Exogenous dendritic cells aggravate atherosclerosis via P-selectin/PSGL-1 pathway

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Abstract: Studies have found that a large number of inflammatory cells, P-selectin, and mature dendritic cells (DCs) are expressed in the damaged and shoulder parts of atherosclerotic plaque, which demonstrates that P-selectin and mature DCs participate in the immune inflammatory response leading to the development of atherosclerosis. However, it is unclear how the above factors interact in this setting. In this study, we investigated the role of P-selectin and its receptor, P-selectin glycoprotein ligand (PSGL)-1 in atherosclerosis, with the finding that DC surface marker expression was consistently high in the P-selectin group while consistently low in the PGSL-1 + DCs group, with CD40 and CD86 expressed by 3.84% and 2.05% for the latter. The highest expression of CD80, CD83, and MHC II was discovered in the DC group, at 7.49%, 3.68%, and 8.98%, respectively. Results of this study are similar to those obtained previously by Ye et al. (2017), which showed larger atherosclerotic lesions in mice that received exogenous DCs, compared with those treated with PBS. In this study, the greatest level of atherosclerosis, fibrosis, and lipid deposition was also seen in mice that received exogenous DCs.

Introduction

Atherosclerosis (AS) is a chronic and progressive immune inflammatory cardiovascular disease (Ross, 1999; Liuzzo, 2001; Bobryshev, 2005; Bauriedel et al., 2006; Hansson and Libby, 2006; Mayerl et al., 2006; Webb and Moore, 2007; Hansson, 2009; Bobryshev, 2010; Libby, 2012a; Moore et al., 2013; Tabas and Bornfeldt, 2013; Gistera and Hansson, 2017). Lipid deposition in the subendothelial space of the arteries initiates a congenital, adaptive immune reaction, triggering a chronic and progressive inflammatory response (Lahoute et al., 2011). Low-density lipoprotein (LDL) also plays a role in the pathogenesis of atherosclerosis, with increasing levels of oxidized LDL risking patients' life with atherosclerotic cardiovascular disease (Napoli, 1997; Murphy et al., 2008; Ding et al., 2014; Ding et al., 2014; Writing Group Members et al., 2016; Schaftenaar et al., 2016; Gao and Liu, 2017).

Dendritic cells constitute a family of special antigen-presenting cells (APCs) that play a critical role as a link between innate and adaptive immune reactions, controlling the function of both B and T lymphocytes which mediate immunity (Banchereau and Steinman, 1998). In both pathological and normal intima, DCs are present in an immature state, and the process of maturation is triggered by endogenous and exogenous antigens in response to inflammatory stimulation. Upon maturation, DCs display reduced antigen capture and uptake capacity with a mounting tendency of antigen presentation and surface expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules such as CD40, CD80, CD83, and CD86 (Mellman and Steinman, 2001; Chen, 2004). In the process of antigen presentation, a co-stimulator is released to stimulate the activation of effector T cells and eventually induce the immune inflammatory response (Koltsova et al., 2012). Therefore, the maturation of DCs is a key process during the activation of naïve T cells and the mediation of the inflammatory cascade.

DCs also act at all stages of the formation of atherosclerosis in multiple ways such as lipid accumulation, foam cell formation and pro-inflammatory cytokine secretion (Bobryshev and Watanabe, 1997; Choi et al., 2009; Kruth, 2011; McLaren et al., 2011; Chistiakov et al., 2014). The specific direction of effector T cells in vivo is closely related to the current intracellular environment. Among them, regulatory T cells (Treg) majorly regulate immune tolerance, which accounts for the proportion of CD4+ T cells, indicating the degree of immune tolerance. CD4+ T cells mostly distribute in the spleen and peripheral blood, with
the proportion of T cells in them being almost the same. Clearly, the primary role of CD4+ T cells is to participate in and regulate the in vivo process of immune inflammation, nevertheless, the mechanisms that initiate Treg responses remain undetermined. It is believed that DCs take a vital part in this process (Pulendran et al., 2010).

Tregs are a special class of immunoregulatory T cells that were once considered as immunosuppressive T cells that can regulate autoimmune tolerance and the balance of endogenous and exogenous pathogens. Tregs are intermediate cells with bi-directional differentiation as a key in regulating immune tolerance and inflammatory response. In addition to Tregs, T cells such as Th1 and Th17 also secreting immunosuppressive factors (IL-10, TGF-β, and others), and these factors directly suppress immune responses. The mechanism is primarily to relieve the inflammatory response by maintaining immune tolerance to auto-antigens (Weber et al., 2008; Hristov and Weber, 2011).

