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In vitro CELLULAR SENESCENCE MICROENVIRONMENT INDUCES T CELL SENESCENCE

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Abstract: Senescent cells promote their own growth arrest in an autocrine manner and can also transmit senescence phenotypes to their surrounding cells in a paracrine manner, inducing the senescence of these cells. But, no appropriate T-cell senescence model may be, to date, applied to relevant researches. To explore whether the cellular senescence microenvironment triggers T-cell senescence *in vitro*, HUVEC-12 cells were pretreated with D-gal. They exhibited the hallmark features of cellular senescence, including the reduced proliferation, the decreased superoxide dismutase (SOD) viability, the accumulation of malondialdehyde (MDA) content and the increased senescence-associated β-galactosidase (SA-β-gal) activity. When the mouse or human-derived T cells were exposed to this senescent HUVEC-12 cell environment, their viability was a little affected, but about 20% of senescent T cells could be formed, which was less than the D-gal-induced senescent T cells. This lays some foundation for establishment of *in vitro* T-cell senescence models.

Keywords: D-gal, T cell, senescence, microenvironment, in vitro

1. Introduction

A diverse range of stress-inducing events can trigger cellular senescence. Potential factors that can contribute to the activation of tumor suppressor networks, such as p53, p16 (Cdkn2a), and p21 (Cdkn1a), include active oncogene signaling, DNA damage, and administration of radiotherapy or chemotherapy, resulting in cell cycle arrest [1, [2]. The senescence microenvironment is frequently characterized by an increase in the production of cytokines and an augmentation of cellular secretion, commonly referred to as the senescence-associated secretory phenotype (SASP) [3]. The SASP is postulated to serve as a mechanism via which senescent cells establish communication with the immune system,

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potentially aiding their elimination [4]. As an illustration, the release of SASP factors [5], including IL-1α, IL-6, IL-8, and TGF-β from senescent cells activated by Ras results in detrimental effects on both neighboring cells and surrounding tissues [6]. The process of cellular senescence leads to tissue senescence through paracrine actions [7], concurrently exacerbating tissue inflammation and facilitating the development of carcinogenesis [8]. Nevertheless, a limited body of research exists about the impacts of the senescent milieu on T cell senescence. The precise mechanisms and functions of the senescent microenvironment remain ambiguous.

The phenomenon of aging induced by D-gal, an aldose, has garnered significant attention [9]. It reduces sugar in the human body and may also be found in various food sources [10]. The production of D-gal-induced reactive oxygen species (ROS) [11], advanced glycation end products (AGEs) [12], osmotic stress [13] and redox imbalance in the body has the potential to ultimately induce cellular or organismal senescence [14]. On other hand, investigating the senescent milieu in vitro has posed challenges in the field of inquiry. The present investigation aimed to explore potential of the in vitro senescent milieu in inducing senescence of T lymphocytes, which is not reported at present. Hence, D-gal was utilized to establish a senescent cell model of the HUVEC-12 stable cell line for duplicating a senescent microenvironment. The results showed that 20% of the mouse or human-derived T cells appear senescent under the HUVEC-12 cell senescence environment, implying that other cell senescence microenvironment merits to be further studied for obtaining better models of inducing T cell senescence.

2. Materials and methods

2.1 Cell culture

HUVEC-12, CTLL-2 and Hut 78 cell lines were provided by Institute of Tissue Transplantation and Immunology in Jinan University. HUVEC-12 cells were grown in DMEM supplemented with 10% FBS. CTLL-2 cells were cultured in high-glucose DMEM supplemented with 10% FBS and 0.5 μg/ml mIL-2, and Hut 78 cells in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS.

2.2 Cell viability assay

Using a CCK-8 Cell Counting Kit (C0039, Beyotime, China), the viability of the cells was determined. The cells used for the D-gal assay were cultured for 4 hours in 96-well plates. According to the experimental design, the proper substance was then deposited in the proper well. In each well, 10 μL of CCK-8 solution was added after D-gal treatment. The cells were then incubated for two hours in a 37°C cell culture incubator with 5% CO2. Finally, optical density (OD) at 450 nm was determined and it was figured out how many viable cells were directly related.

