Improved function and balance in T cell modulation by endothelial cells in young people

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Abstract

Elderly individuals exhibit unbalanced bone marrow (BM) effector T cell subset differentiation, such as increased T helper (Th)-1 and T cytotoxic (Tc)-1 cell frequencies, but the underlying mechanism still unclear. Endothelial cells (ECs) , which are instructive components of the BM microenvironment, exhibit the phenotype of semi-professional antigen-presenting cells and regulate T cell recruitment and activation. Thus, we compared the frequency and function of BM ECs, especially their capacity to regulate effector T cell subsets, between young and old healthy individuals, and explored the underlying mechanism of this immunomodulatory discrepancy. Although the young and old EC percentages were comparable, young ECs showed less reactive oxygen species and better migratory and tube-forming abilities than old ECs. Notably, young ECs regulated T cells to differentiate into fewer Th1 and Tc1 cells than old ECs. Reduced T cell activation molecules and inflammatory cytokines in young BM ECs may be the possible mechanism.

Introduction

The immune system undergoes a series of physiological functional declines with ageing, which are termed immunosenescence and partly result in poor responses to vaccines and increased incidence of severe infectious diseases and cancer in the elderly population[1, 2]. Some studies have shown that the adaptive immune system, especially T cells, is more susceptible to ageing than the innate immune system[3, 4]. Our and other previous studies showed decreased frequencies of bone marrow (BM) lymphoid progenitor cells and naïve T cells but increased frequencies of memory T cells, as well as unbalanced effector T cell subset differentiation, such as increased T helper (Th)-1 and T cytotoxic (Tc)-1 cells, in elderly individuals[1, 2, 5]. Emerging evidence has indicated that decreased BM lymphoid progenitor cells and involution of the thymus may be responsible for the alterations in T cell subsets with ageing[6-8]. However, the underlying mechanism of the more balanced differentiation of effector T cell subsets in young individuals than in elderly individuals remains to be clarified.

The BM, as both a primary and secondary lymphoid organ, plays an essential role in regulating the development and differentiation of T cells[9]. Endothelial cells (ECs), which line the interior of blood and lymphatic vessels, are instructive components of the BM microenvironment[10]. In addition to supporting self-renewal and lineage-specific differentiation of haematopoietic stem cells through the release of specific angiocrine factors[11-13], ECs also play crucial immunological roles, particularly in T cell recruitment and activation[14, 15]. Emerging evidence has suggested that ECs exhibit a semi-professional antigen-presenting cell phenotype by expressing classic innate immune receptors such as Toll-like receptors (TLRs), NOD-like

receptors, and inducible costimulatory ligands[16-18]. Although controversial, some studies have suggested that ECs express costimulatory molecules such as CD80 and CD86[19, 20]. Activation of EC TLRs by lipopolysaccharide (LPS) or lipopeptide upregulated the secretion of specific cytokines, chemokines and adhesion molecules and increased the recruitment and activation of T cells[14, 15]. In addition, recent studies demonstrated that ECs were able to suppress T cell proliferation and modulate T cells to produce fewer proinflammatory cytokines through tumour necrosis factor and its receptor 2 signalling pathway[10]. Moreover, liver sinusoidal ECs have been shown to modulate naïve $CD8^+$ T cells to secrete less interferon-γ (IFN-γ) and interleukin (IL)-2 by cross-presentation to $CD8^+$ T cells, which then induces immune tolerance[21, 22]. However, whether the frequency and functions of BM ECs, especially their capacity to regulate effector T cell subsets, differ between young and old individuals remains to be clarified.

Therefore, the current study was performed to evaluate the frequency and reactive oxygen species (ROS) levels of BM ECs among young, middle-aged, and old healthy individuals. Moreover, we investigated whether the function of BM ECs, especially their capacity to modulate the differentiation of effector T cell subsets, differs between young and old individuals and the underlying mechanism of this immunomodulatory discrepancy. Our study may contribute to improving the understanding of T cell subset alterations with ageing.

Materials and methods

Healthy donors

In the study, a total of 45 healthy adult individuals who routinely received BM examination as allo-HSCT donors from July 1, 2020 to March 1, 2021 at Peking University Institute of Hematology were enrolled. Donors aged <30 years, 30-45 years, and >45 years were divided into the young, middle-aged and old groups respectively, with 15 donors per group. The donor characteristics, including age, sex, and routine peripheral blood parameters were evaluated among young, middle-aged and old donors.

Peking University People's Hospital Ethics Committee consented the study. Moreover, all subjects signed informed consent before enrollment, in conformity to the Declaration of Helsinki.

