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ORIGINAL ARTICLE

CD5L Secreted by Macrophage on Atherosclerosis Progression Based on Lipid Metabolism Induced Inflammatory Damage

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Abstract

To explore the molecular mechanism of exosomal protein CD5L secreted by macrophage to promote the progression of atherosclerosis. Twenty cases of patients with atherosclerosis (AS) and 20 cases of healthy subjects were collected. Morphological properties of exosomes were identified by transmission electron microscopy, and the marker proteins CD63 and CD81 of exosomes were measured by Western blot. The secretion of inflammatory factors in the blood supernatant were analyzed by ELISA. Atherosclerosis cell models were established by transwell and separated into three groups: first group was treated with exosome inhibitor (GW4869), second group was injected with CD5L protein and third group was model control. Morphological properties of exosomes were identified by transmission electron microscopy, and the marker proteins CD63 and CD81 of exosomes were measured by Western blot. The levels of TNF-α, IL-1β, IL-6, IL-13, IL-17A, IL-31 in the cells were analyzed by ELISA. Analysis of the expression and distribution of IL-17RA in vascular smooth muscle cells by immunofluorescence. The proteins of CD63, CD81, CD5L were high expressed in AS group compared to healthy subject group. Cell test results showed that protein levels of CD63, CD81, CD5L in AS group were much higher than that in normal group. Immunofluorescence showed that the expression level of IL-17RA in cell membrane was the highest in the AS model group, and the expression of IL-17RA was decreased in GW4869 group and CD5L group. Expression of inflammatory factors in AS was much higher than that in GW4869 group and CD5L group. The exosomal protein CD5L secreted by macrophage promotes the development of atherosclerosis based on lipid metabolism-induced inflammatory damage of vascular smooth muscle cells.

Keywords Atherosclerosis · Exosomes · Protein CD5L · Inflammatory damage · Vascular smooth muscle cells · Mechanism

Introduction

Atherosclerosis (AS) is usually regarded as a chronic inflammatory disease. The common characteristics of arteriosclerosis are thickening and hardening of the arterial wall, weakening of elasticity and narrowing of the lumen (Cui et al. 2020). At present, the pathogenesis of AS is still inconclusive. The occurrence of inflammation and abnormal lipid metabolism are the common basis of pathophysiological changes during the development of atherosclerosis. Virchow proposed in 1863 that the lesions of AS are mainly caused by increased plasma lipid levels (Wang et al. 2018), and their essence was the occurrence of inflammation caused by lipid attaching to artery walls with plasma. Inflammatory response is the main reason that stimulates the differentiation of fibrous tissue and causes the formation of atherosclerotic plaque (Kosmala and Marwick 2017).

With the development of biological function research, more and more studies have confirmed that the injury of vascular smooth muscle cell (VSMCs) is one of the important pathogenesis of atherosclerosis. In healthy arteries, vascular smooth muscle cells, located in the middle layer, are responsible for artery contraction and production of extracellular matrix (ECM), and play an important role in maintaining arterial elasticity to better adapt to changes in hemodynamics. In atherosclerosis, VSMCs are involved in remodeling of arterial wall to maintain blood flow in affected vessels

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due to atherosclerotic changes (Chistiakov et al. 2015). The development of AS is largely due to the accumulation of lipids and inflammatory substances in the vascular wall, resulting in apoptosis of cells such vascular smooth muscle cells (Basatemur et al. 2019). The pathological mechanism of atherosclerosis is mainly that abnormal lipid metabolism induces inflammatory activation, damages vascular smooth muscle cells, and leads to vascular smooth muscle injury, lipid accumulation, platelet aggregation, inflammatory cell aggregation, and formation of plaque.

Exosomes are nanoscale membrane particles secreted by cells, with the diameter of 30–120 nm. Exosomes form in the endosome system and are released into the extracellular domain in the form of exocrine secretion after fusion with the membrane. Exosomes can convey cellular information and participate in the regulation of cell physiological activities in AS. Exosomes as a subgroup of cell-released extracellular vesicles, promote the exchange of peptides, microRNA, mRNA and mitochondrial DNA in cells and tissues, and can be used as diagnostic markers of various cardiovascular diseases (Villasante et al. 2016). There are large of exosomes derived from immune cells, platelets, vascular smooth muscle cells in lesion of AS (Libby et al. 2019). Studies have shown that exosomes play an essential role in various stages of AS occurrence and development, and mediate inflammation, oxidative stress and apoptosis in AS (Abels and Breakefield 2016; Wang et al. 2019).