2.3 Modelling the cellular senescence microenvironment

To induce senescence in HUVEC-12 cells, a pre-treatment of 10 mg/ml D-gal (G100367, Aladdin, China) was delivered to the cells for 48 hours. HUVEC-12 cells that had achieved senescence were utilized as a representative model for senescent cells inside the senescent milieu. The senescent cells were grown alongside normal CTLL-2 cells or Hut 78 cells. Following the designated co-culturing period, T-lymphocytes in suspension were collected to conduct a status assessment.

2.4 Senescence-associated β-galactosidase staining

T cell senescence was assessed using a β-galactosidase staining kit (KGPAG001, Keygen, China). In brief, cells were initially subjected to two rounds of rinsing with PBS and subsequently exposed to β-galactosidase fixative for 15 minutes at room temperature. Subsequently, the cells were subjected to three successive rinses using PBS for 3 minutes per rinse. They were further treated with a staining solution comprising 10 μL of β-galactosidase staining solution A, 10 μL of solution B, 930 μL of solution C, and 50 μL of X-Gal solution. Afterwards, the cells were incubated for one night at 37°C. In the end, the cellular specimens were analyzed using an inverted microscope. Presence of β-galactosidase positive signals was observed within cytoplasm, appearing as small and evenly distributed blue particles.

2.5 SOD detection

Cellular concentration of superoxide dismutase (SOD) was assessed using a Total

Superoxide Dismutase Assay Kit with WST-8 (S0101S, Beyotime, China). Based on the manufacturer's instruction, WST-8/enzyme working solution, reaction initiation working solution and SOD assay buffer were put into a 96-well plate in turn. Absorbance assay was performed at a scheduled time for activity of SOD by measuring the stably formed water-soluble product due to the reaction involved in WST-8, which revealed a direct relationship between optical density at 450 nm and cellular viability via the standard curve of enzyme.

2.6 Malondialdehyde (MDA) content detection

Concentration of malondialdehyde (MDA) in cells was quantified utilizing a MDA Assay Kit (T01013, LEAGENE, China). Procedures delineated in the product documentation facilitated preparation of TBA stock solution and MDA working solution. Furthermore, the standard chemicals were diluted following the stated criteria. An appropriate amount of MDA standard (1 mmol/L) was taken and diluted to 1, 2, 5, 10 and 20 μM with a gradient of appropriate solutions. Levels of MDA in sample solutions were measured using a standard curve. Absorbance values of each group at 450 nm were determined by a microplate reader (BIO-RAD, USA).

2.7 Statistical analysis

The data were all shown as mean ± standard deviation (SD). GraphPad Prism 8 was used for statistical analysis with the Student t test and one-way analysis of variance used for both paired and unpaired data.

3. Results

3.1 Establishment of HUVEC-12 cell senescence microenvironment by D-gal

Effects of D-gal on proliferative viability of HUVEC-12 cells were firstly investigated using CCK-8 assay at different concentrations and action time. The results showed that D-gal could inhibit the proliferative activity of HUVEC-12 cells in a time-dependent manner (Figure 1a). The D-gal (10 and 30 mg/ml) treatment of HUVEC-12 cells significantly reduced SOD activity compared to the control group (Figure 1b). The 10 and 30 mg/ml D-gal treatment caused a substantial rise in MDA levels compared to the control group (Figure 1c). As shown in Figure 1d-e, after exposed to 10 mg/ml D-gal for 48 hours the SA-β-gal-

positive cells increased to 71.93% compared to the control group. Live/Dead staining assay demonstrated that 10 mg/ml D-gal had no significant impact on the senescent death of HUVEC-12 cells within 48 hours, but 30 mg/ml D-gal could induce their remarkable senescent death, compared to the control group (Figure 1f-g). These results indicate that HUVEC-12 cell senescence model is successfully established by D-gal.