Characterization of BM ECs

BM mononuclear cells (BMMNCs) were isolated with Ficoll-Paque solution (HaoYang, Tianjin, China). The following multicolour flow cytometry panel were applied to quantify ECs in BM: anti-CD34-PE/Cy7, anti-CD309-PE, and anti-CD133-APC (BD Biosciences, CA, USA). ECs were identified as $CD34^+CD309^+CD133^+$, and the relative EC frequency was expressed as a fraction of BMMNCs[23-27]. Samples were collected on a BD LSRFortessa flow cytometer and the compensation adjustion as well as data analysis were accomplished via a BD LSRFortessa software.

Measurement of intracellular ROS

To examine EC ROS level, 10 μmol/l DCFH-DA (Beyotime, Beijing, China) was added into the BMMNC suspension that stained with EC markers at 37°C for 15 minutes[23-27]. EC ROS levels were examined by flow cytometry and expressed as the mean fluorescence intensity (MFI).

Culture of primary BM ECs

As previously reported [23-27], BMMNCs were extracted and seeded to the cell culture plates (Corning, NY, USA) pre-coated with fibronectin (Sigma, MO, USA). EGM-2-BulletKit (Lonza, MD, USA) medium with extra 10% foetal bovine serum (FBS) (Gibco, MA, USA) was added to the cell plates and was renewed on the fourth day to supplement nutrition for cells.

DiI-Ac-LDL uptake and FITC-UEA-1 binding assay

As previously reported[23-27], DiI-Ac-LDL (Life Technologies, MD, USA) and FITC-UEA-I (Sigma, MO, USA) was added to anchorage-dependent cells that had been cultured for 7 days, and then cells were observed

under a fluorescence microscope (Olympus, Tokyo, Japan). Double-positive-stained cells were considered as ECs, and the EC numbers on the 3 random views per well were counted manually [23-27].

Tube-formation assay

250 μl Matrigel (Corning) was added to each well of a 24-well plate for overnight. Then, 7-day-cultured ECs were plated with a density of 5×10^4/well above Matrigel and cultured in 500 μl EGM-2 medium in an incubator[23-25]. After 48 hours, tube formed by ECs were observed with a light microscope (Olympus) and 3 photographs per well were taken randomly[23-25]. ImageJ (National Institutes of Health, MD, USA) was used to quantify the relative tube length.

Migration assay

After 7 days of culture, the cells were detached, and 5×10^4 cells were resuspended in the upper chamber of a Transwell chamber (pore size 8 μm, Corning) with 200 μl EBM-2 basal medium (Lonza), while 500 μl EGM-2-BulletKit containing 10% FBS was added to the lower chamber[23-27]. After 24 hours, cells that migrated from the upper chamber to the lower surface of the membrane were fixed and stained as previous protocols[23-27]. For quantification, the stained cells were observed and counted manually in 3 random views per well with a phase-contrast microscope (Olympus).

Coculture of ECs and $CD3^+$ T cells

$CD3^+$ cells were sorted from BMMNCs with a CD3 MicroBead kit (Miltenyi Biotec, NRW, Germany), and were cocultured with 7-day primary cultured ECs in a direct contact way at a 1:10 number ratio. The previous medium for ECs was changed to 1 ml RPMI medium (Gibco) containing 10% FBS per well for coculture.

Immunophenotype analysis of the cocultured T cell subsets

After 3 days of coculture, $CD3^+$ T cells were treated with cell stimulation cocktail (500×, eBioscience, CA, USA) as the manufacturer's instructions to induce $CD3^+$ T cells activation and cytokine secretion. Then, we evaluated the IL-4, IFN-γ, IL-17 and Foxp3 levels by flow cytometry using the hereinafter monoclonal antibody panel: CD3-APC-H7, CD8-V500, CD25-PE-Cy7, IL-4-PE, IFN-γ-Percpcy5.5, IL-17-FITC, and Foxp3-APC (BD Biosciences)[5, 28-30]. The immunophenotyping of T cell subsets including Th1, Th2, Th17, Tc1, Tc2 and regulatory T cells (Tregs) were in keeping with our previous studies[5, 28-31].

RNA sequencing

To search for the regulatory mechanism involved in the improved ability of young donor ECs to support T cell differentiation, we performed RNA sequencing (RNA-seq) in 7-day-cultured ECs from young and old donors. RNA extraction and analysis of differential gene expression and Gene Oncology (GO) enrichment between the young and old donor EC groups were completed as previously reported[27, 32].

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from 1×10^7 7-day-cultured ECs using Trizol reagent (Life Technologies). qRT-PCR was carried out with the QuantiTect SYBR Green RT-PCR kit (Qiagen, NRW, Germany) according to the manufacturer's instructions. The relative mRNA levels of TLR1, TLR2 and TLR4, CCR2, CCR5, CXCL10, PRF1 and the NLRP3 inflammasome were analysed after normalization to 18s mRNA levels.