The exosome protein CD5L secreted by macrophage, a protein that inhibits macrophage apoptosis, has the function of regulating lipid metabolism and participates in the inflammatory process of atherosclerosis. Therefore, this study explored the mechanism of the protein CD5L involved in inflammatory signaling molecules to regulate inflammatory damage of vascular smooth muscle cells and promote the progression of atherosclerosis.

Materials and Methods

Materials

SYBR Green, PCR Kit were purchased from Innochem (Beijing, China); Trizol (invitrogen); phosphate buffer saline (PBS), glutaraldehyde, 3% uranium acetate were purchased from Gibco (Shanghai, China); Anti-CD63 (1:1000), Anti-CD81 (1:1000) were purchased from Therm; hematoxylin–eosin, 4% paraformaldehyde, anticoagulation, exosome inhibitor GW4869, CD5L protein were purchased from Inochem (Beijing, China); Transmission electron microscope (TEM; Tecnai G2 F20 S-TWIN, FEI, Hillsboro, OR, USA); Real-time PCR detector (ABI company); Cryogenic refrigerating centrifuge TG-16 M (Shanghai Luxiangyi centrifuge Instrument Co. LTD); Synergy HTX Multi-Reader (BioTek, USA).

Methods

Morphological Characteristics of Exosomes

Samples Twenty cases of AS patients (average age: 63.8 ± 5.6 years; 12 males and 8 females) and 20 cases of healthy subjects (NC) (average age: 60.22 ± 7.6 years; 12 males and 8 females) were collected from the physical examination center of our hospital. Inclusion criteria: Carotid ultrasound showed abnormal intima-media thickness (≥ 0.9). The atheromatous plaque is locally elevated. Exclusion criteria: valvular heart disease, severe arrhythmia, diabetes, malignancy, and severe hepatic and renal insufficiency.

Venous blood from the elbow (5 mL) of every subject was collected in EP tube without anticoagulant and was treated within 1 h. After extraction, the blood samples were cooled at 4 °C for 1 h, and then centrifuged at 5000 rpm/min for 10 min. The supernatant (serum) was resuspended with 100 μL PBS, and transferred to a sterile EP tube and stored at –80 °C. This research was performed according to the protocol agreed by the clinical Research Ethics Committee of our hospital (No. 201902063). The animal experiment was agreed with Animal Ethics Committee ((No. 20190131).

Transmission Electron Microscopy Identification The separated and purified exosome suspension was diluted with PBS to a concentration of 100 μg/mL and fixed with glutaraldehyde. Twenty μL exosome solution dropped onto the copper mesh. After about 20 s, use a filter paper to absorb the excess exosome solution from the edge of the copper mesh. Drop 3% uranium acetate dye on cling film, negative dye for 5 min at room temperature, and dry by incandescent lamp. The copper mesh was placed on the carrier rod in the sample room of transmission electron microscope for observation and photography.

Detection of Protein Molecular Markers of Exosomes

A one hundred μL separated and purified exosomes were taken and added the same volume of RIPA lysis buffer. The mixture was lysed at 4 °C for 30 min, and centrifuged at 13,000 rpm/min for 20 min. The supernatant was added loading buffer (5×) to boil for 10 min. 12% SDS polyacrylamide gel was prepared according to the target molecular protein. Follow the western blot procedure above to perform sample loading, electrophoresis, membrane transfer and sealing successively. Rabbit anti-CD63 (1:1000) and rabbit anti-CD81 (1:1000) were incubated at 4 °C for 12 h.

The corresponding secondary antibody was incubated and exposed after the film being washed the next day.

