR3 peptides for the surface molecules of human Ad-MSCs. B. The

affinity of 5 CDR3 peptides for the intracellular molecules of human Ad-MSCs. Blank, irrelevant (biotin-SPGH), and secondary antibody controls were included. Data are shown as the mean ± SD; n=3. C. SDS-PAGE and silver staining were performed to identify proteins pulled down by TRA CDR3 peptide clone 2. The unique protein band (in the green rectangle) was excised and subjected to mass spectrometry identification. D. Information on the identified proteins. Access: protein ID in the UniProt database.

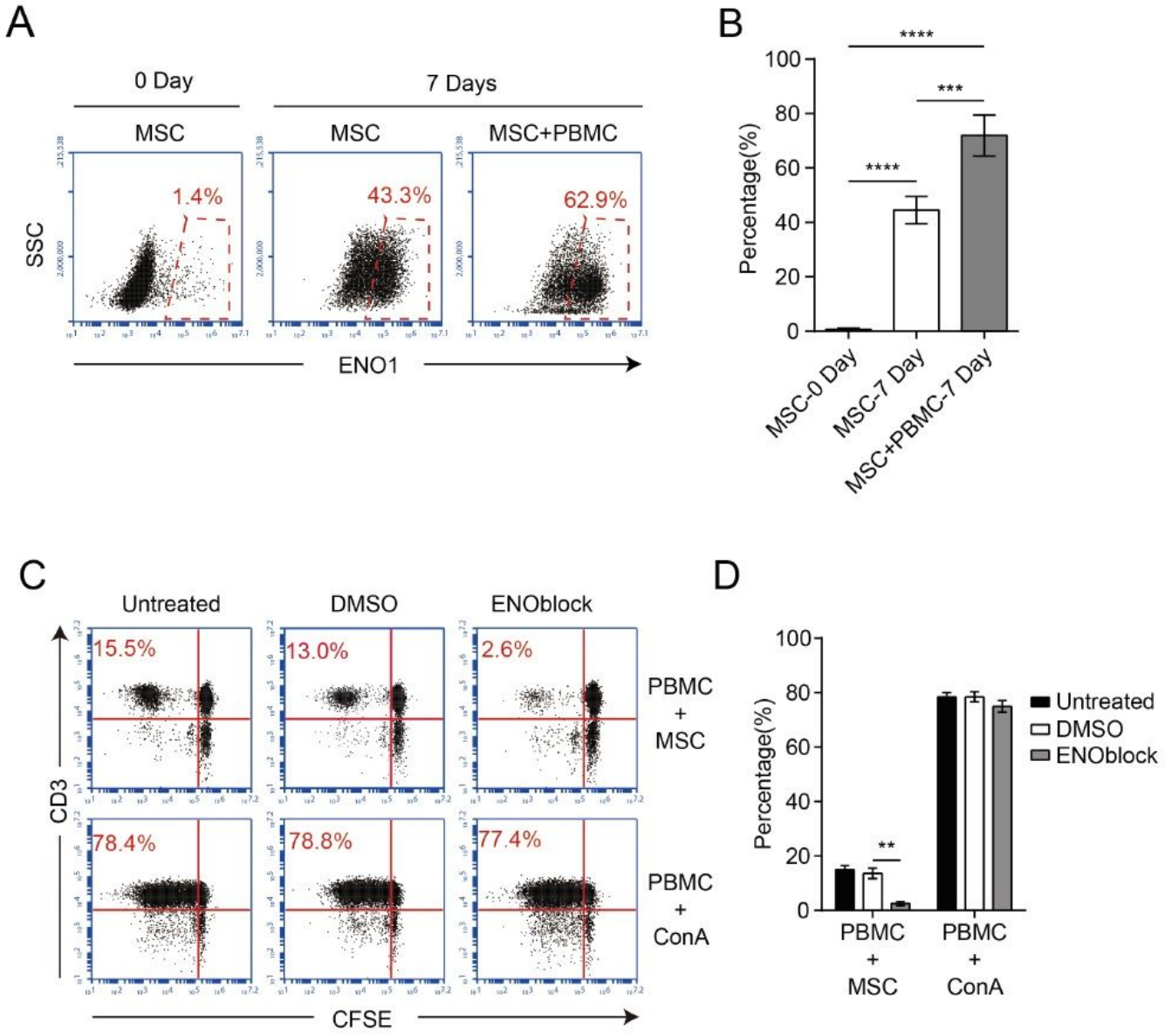


Figure 5

Verification of ENO1 as a potential immunogenic molecule of human adipose-derived MSCs A. The expression level of ENO1 on the surface of human Ad-MSCs in different conditions. B. Quantification of panel (A); n=5, mean ±SD, ***, P<0.0005, ****, P<0.0001, Student's t test. C. Flow cytometry and one-way MLR were performed to evaluate the influence of ENOblock (10 μM) on the proliferation of CD3+ T cells stimulated with human Ad-MSCs in vitro. Positive control: PBMC+ConA. D. Statistics: the proliferation of CD3+ T cells shown in panel (C); n=3, mean ±SD. *, P < 0.05, Student's t test.

Supplementary Files

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