Analysis of T cell activation molecules in ECs by flow cytometry

BMMNCs were cultured in EGM-2-BulletKit medium and then incubated with 100 ng/ml LPS for 4 hours. Afterwards, to test the levels of T cell activation molecules in ECs, the cells were collected and stained with the previously mentioned EC markers and the following multicolour flow cytometry panel at room temperature for 15 minutes: anti-TLR4-BV786, anti-CCR5-BUV737, anti-CD80-BV650, and anti-CD86-BB515 (BD, Biosciences).

Statistical analysis

Statistical analysis of two groups and three groups were completed via Mann-Whitney U test and one-way ANOVA, respectively, by GraphPad Prism (GraphPad Software, CA, USA). P -values <0.05 were considered statistically significant.

Results

Donor characteristics

A total of 45 healthy donors including 29 males and 16 females were recruited in the study cohort. The median age of these subjects was 38 years, with a range from 18 to 64 years old. Table S1 showed the characteristics of donors in the young, middle-aged and old groups. The counts of peripheral blood cells were not statistically significant among the three groups.

Comparable frequencies but lower ROS levels in BM ECs from young donors

To clarify whether BM EC frequencies differ in young, middle-aged and old individuals, we measured BM ECs ($CD34^+CD309^+CD133^+$) by flow cytometry. Fig. 1 shows the gating strategy of BM ECs and the percentages as well as intracellular ROS levels of BM ECs among the three age groups. The percentages of primary BM ECs were comparable in the three groups (Fig. 1B, 0.12% ± 0.01% vs. 0.11% ± 0.01%, P = 0.98; 0.12% ± 0.01% vs. 0.11% ± 0.009%, P = 0.74), whereas the ROS levels in BM ECs from young donors were significantly lower than those from middle-aged donors (Fig. 1C, 1661 ± 259 vs. 2462 ± 253,P =0.02) and old donors (Fig. 1C, 1661 ± 259 vs. 2715 ± 315,P =0.005).

Characterization of primary cultured BM ECs

On the 7th day of culture, anchorage-dependent spindle-shaped cells were trypsinized and then confirmed by flow cytometry using the $CD34^+CD309^+CD133^+$markers. In addition, the spindle-shaped cells with double-positive staining of DiI-Ac-LDL and FITC-UEA-I were functionally determined to be ECs (Fig. 2C).

Improved migration and tube formation functions of BM ECs from young donors

On the 7th day of culture, BM ECs from young donors showed significantly higher abilities of tube formation (Fig. 2A, 9476 ± 746.1 vs. 5748 ± 950.1, P =0.02) and migration (Fig. 2B, 146.6 ± 5.3 vs. 110.8 ± 5.2, P =0.002) than those of cells from old donors. No significant difference was found in double-positive staining between the young and old groups (Fig. 2C, 62.2 ± 2.1 vs. 59.3 ± 1.3, P=0.48), which was in accordance with the flow cytometric EC frequency results.

ECs from young donors regulated T cells toproduce fewer proinflammatory cytokines

To investigate the effect of ECs from individuals with different ages on T cell cytokine production capacity, $CD3^+$ T cells from middle-aged donors were cocultured with ECs from young and old donors for 3 days, respectively, and then T cell subsets were examined via flow cytometry.

Fig. 3A shows the schematic diagram of the study design for the coculture of ECs with $CD3^+$ T cells. After coculture, significantly fewer type 1 T cells, including Th1 cells (Fig. 3B, 4.60% ± 1.21% vs. 17.46% ± 4.28%, P =0.001) and Tc1 cells (Fig. 3E, 5.09% ± 0.73% vs. 19.3% ± 2.86%, P =0.0006), were observed in young donors than in old donors. No statistic differences were found in the Th2 (Fig. 3C) and Tc2 cell frequencies (Fig. 3F) between the two groups. Therefore, the ratios of Th1/Th2 cells (Fig. 3D, 1.15 ±0.35 vs. 3.68 ± 0.94, P =0.01) and Tc1/Tc2 cells (Fig. 3G, 1.33 ± 0.36 vs. 3.43 ± 0.79, P =0.02) were notably less in young group than in old group. No significant differences were observed in $CD4^+/CD8^+$ T cell ratio (Fig. 3H), Th17 (Fig. 3I) or Tregs (Fig. 3J) between the two groups.