Various families of adhesion molecules are involved in a cascade of interactions that lead to leukocyte binding and adherence to the endothelial and epithelial surface (Hogg and Landis, 1993). As a member of the selectin family in adhesion molecules, P-selectin is significantly involved in immune inflammation. P-selectin glycoprotein ligand-1 (PSGL-1) on the surface of mature DCs and P-selectin forms a mutual receptor relationship. P-selectin/PSGL-1 exhibits mutual recognition and mediates the maturation and migration of DCs, activating platelets and promoting thrombosis. P-selectin and PSGL-1 receptors can not only bind to each other, but also to ligands and receptors, and act in the maturation process of DCs. Both receptors increase the upregulation of TLR-4 receptors by stimulating the release of costimulatory factors of DCs, causing the presentation of antigens, activating downstream signaling pathways, and promoting the cascade effect of inflammatory immune responses (Hoshino et al., 2002). Burger and Wagner (2003) also found that the expression of P-selectin on the platelet surface of acute myocardial infarction patients was significantly higher than that of patients with stable angina pectoris, suggesting that P-selectin may be associated with the stability of atherosclerotic plaque.

As previously mentioned, upon the occurrence of immune inflammatory response, mature DCs can highly express cytokines and a variety of adhesion molecules such as CD40, CD80, CD83, and CD86, which can induce conglutination with platelets, endothelial cells and leukocytes, prior to increasing the expression of the TLR-4 signaling pathway, promoting an increase in the maturity of DCs and activating the process downstream (Lien et al., 2000; Kleemann et al., 2008; Ait-Outella et al., 2011; Boschuizen and de Winther, 2015). Immune inflammatory response signaling pathways cause an inflammatory cascade.

DCs, macrophages, T cells, and other immune cells can be found in atherosclerotic plaques, and it has been shown that these cells are involved in the entire process of AS occurrence and progression. Mature DCs, as the most powerful antigen-presenting cells in vivo, are the liaison between the innate and adaptive immune responses of the body, and the only antigen-presenting cells in the body that can activate the initial T lymphocytes (Joffre et al., 2009). Only if the antigen is captured by DCs and processed for modification can it receive a second signal molecule through the mature DC’s surface Toll-like receptor-4 (TLR-4) and initiate the downstream signaling pathway (particularly the classical TLR-4/MyD88/NF-kb pathway) (Hoshino et al., 2002). Next, it would begin to release a large number of inflammatory factors and trigger the inflammatory response cascade; it is possible to promote the maturity of immature DCs in the body through positive feedback, which is one of the purposes of this study.

In our previous animal studies (Ye et al., 2017), it has been demonstrated that mature DCs increased significantly after subcutaneous injection of mature exogenous wild type DCs into ApoE−/− knockout mice. However, it’s still unclear how exogenous DCs participate in the occurrence, development, and interactions of immune inflammation after the entrance in animal bodies.

Hence, in this study, P-selectin or exogenous mature DCs were injected into ApoE−/−/P−/− and ApoE−/− /PSGL−/− double knockout mice, and NS was administrated to a control group. Investigation was made to determine how the initiation and progression of atherosclerosis occurs through the P-sel/PSGL-1 pathway, how the differentiation of immature T cells and Treg occurs, and how immunoregulation and immune tolerance occurs after the administration of P-selectin and mature DCs pathway in the body.

**Materials and Methods**

The principal method of generating bone marrow-derived dendritic cells (BMDCs) was adapted from a publication by Son et al. (2002), with minor modifications. Bone marrow from C57BL/6 wild type mice was cultured with 1,000 U/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 (R & D Systems, Minneapolis, MN, USA). Then, magnetic beads were applied to select CD11c+ DCs (Millenyi Biotec, Auburn, CA, USA), according to the manufacturer’s instructions. To stimulate the DCs, 1 µg/mL of lipopolysaccharide (LPS; Sigma-Aldrich, St. Louis, MO, USA) was added to generate mature DCs from day 8 to day 9. Fluorescence-activated cell sorting (FACS, FACS Aria II; Becton Dickinson Immunocytometry Systems) was adopted to analyze the expression of surface molecules on the DCs.

The ApoE−/- mice on a C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed under specific pathogen-free conditions at Dalian Medical University, and they were injected with DCs or PBS every week for a total of 12 weeks. The study procedures complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (No. 8523, revised in 1996; NIH Publications). In addition, the mice were used in accordance with protocols approved by the Institutional Animal Care and Use Committee of Dalian Medical University (L2014030).