Content of TNF-α, IL-1β, IL-6, IL-13, IL-17A and IL-31

Blood samples were collected of 20 healthy subjects and 20 patients with atherosclerosis, and the contents of inflammatory factors in the blood supernatant were analyzed by ELISA. The specific procedure was carried out according to the instructions. Absorbance (OD value) was measured at 450 nm with an enzyme micrometer. The contents of TNF-α, IL-1β, IL-6, IL-13, IL-17A and IL-31 in the samples were calculated using the standard curve.

Establishment of Rat Model

Mice were fed with high fat diet to establish atherosclerosis mouse model. There were 12 mice in each group. Three model groups were established. At the same time, one group of mice was set as normal group. In addition, one model group was given exosome inhibitor (GW4869) and the other model group was given CD5L antibody by tail vein injection. After seven days of treatment, the blood and tissue were collected, and the morphological characteristics of exosomes in the supernatant were identified by transmission electron microscopy.

Detection of Protein CD5L, CD63 and CD81

Western blot was used to analyze the differential expressions of CD5L in samples, and surface specific marker proteins CD63 and CD81. The specific procedure was described in *Detection of Protein Molecular Markers of Exosomes*.

Immunofluorescence Assay

Immunofluorescence was used to analyze the expression and distribution of IL-17RA qin vascular smooth muscle cell of mice.

Inflammatory Factors were Analyzed by ELISA

Inflammatory factors including TNF-α, IL-1, IL-6, IL-13, IL-17A and IL-31 in the blood were analyzed by ELISA, and the specific procedure was described as 2.2.3.

Data Analysis

All analyses were performed using Prism version 8.0 (GraphPad Software Inc., La Jolla, CA, USA). Statistical comparisons of repeated measures and multiple groups were determined by One-way ANOVA with the Tukey's post hoc test. The statistical comparisons between two groups of continuous variables were determined by two-tailed Student's t-test. Data were presented as mean ± SD. $P < 0.05$ was considered statistically significant.

Results

Morphological Characteristics of Exosomes

No exosomes were detected in blood samples of normal healthy subjects (NC group), but exosomes were significantly measured in blood samples of patients with atherosclerosis (AS group). The exosomes observed by TEM were usually saucer shaped or hemispherical in one depression, as shown by the arrow in Fig. 1.

Detection of Protein Molecular Markers of Exosomes

Exosome biomarker protein CD63 and CD81 of the collected exosomes were detected by Western blot (Erozenci et al. 2020). As shown in Fig. 2, CD63 and CD81 were expressed in the extracted exosomes, which proved this method could successfully obtain exosomes. The expression of exosomes surface specific marker proteins CD63 and CD81 obviously increased in AS group compared with NC group, $^{*}P < 0.05$. However, no exosomal protein CD5L

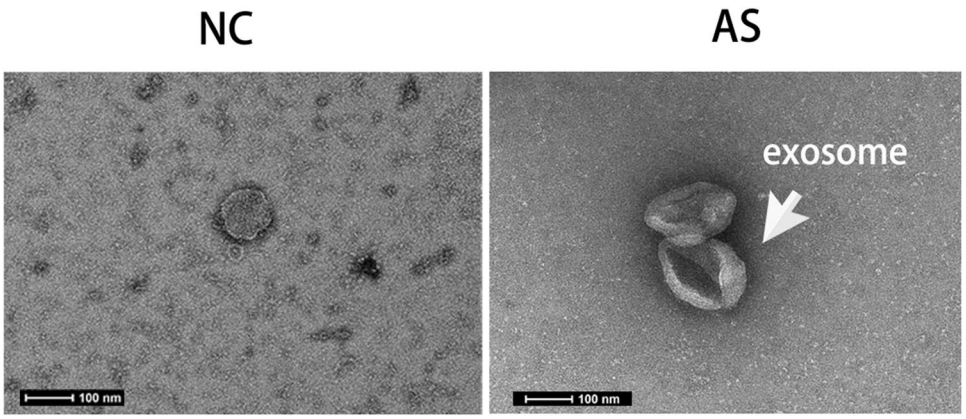

Fig. 1 Scanning electron microscopy of exosomes. Left: NC group. Right: AS group, the exosome was shown in white arrow

expression was found in blood samples of normal subjects. The expression of CD5L protein was obvious in AS group and exhibited a significant difference $^{***}P<0.001$.