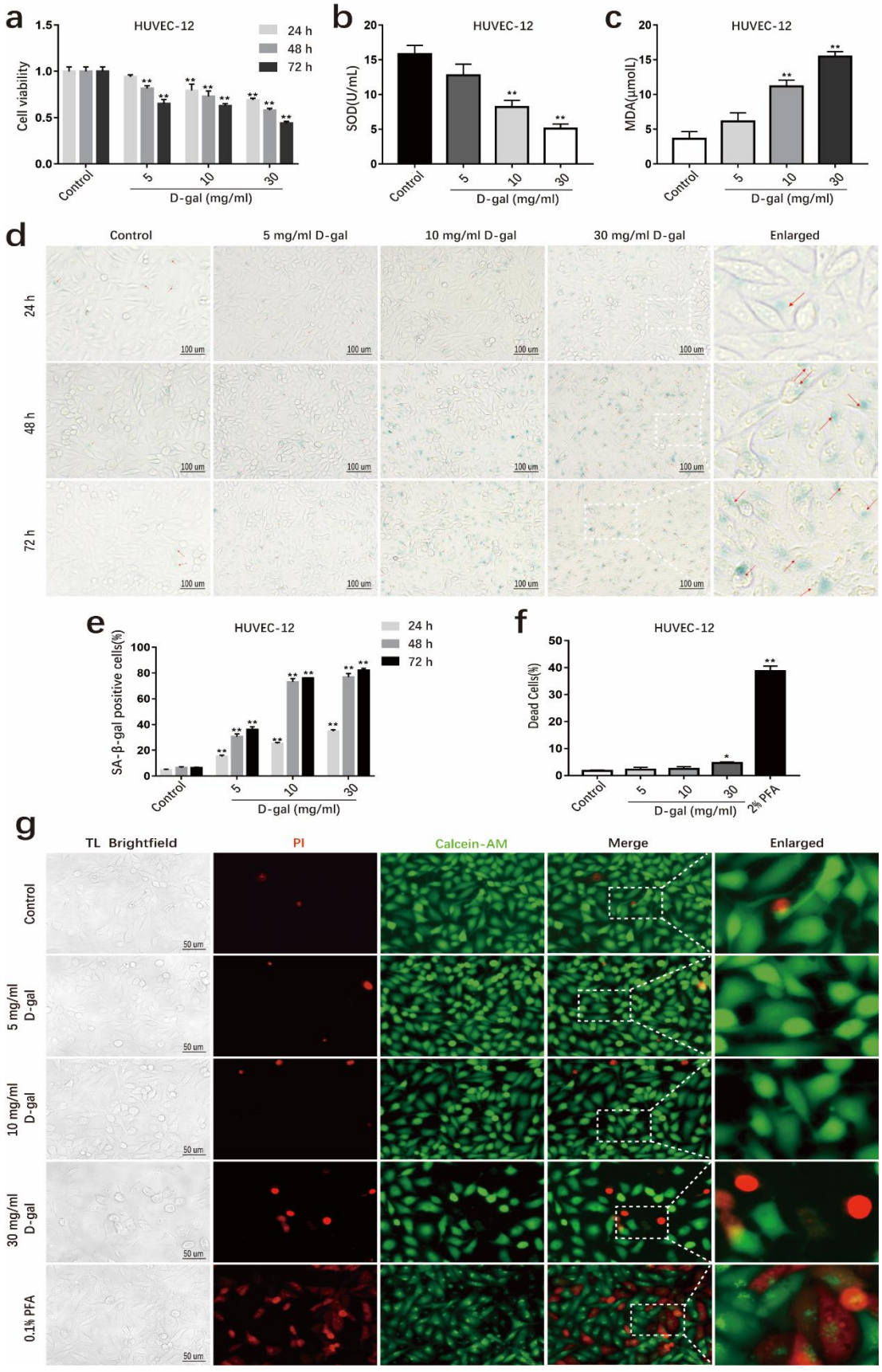

Figure 1. D-gal induces senescence in HUVEC-12 cells. (a) The HUVEC-12 cells were