Upregulated cytokine-mediated signalling pathway and T cell activation regulatory genes in old ECs

To clarify the possible mechanism by which ECs from young and old donors cause different effects on T cell subsets, RNA-seq analyses were performed in ECs from 3 young donors and 3 old donors that were cultured for 7 days (Fig. 4A). Among the 30,331 genes in the volcano plot (Fig. 4B), 975 genes had lower

expression, and 688 genes had higher expression in the old ECs compared to the young ECs. GO term enrichment analysis indicated that genes related to neutrophil activation involved in the immune response were upregulated in old ECs (Fig. S1), suggesting that old ECs participated in more immune cell activation than young ECs. GO enrichment analysis also showed the top 10 biological processes enriched by upregulated genes in ECs from young and old donors, and the results indicated that the cytokine-mediated signalling pathway and regulation of the T cell activation process were significantly upregulated in the old group (Fig. 4C). The size of each circle indicates the ratio of differentially expressed gene counts to the total gene counts of the term. Heatmaps show that the expression of genes involved in these two biological processes, including TLR1, TLR2, costimulatory molecules such as CD86, chemokines and chemokine receptors such as C-X-C chemokine ligand 10 (CXCL10), C-C chemokine receptor (CCR) 2 and CCR5, and inflammatory cytokines such as perforin 1 (PRF1) and NOD-like receptor protein 3 (NLRP3), was upregulated in old ECs compared with young ECs (Fig. 4D).

To confirm the RNA-seq results, we further compared the relative mRNA expression of the aforementioned genes in young and old BM ECs by qRT-PCR. Table S2 shows the forward and reverse gene primers that were used for qRT-PCR. In accord with the RNA-seq results, the mRNA levels of TLR family members, including TLR2 (Fig. 4E, 1.78+-0.36-fold, P=0.04) and TLR4 (Fig. 4E, 1.92+-0.32-fold, P =0.01), CD80 (Fig. 4E, 1.3+-0.01-fold, P =0.01), CCR5 (Fig. 4E, 3.46+-1.14-fold,P =0.04), CXCL10 (Fig. 4E, 4.48+-1.29-fold, P =0.02), PRF1 (Fig. 4E, 2.24+-0.35-fold, P =0.004) and NLPR3 (Fig. 4E, 1.99+-0.46-fold, P =0.04), were significantly higher in ECs derived from the old donors than in ECs from the young donors.

Lower expression of TLR4, CCR5 and CD80 in ECs from young donors

To confirm the RNA-seq and qRT-PCR results and to further explore probable immunoregulatory mechanisms of ECs, we determined the expression of TLR4, CCR5, and costimulatory molecules such as CD80 and CD86 in young and old BM ECs by flow cytometry after 4 hours of LPS stimulation. Fig. 5 shows the representative gating strategy for the expression of TLR4, CCR5, CD80 and CD86 (left panel) and their expressed frequencies (right panel) in ECs from the young and old donor groups. The frequencies of $TLR4^+$ ECs (Fig. 5A, 4.16% +-0.82% vs. 10.39% +- 1.32%, P =0.0001), $CCR5^+$ ECs (Fig. 5B, 4.8% +-0.89% vs. 10.05% +- 1.47%, P =0.001), and $CD80^+$ ECs (Fig. 5C, 5.62% +-0.78% vs. 10.67% +- 1.25%, P =0.002) in young donors were markedly lower than those in old donors. The percentage of $CD86^+$ ECs (Fig. 5D, 13.51% +-0.98% vs. 16.53% +- 1.22%, P =0.08) was lower in young donors than in old donors, although the result was not statistically significant.

Discussion

In the current study, we found that although the percentages of BM ECs in young and old donors were comparable, BM ECs from young donors had significantly lower ROS levels and better migratory and tube-forming abilities than those from old donors, which was in accordance with previous murine and human circulating blood studies about EC ageing[33, 34]. Moreover, young ECs regulated T cells differentiation into more balanced Th1, Th2, Tc1 and Tc2 cells than old ECs. Reduced levels of T cell activation molecules and inflammatory cytokines in young BM ECs may be the possible mechanism underlying of the balanced immunomodulatory effects of young ECs (Fig. 6). Studies have shown that ECs can stimulate purified T cell proliferation without the involvement of other antigen-presenting cells, as ECs express major histocompatibility complex class I and II molecules and innate immune receptors and secrete inflammatory mediators[18, 35, 36]. Human ECs were found to induce programmed death ligand-1 expression on Tregs and thus enhance the suppressive ability of Tregs[15]. One murine study demonstrated pretreatment of EC with palmitoyl-3-cysteine-serine-lysine-4 (TLR1/2 ligand) reverted the suppressive properties of T cells and triggered virus-specific $CD8^+$ T cell immunity[17]. Notably, destruction of the EC barrier triggered T cell costimulation and led to the activation of immune effectors such as Th1 and Tc1 cells and the release of inflammatory mediators to destroy infected cells[14]. In the present study, young BM ECs exhibited lower expression of T cell activation receptors such as TLRs, fewer costimulatory molecules such as CD80, fewer chemokine receptors and ligands such as CCR5 and CXCL10, and fewer inflammatory cytokines such as PRF1 and NLRP3 than old BM ECs. Therefore, combining previous reports[14-18, 35, 36] and the current

study, we hypothesize that the different immunomodulatory effects of ECs from young and old individuals may lead to T cell subset alterations with ageing. Furthermore, the increased TLR signalling pathways and costimulatory molecules and chemokines in ECs from older individuals might be one of the underlying reasons for the imbalance in effector T cell subsets.