Contents of TNF-α, IL-1β, IL-6, IL-13, IL-17A and IL-31

Contents of TNF-α, IL-1β, IL-6, IL-13, IL-17A and IL-31 was shown in Table 1 and Fig. 3. There was a significant difference, $^{**}P<0.01$, compared with normal group.

Morphological Characteristics and Markers Detection of Exosomes

Exosomes were photographed in the blood of mice model group, as shown by the arrows in Fig. 4. Exosomes were not observed in normal group, GW4869 group and CD5L group. Exosome surface specific marker proteins CD63 and CD81 were also not detected in the results of Western blot (Fig. 5).

Immunofluorescence Assay

Immunofluorescence was used to analyze the expression and distribution of IL-17RA in vascular smooth muscle cell of mice, as shown in Fig. 6. The expression level of IL-17RA in cell membrane was the highest in the AS model group, and the second group was NC group. The expression of IL-17RA was decreased in GW4869 group and CD5L group.

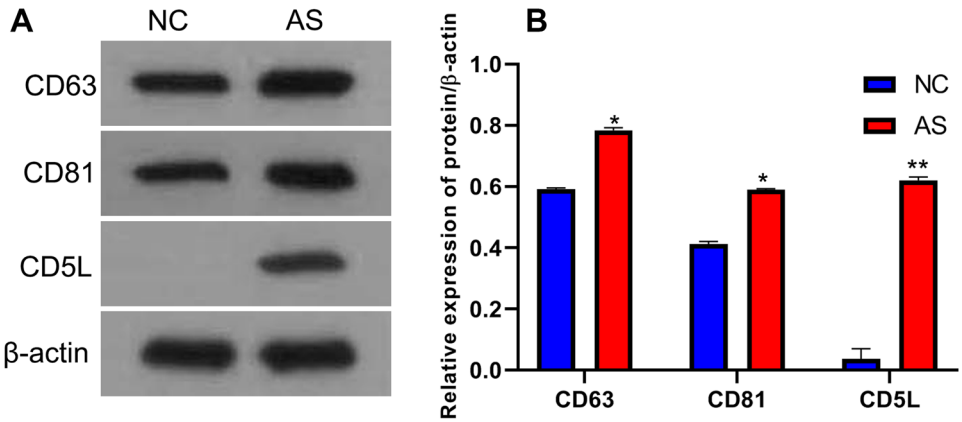

Fig. 2 Protein levels of CD63, CD81, CD5L in normal group and AS group determined by Western blotting (mean ± SD; $n=3$). **a** Gray value; **b** Statistic analysis. Compared with NC group, $^{*}P<0.05$, $^{***}P<0.001$

Table 1 Contents of TNF-α, IL-1β, IL-6, IL-13, IL-17A and IL-31 in normal group and AS model group

Name	Normal group	Intra-assay CV%	AS model group	Intra-assay CV%	Intra-assay CV%
TNF-α	133.32 ± 11.29 ng/L	0.085	196.14 ± 11.17 ng/L	0.057	0.032
IL-1β	5.95 ± 0.61 ng/L	0.103	10.07 ± 1.66 ng/L	0.165	0.046
IL-6	1.75 ± 0.22 ng/L	0.126	4.67 ± 0.19 ng/L	0.041	0.124
IL-13	11.18 ± 0.51 ng/L	0.046	19.23 ± 1.89 ng/L	0.098	0.068
IL-17A	4.96 ± 0.30 ng/L	0.060	8.70 ± 0.67 ng/L	0.077	0.021
IL-31	21.92 ± 1.48 ng/L	0.068	41.57 ± 5.04 ng/L	0.121	0.073