exposed to D-gal at the concentrations of 0, 5, 10 and 30 mg/ml for 24, 48 and 72 hours, and their viabilities were analyzed by CCK-8 assay. (b) The HUVEC-12 cells were exposed to D-gal at the concentrations of 0, 5, 10 and 30 mg/ml for 48 hours, and their SOD activities were analyzed. (c) The HUVEC-12 cells were exposed to D-gal at the concentrations of 0, 5, 10 and 30 mg/ml for 48 hours, and their MDA contents were measured. (d-e) The HUVEC-12 cells were exposed to D-gal at the concentrations of 0, 5, 10 and 30 mg/ml for 48 hours before the proportion of SA-β-gal positive cells was calculated under an inverted fluorescence microscope via SA-β-gal staining (× 100). (f-g) The HUVEC-12 cells were exposed to D-gal at the concentrations of 0, 5, 10 and 30 mg/ml for 48 hours, and the percentage of dead cells by the Calcein-AM/PI staining was analyzed (× 200). The live cells appear green, but the dead cells do red. The data are expressed as means ± SD of three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the control group. TL, transmissive lighting; PI, propidium iodide; PFA, paraformaldehyde.

3.2 Cellular aging microenvironment induces the mouse-derived T-cell senescence

HUVEC-12 cells were first pre-treated with 10 mg/ml of D-gal for 48 h to induce their senescence. The senescent cells were co-cultured with normal CTLL-2 cells for 24 hours to ascertain whether the senescent HUVEC-12 cells-offered microenvironment induces senescence in CTLL-2 cells. The experiment was divided into the following groups CTLL-2, HUVEC-12 + CTLL-2, Aged-HUVEC-12 + CTLL-2, and Aged-CTLL-2. The CTLL-2 group acts as the normal control without any treatment. The HUVEC-12 + CTLL-2 group means that the normal CTLL-2 cells were co-cultured with the normal HUVEC-12 cells. The Aged-HUVEC-12 + CTLL-2 group means that the normal CTLL-2 cells were co-cultured with the senescent HUVEC-12 cells. The Aged-CTLL-2 group was the D-gal-treated CTLL-2 cells. After the cell co-culture, the suspended CTLL-2 cells were separated for further tests. CCK-8 test was used to examine viability of CTLL-2 cells. The Aged-HUVEC-12 + CTLL-2 group had the lower cell viability (Figure 2a). However, SOD activity and MDA content did not significantly change compared to the CTLL-2 group (Figure 2b-c). Senescence enhances SA-β-gal activity in vivo, resulting in X-Gal-stained

blue crystals visible as blue cells under a light microscope. As shown in Figure 2d, the SA-β-gal cellular senescence staining demonstrated a 11.77% increase of the positive cells in the Aged-HUVEC-12 + CTLL-2 group. These findings indicate that the microenvironment offered by the senescent HUVEC-12 cells can induce limited CTLL-2 cells to undergo senescence.