T cell subset disparities have been found to affect the incidence of acute graft-versus-host disease (aGVHD), a common and life-threatening complication after allogeneic haematopoietic stem cell transplantation[37-41]. Numerous studies have shown that the occurrence of aGVHD was related to increased frequencies of donor Th1 and Tc1 cells and reduced frequencies of Th2 and Tc2 cells[37, 38, 41]. Furthermore, we recently reported that young donors had lower percentages of Th1 and Tc1 cells than old donors[5], which could partly explain why patients receiving transplants from young donors showed less aGVHD than those who received transplants from old donors[42, 43]. However, the in-depth reason why the aGVHD incidence was affected by donor age remains to be explored. It is worth noting that our study and other previous studies indicated that the number and function of ECs were damaged, EC ROS levels were increased in aGVHD patients, and EC destruction could predict the severity of aGVHD[23, 44]. Moreover, our current data demonstrated that ECs from younger donors could instruct T cells to generate more balanced Th1/Th2 and Tc1/Tc2 cell ratios. Thus, we hypothesize that the different effects on T cells by ECs from donors of different ages might affect the occurrence of aGVHD in patients after allo-HSCT.

We are aware, however, that the mechanism by which T cell subsets change with ageing is heterogeneous. It would be more rigorous and informative to further explore whether T cell activation molecules in BM ECs in donors of different ages directly impact effector T cell subset differentiation.

In summary, the current study indicated lower EC ROS levels, better BM EC functions, and more balanced T cell modulating effects of BM ECs from young donors than from old donors. Our preliminary data showed that lower TLR signalling and costimulatory molecules as well as chemokine and receptor expression, and less inflammatory cytokine secretion in the ECs of young individuals might cause young ECs to induce more balanced T cell subset differentiation than old ECs. Although further validation is required, our results may provide a new prospective to better understand the mechanism of effector T cell subset alterations with ageing.

Data availability statement

The data presented in this manuscript are included in the paper and its Supplementary information.

Conflict of interest disclosure

The authors declare no commercial or financial conflict of interests.

Author contributions

YK designed the study and supervised the manuscript preparation. SQT and WLY performed the research and analysed the data, and contributed equally to this work. WLY and YK wrote the manuscript. All other authors participated in the collection of patient data. All of the authors agreed to submit the final manuscript.

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Figure Legends

Fig 1. Comparable frequencies but lower ROS levels in BM ECs from young donors than from old donors. Representative gating strategy for BM ECs ($CD34^+CD309^+CD133^+$) from healthy donors **(A)** . Frequencies of BM ECs in BMMNCs**(B)** and intercellular ROS levels in BM ECs **(C)** among young, middle-aged and old donors. The data are showed as the mean +- standard error of the mean (SEM).

Fig 2. Better migration and tube formation functions of BM ECs from young donors than from old donors. Representative tube formation images and tube length of 7-day primary cultured BM ECs (magnification x4) in the young and old donor groups **(A)** . Typical figures of the transwell migration assay and counts of the migrated BM ECs (magnification x10) from young and old donors**(B)** . Representative images of double-positive-stained ECs (yellow) with DiI–Ac-LDL uptake (red) and FITC–UEA-I binding (green) and the double-positive-stained cell numbers (magnification x10) in the young and old donor groups **(C)** .

Fig 3. ECs from young donors regulated T cells to produce fewer proinflammatory cytokines. Schematic diagram of the study design of the coculture of ECs and $CD3^+$ T cells and the measurement of effector T subsets after 3 days of coculture **(A)** . The frequencies of BM Th1 ($CD3^+CD8^-IFN\text{-}\gamma^+$)**(B)** , Th2 ($CD3^+CD8^-IL\text{-}4^+$)**(C)** , Tc1 ($CD3^+CD8^+IFN\text{-}\gamma^+$)**(E)** , Tc2 ($CD3^+CD8^+IL\text{-}4^+$)**(F)** , Th17 ($CD3^+CD8^-IL\text{-}17^+$)**(I)** and Tregs ($CD3^+CD8^-CD25^+Foxp3^+$)**(J)** in young and old donors. The ratios of BM Th1/Th2**(D)** , Tc1/Tc2 **(G)** , $CD4^+/CD8^+$ T cells **(H)** in young and old donors.