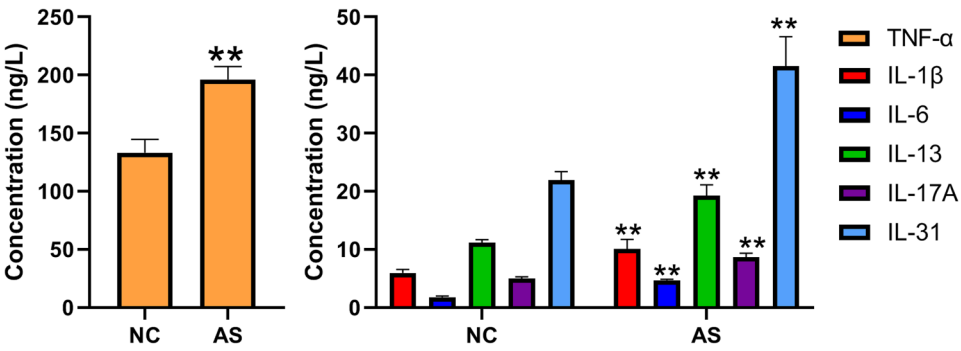

Fig. 3 Contents of TNF-α, IL-1β, IL-6, IL-13, IL-17A and IL-31 (ng/L). Compare with NC group, $^{**}P<0.01$

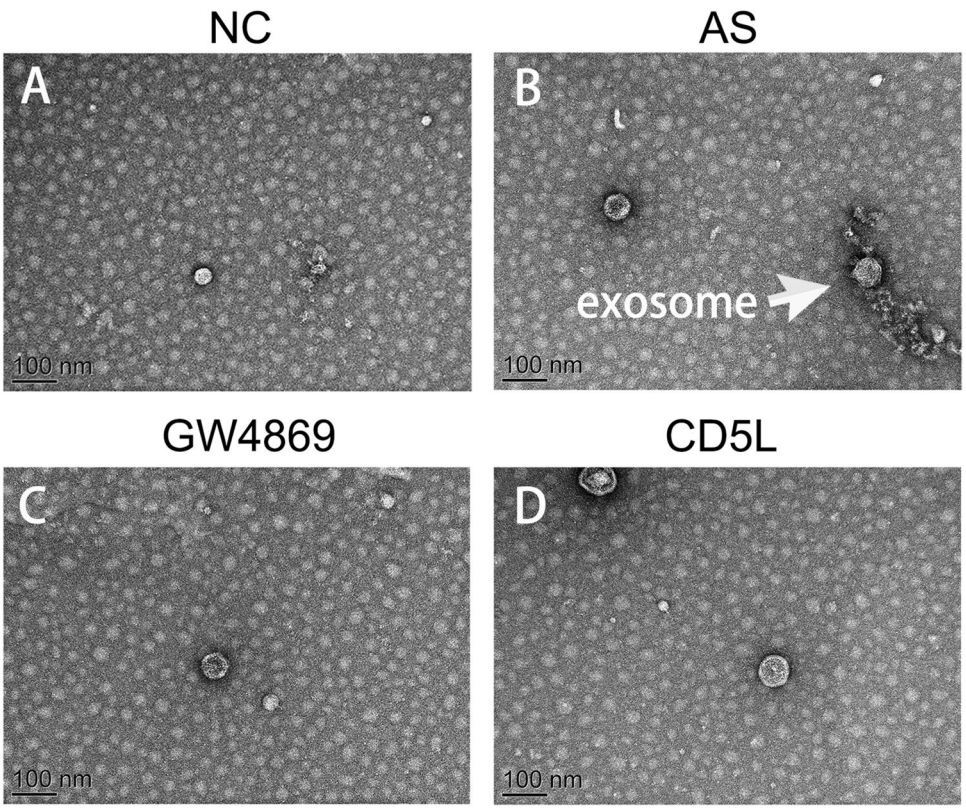

Fig. 4 Scanning electron microscopy of exosomes. **A** NC group. **B** AS group, **C** GW4869 group; **D** CD5L group. The exosome was showed in white arrow