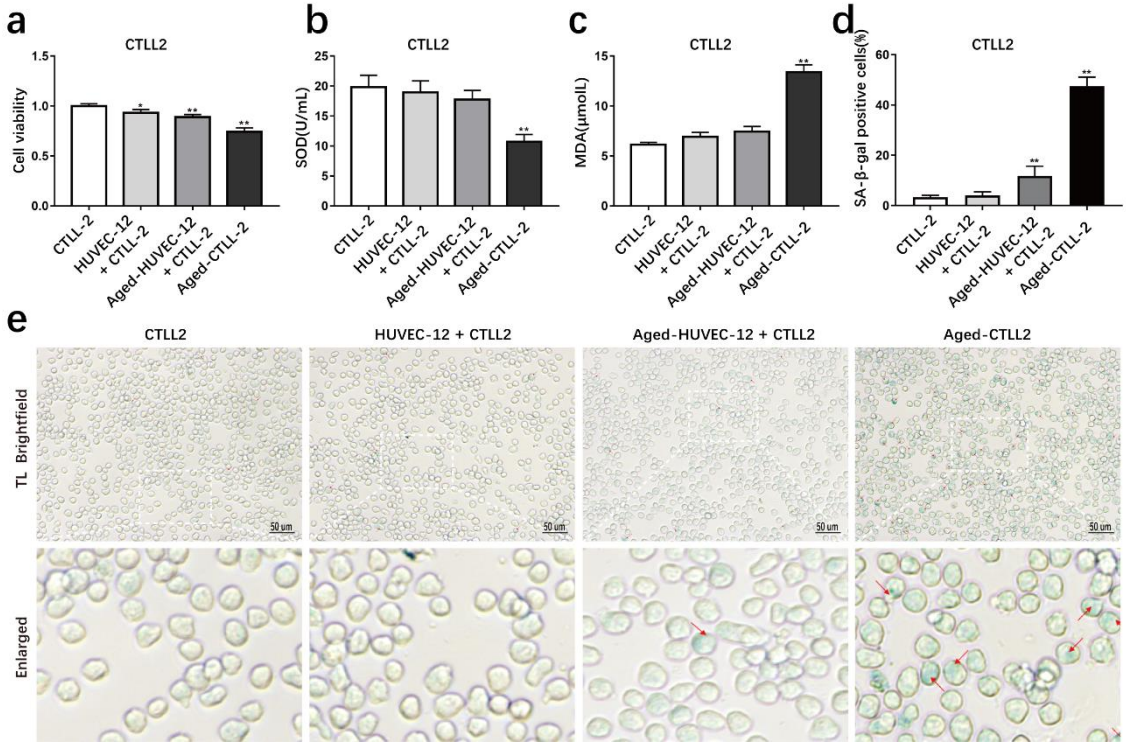

Figure 2. Impact of senescent HUVEC-12 cells on the senescence of mouse CTLL-2 cells. (a) The CTLL-2 cells were incubated with the senescent HUVEC-12 cells for 24 hours, and their viability was determined using the CCK-8 assay. (b) The CTLL-2 cells were cultured together with the senescent HUVEC-12 cells for 24 h, and analyzed for their SOD activity. (c) After 24 hours of co-culture with the senescent HUVEC-12 cells, the MDA content in the CTLL-2 cells was analyzed.

(d-e) There were 24 hours of co-culture of the senescent HUVEC-12 cells with CTLL-2 cells, and SA-β-gal staining was used to determine the proportion of SA-β-gal positive cells (× 200). The data are expressed as means ± SD of three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the control group. TL, transmissive lighting.

3.3 Cellular aging microenvironment induces the human-derived T-cell senescence

HUVEC-12 cells were pre-treated with 10 mg/ml D-gal for 48 h to create senescent cells, which was used to mimic the cellular senescent microenvironment offered to normal Hut 78 cells. The experiments were divided into the following groups Hut 78, HUVEC-12 + Hut 78, Aged-HUVEC-12 + Hut 78, and Aged-Hut 78. After the cell co-culture, the suspended Hut 78 cells were separated for further tests. CCK-8 test was used to examine viability of Hut 78 cells. As shown in Figure 3a, The Aged-HUVEC-12 + Hut 78 cells had the similar cell viability to the HUVEC-12 + Hut 78 cells. The intracellular SOD activity and MDA content in the Aged-HUVEC-12 + Hut 78 cells did not significantly change compared to the Hut 78 cells (Figure 3b-c). According to Figure 3d-e, the Aged-HUVEC-12 + Hut 78 group had a higher proportion of SA-β-gal-positive cells (24.73%) compared to the Hut 78 and HUVEC-12 + Hut 78 groups, respectively. There results indicate that the cellular senescence microenvironment can promote senescence in a limited proportion of Hut 78 cells.