Fig 4. Upregulated genes related to the cytokine-mediated signalling pathway and the regulation of T cell activation in BM ECs from old donors. Schematic diagram of the study design for RNA-seq analysis of ECs from donors **(A)** . Volcano plots of the downregulated genes (red) and upregulated genes (green) in the old group and comparable genes (blue) between the two groups **(B)** . GO enrichment analysis shows the top 10 biological processes enriched by upregulated genes in ECs from young and old donors **(C)** . Heatmaps show genes expression involved in the regulation of T cell activation and cytokine-mediated signalling pathways, including TLR1, TLR2, TLR4, CD80, CD86, CXCL10, CCR2, CCR5, PRF1, and NLRP3, in young and old ECs (scaled by row) **(D)** . The relative mRNA expression levels of aforementioned genes in ECs from young and old donors tested by qRT-PCR **(E)** . $^*P < 0.05$, $^{**}P < 0.005$.

Fig 5. Lower expression of TLR4, CCR5 and CD80 in ECs from young donors than from old donors. The representative gating strategy and frequencies of $TLR4^+$ ECs **(A)** , $CCR5^+$ ECs**(B)** , $CD80^+$ ECs **(C)** , and $CD86^+$ ECs **(D)** from the young and old donors with LPS stimulation.

Fig 6. Graphical abstract of the current study. BM ECs from young donors had lower ROS levels, better functions, and more balanced T cell modulating effects than ECs from old donors. Lower TLR signaling and costimulatory molecules, and less inflammatory and chemotaxis cytokine secretion in ECs from young donors might cause young ECs to induce more balanced T cell subset differentiation than old ECs.