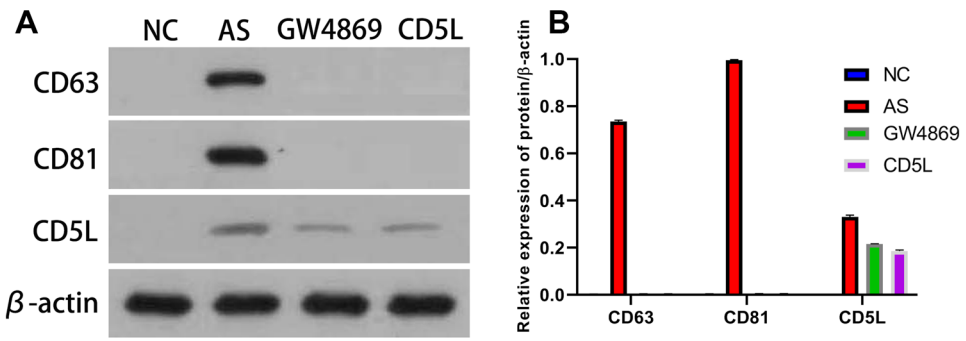

Fig. 5 Protein levels of CD63, CD81, CD5L in normal group and AS group determined by Western blotting (mean ± SD, $n = 3$). **A** Gray value; **B** Statistic analysis

Expression of Inflammatory Factors in the Blood of Rats

TNF-α in normal group was 258.01 ± 4.57 ng/L, AS group was 519.98 ± 83.59 ng/L, GW4869 group was 462.86 ± 47.23 ng/L, CD5L group was 391.30 ± 24.84 ng/L, compared with NC group, $^{**}P < 0.01$, compared with AS group, $^{\#}P < 0.01$; IL-1β in normal group was 91.38 ± 23.90 ng/L, AS group was 181.12 ± 1.66 ng/L, GW4869 group was 154.70 ± 35.48 ng/L, CD5L group was 129.32 ± 42.07 ng/L, $^{**}P < 0.01$, compared with AS group, $^{\#}P < 0.01$; IL-6 in normal group was 42.49 ± 16.61 ng/L, AS group was 89.15 ± 30.32 ng/L, GW4869 group was 82.06 ± 21.47 ng/L; CD5L was 82.06 ± 21.47 ng/L; compared with NC group, $^{*}P < 0.05$, $^{**}P < 0.01$, compared with AS group, $^{\#}P < 0.01$; IL-13 in normal group was 16.64 ± 5.15 ng/L, AS group was 30.32 ± 8.68 ng/L; GW4869 group was 24.27 ± 5.05 ng/L; CD5L group was 25.00 ± 8.05 ng/L; compared with NC group, $^{**}P < 0.01$, compared with AS group, $^{\#}P < 0.01$; IL-17A in normal group was 33.49 ± 15.55 ng/L, AS group was 82.15 ± 27.00 ng/L, GW4869 group was 70.38 ± 20.56 ng/L; CD5L group was 68.49 ± 23.77 ng/L; compared with NC group, $^{**}P < 0.01$, compared with AS group, $^{\#}P < 0.01$; IL-31 in normal group was 266.12 ± 66.07 pg/mL, AS group was 492.48 ± 190.85 pg/mL, GW4869 group was 492.48 ± 190.85 pg/mL, CD5L group was 437.39 ± 129.75 pg/mL; compared with NC group, $^{**}P < 0.01$, compared with AS group, $^{\#}P < 0.01$, and there is a significant difference (Fig. 7).