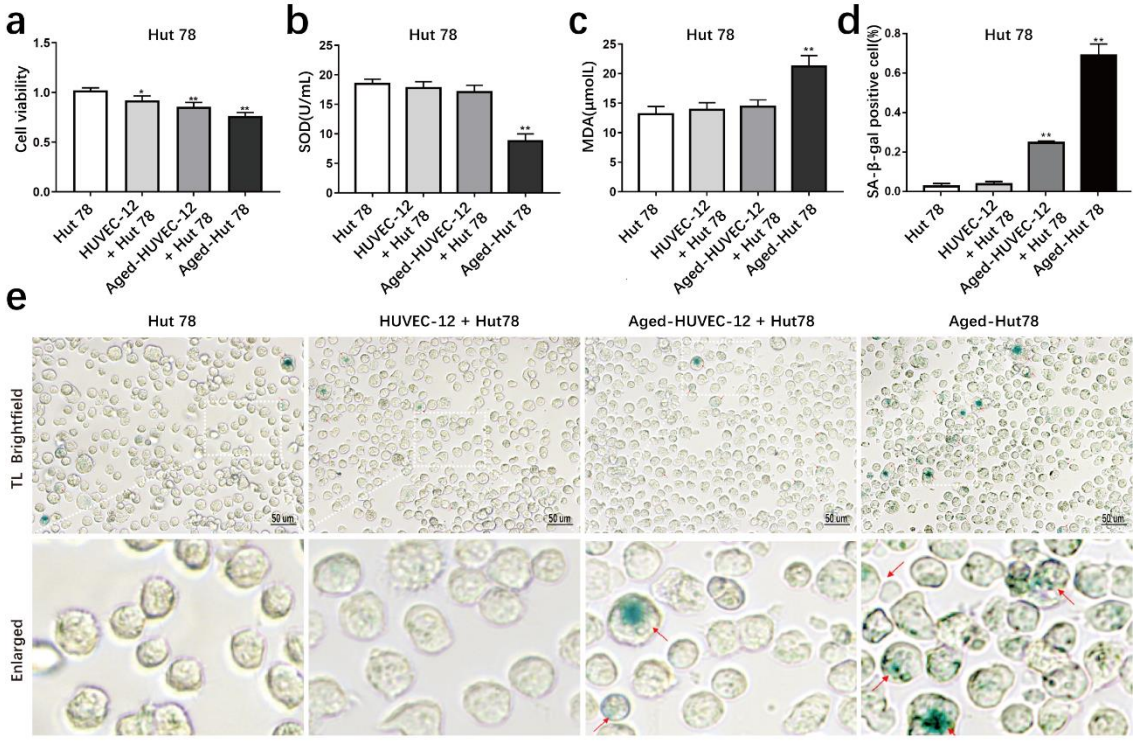

Figure 3. Impact of senescent HUVEC-12 cells on the senescence of human Hut 78 cells. (a) The Hut 78 cells were cultured together with the senescent HUVEC-12 cells for 48 hours, and their viability was determined using the CCK-8 assay. (b) The Hut 78 cells were

cultured with the same cells for 48 hours and then their SOD activity were tested. (c) The Hut 78 cells were similarly treated, and their MDA content was analyzed. (d-e) After co-cultured with the senescent HUVEC-12 cells for 48 hours they were stained by SA-β-gal for determining the proportion of SA-β-gal positive cells (× 200). Data are expressed as means ± SD of three experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the control group. TL, transmissive lighting.

4. Conclusion

No appropriate T-cell senescence model has been harnessed to explore a mechanistic process of occurrence of T-cell senescence for interfering with it, up to now. In theory, the construction of cells by creating an aging microenvironment seems to be closer to the naturally occurring cellular senescence in physiological or pathological states. Based on our perception, D-gal was utilized to duplicate this microenvironment via HUVEC-12 cells, which T cells were provided with for establishing T-cell senescence model. About 11.77 - 24.73% of senescent T cells can occur in the presence of the current aging microenvironment. This opens a new chapter in establishment of in vitro T-cell senescence models.

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