Figure 1

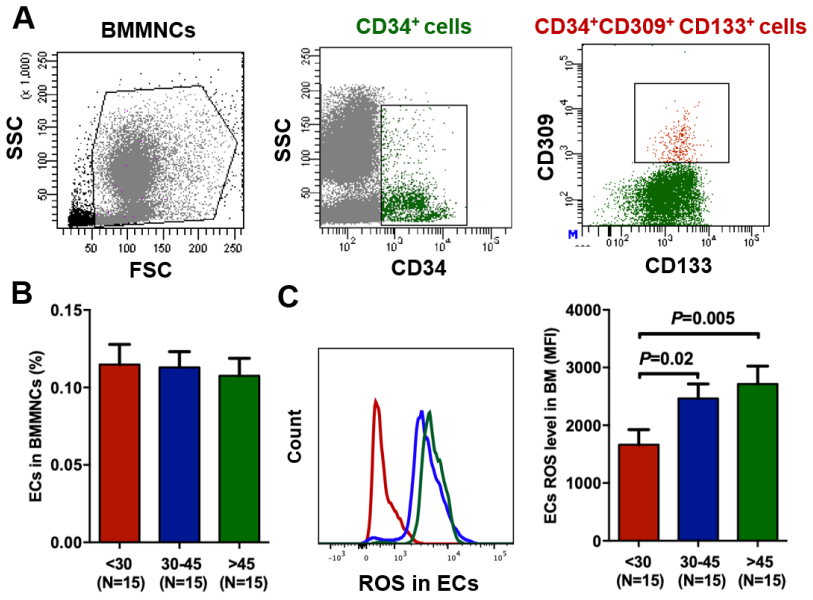

Figure 2

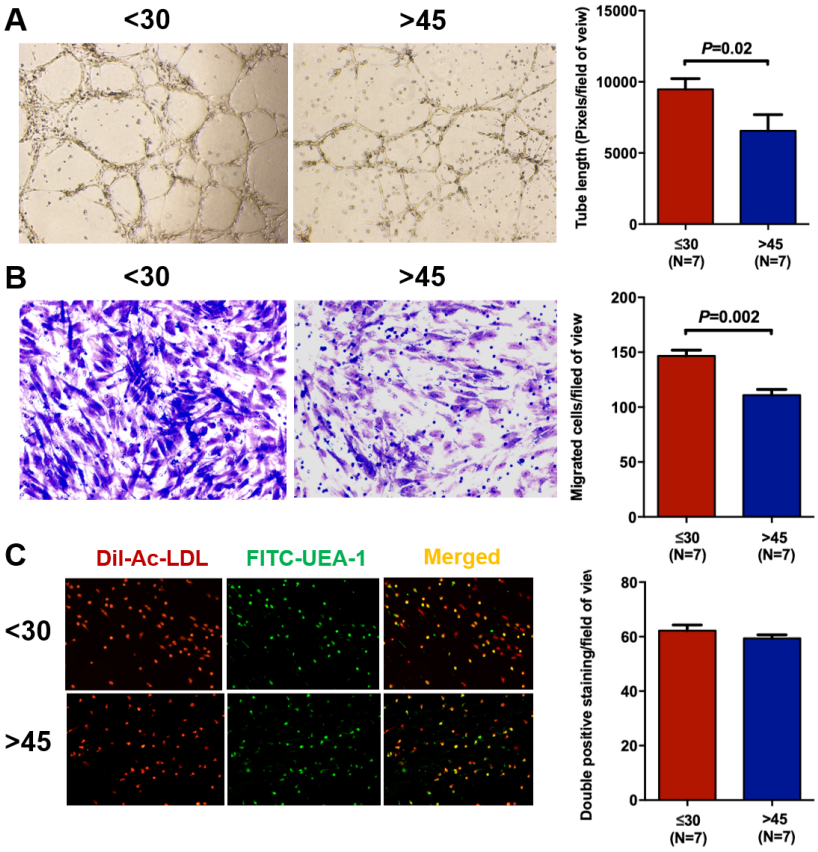

Figure 3