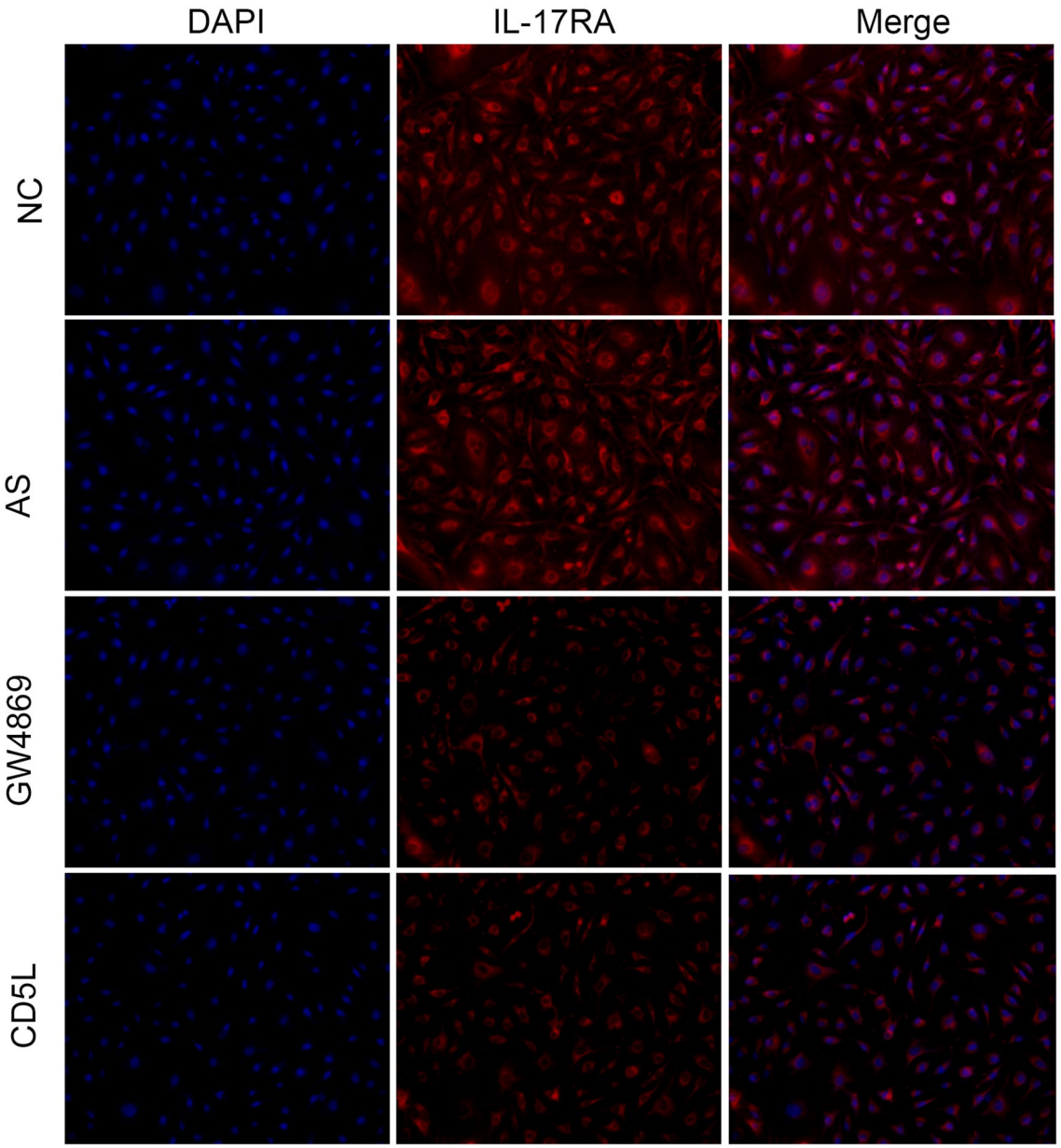

Fig. 6 Analysis of the expression and distribution of IL-17RA in vascular smooth muscle cells by immunofluorescence

Discussion

Exosomes are the kind of extracellular vesicles with the size of 30–150 nm and are widely found in cell culture supernatants and various body fluids, such as blood, lymph, urine, saliva, and latex. Exosomes are involved in intercellular material transport and information transmission and regulate cellular physiological activities (Zhu et al. 2020).

GW4869 is a neutral sphinolipase inhibitor with cellular permeability, specificity and non-competitiveness. It can block the budding of polyvesicles mediated by ceramide and thus inhibit the release of exosomes from polyvesicles, and also is a drug commonly used to inhibit the production of exosomes. No exosomes surface specific marker proteins CD63 and CD81 were detected in the GW4869 group, indicating that GW4869 could significantly inhibit the release of exosomes from polyvesicles.

CD5L protein is identified as a secreted protein produced by macrophages in 1997 (Yang et al. 2021). Subsequently, CD5L protein was called macrophage apoptotic inhibitor due to the discovery of its anti-apoptotic effect on immune cells (Lai et al. 2018). CD5L is a member of the cysteine-rich scavenger receptor superfamily (Lai et al. 2018). In blood, CD5L binds to immunoglobulin M pentmer (Kai

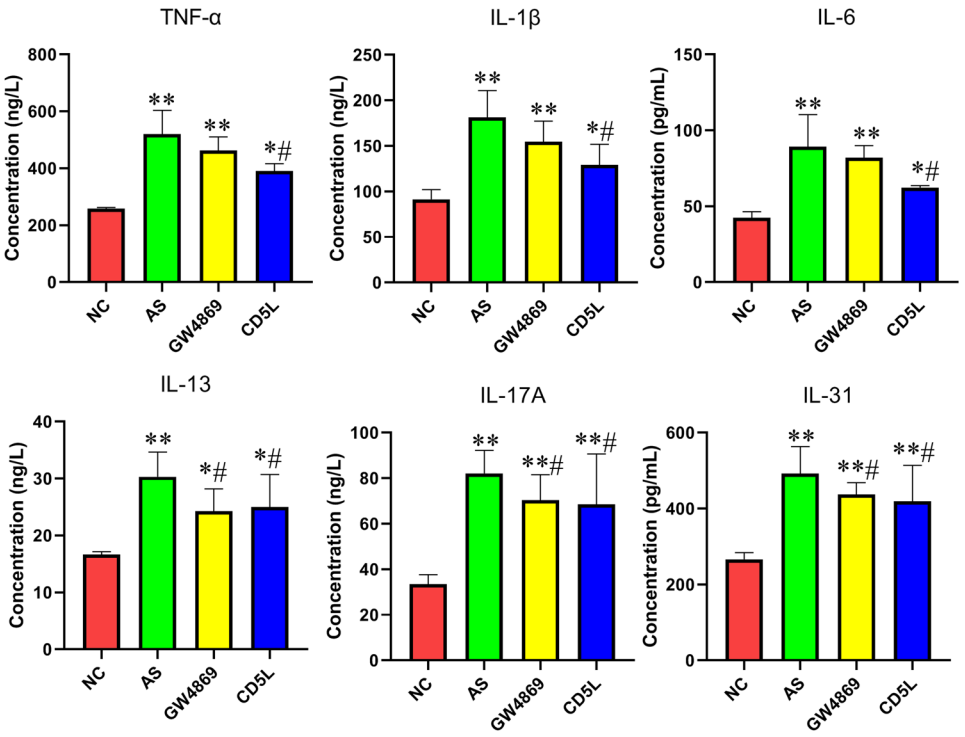

Fig. 7 Contents of TNF-α, IL-1β, IL-6, IL-13, IL-17A, IL-31 (ng/L). Compare with NC group, $^{**}P < 0.01$; compared with AS group, $^{\#}P < 0.05$

et al. 2014), which makes it less easily excreted in urine, and maintains a high concentration in the blood (Arai et al. 2013). CD5L can inhibit CD4/CD8 double-positive T cell death and CD95/Fas cross-linked cell apoptosis and inhibit the apoptosis of NKT and T cells and the apoptosis of macrophages induced by various pathogens (Kuwata et al. 2003).

CD5L is a newly discovered component that plays a key role in the immune system. Subsequently, it was also known as AIM or macrophage apoptosis inhibitor due to its anti-apoptotic effect on leukocytes (Miyazaki et al. 1999). Studies during the past decade have shown that CD5L protein has inflammatory responses that regulate leukocyte range migration and control lipid metabolism balance. Macrophages are the main source of CD5L, and the expression of CD5L is up-regulated in the body under inflammatory conditions, such as cardiovascular diseases and atherosclerotic lesions (Arai et al. 2005; Kurokawa et al. 2010). Figure 5 shows that CD5L is highly expressed in atherosclerosis, indicating that CD5L is involved in the pathogenesis of atherosclerosis.

The sample size is too small to represent the characteristics of clinical big data, and there may be differences in population studies. Moreover, this research is only limited to animal models. There are still certain differences and differences between cells and animals in physiological structure. Therefore, this experiment should further study.

In conclusion, the exosomal protein CD5L secreted by macrophage promotes the development of atherosclerosis based on lipid metabolism-induced inflammatory damage of vascular smooth muscle cells of mice.

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Data Availability The data can be obtained on request.

Declarations

Conflict of interest There are no any competing interests.

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