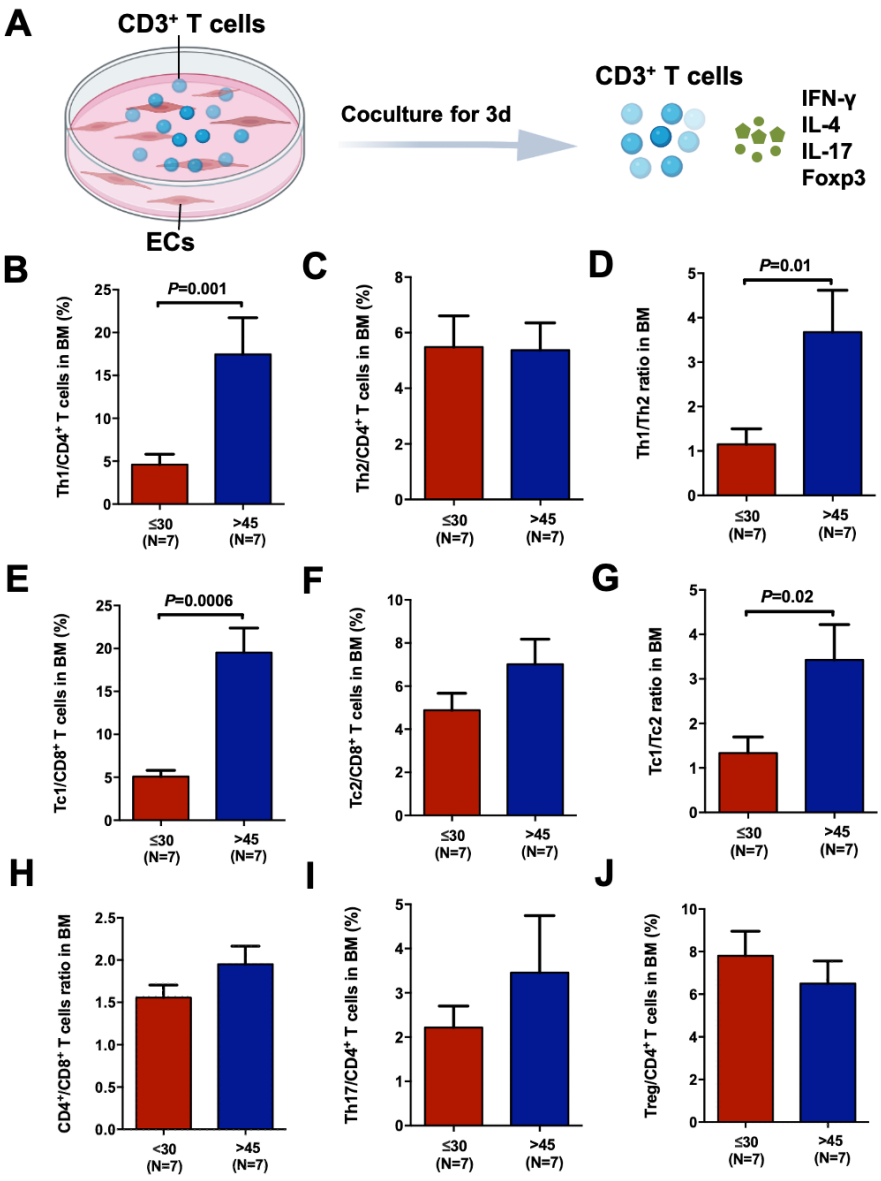

Figure 4

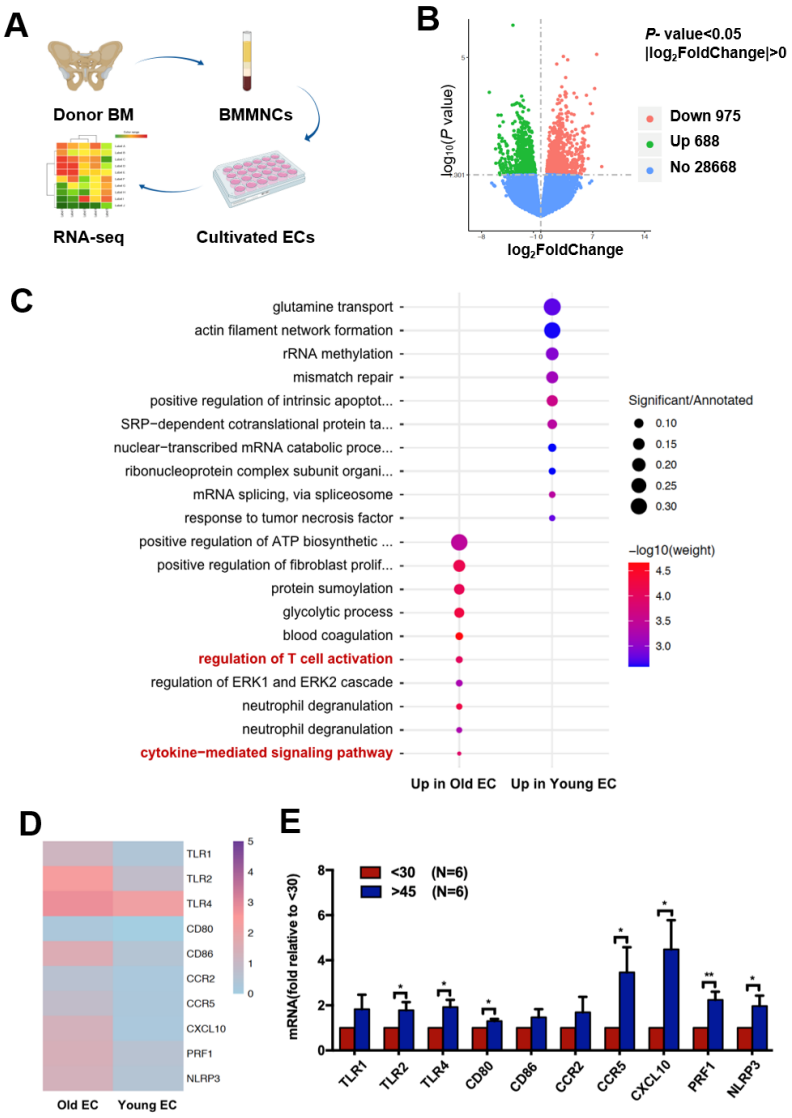

Figure 5

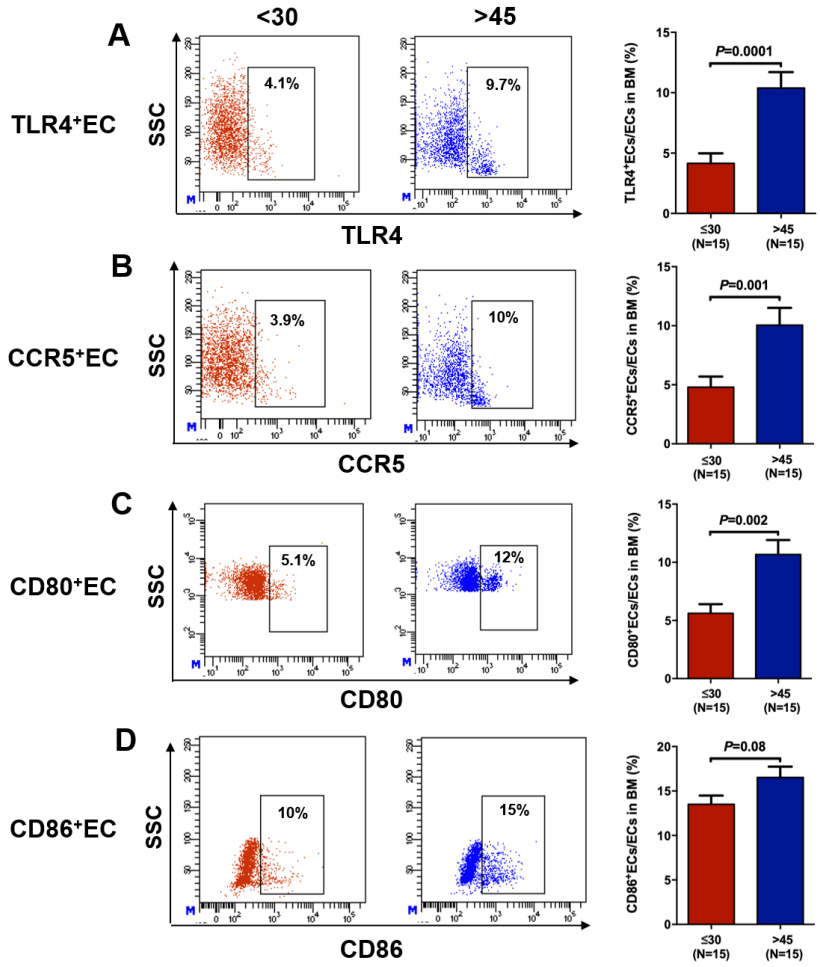

Figure 6