Extraction, culture, and identification of mature DCs from C57BL/6J strain mice in vitro were performed, per protocol.
Technology roadmap
The mice were divided into four groups:

1. ApoE\(^{-/-}\)/P-/- group (n = 6): ApoE\(^{-/-}\)/P-/- mice + high-fat diet. In the 9\(^{th}\) week, 200 \(\mu\)L of NS was injected into the tail vein of the mice, and another 200 \(\mu\)L of NS was subsequently injected subcutaneously into the inner thigh of the mice every week till the 16\(^{th}\) week.

2. ApoE\(^{-/-}\)/P\(^{-/-}\) + P-selectin group (n = 6): ApoE\(^{-/-}\)/P\(^{-/-}\) mice + high-fat diet. In the 9\(^{th}\) week, \(1 \times 10^6\) P-selectin was injected into the tail vein of the mice, and 200 \(\mu\)L of NS was subsequently injected subcutaneously into the inner thigh of the mice every week till the 16\(^{th}\) week.

3. ApoE\(^{-/-}\)/PSGL\(^{-/-}\) + NS group (n = 6): ApoE\(^{-/-}\)/PSGL\(^{-/-}\) mice + high-fat diet. In the 9\(^{th}\) week, 200 \(\mu\)L of NS was injected into the tail vein of the mice, and another 200 \(\mu\)L of NS was subsequently injected subcutaneously into the inner thigh of the mice every week till the 16\(^{th}\) week.

4. ApoE\(^{-/-}\)/PSGL\(^{-/-}\) + DCs group (n = 6): ApoE\(^{-/-}\)/PSGL\(^{-/-}\) mice + high-fat diet. In the 9\(^{th}\) week, \(1 \times 10^6\) wild type exogenous mature DCs were injected into the tail vein of the mice, and \(1 \times 10^6\) wild type exogenous mature DCs were subsequently injected subcutaneously in the inner thigh of the mice every week till the 16\(^{th}\) week.

At the age of approximate 16 weeks, the mice were heavily sedated following a 12-h overnight fast, and blood samples were immediately drawn from the orbits. The animals were then sacrificed. The spleen and aorta (extending from the aortic valve to the femoral bifurcation) were removed and snap frozen at \(-80^\circ\)C for later RNA extraction, as reported previously (Ye et al., 2017). In addition, a sample of the aortic tissue was stored in 10% buffered formalin for histological staining.

Cytokine analysis
Plasma levels of mouse interleukin (IL)-6 and tumor necrosis factor (TNF)-\(\alpha\) were determined by ELISA, following the manufacturer’s instructions (Abcam, Cambridge, UK). Subsequently, plasma or tissue samples were obtained as indicated previously and stored at \(-80^\circ\)C in advance. After the microplate wells were coated with purified rat anti-mouse IL-6 or TNF-\(\alpha\), incubation with a blocking buffer was conducted for 1 hour at room temperature before the wells were washed with PBS. Next, mouse plasma was added to the microplate wells, which were incubated for 2 hours at room temperature. They were subsequently incubated with biotin-labeled anti-mouse for IL-6 and TNF-\(\alpha\) for 1 hour at room temperature. A microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to measure the absorbance at 450 nm.

Statistical analysis
The GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA) was used to perform the statistical analyses. Data were expressed as mean ± standard deviation. The variables of interest were compared among the four treatment groups, using one-way analysis of variance (ANOVA). Pairwise comparisons were then performed with Tukey’s HSD test if the overall ANOVA test was significant. Box plots were generated to illustrate the comparisons among the four treatment groups. It was considered with statistical significance when \(P < 0.05\) was met.

Results
Comparison of DC surface markers among the four groups
The distribution factors of the surface markers of the DCs among the four treatment groups are illustrated using box plots in Fig. 1. Specifically, the expression of Treg is the highest in ApoE\(^{-/-}\)/PSGL1\(^{-/-}\) + NS group and the lowest in ApoE-/- / P\(^{-/-}\) + P group (Fig. 1a); the expression of CD40 and CD86 is the highest in ApoE-/- / P\(^{-/-}\) + P group among the four treatment groups (Figs. 1b and 1e); the expression of CD80, CD83, MHC II, TNF-\(\alpha\), HE and Masson is the highest in ApoE-/- / P\(^{-/-}\) + NS group (Figs. 1c, 1d, 1f, 1h, 1i and 1j); the expression of IL-6 and Oil red is the highest in ApoE-/- / P\(^{-/-}\) + DC group and the lowest in ApoE-/- / P\(^{-/-}\) + NS group (Figs. 1c and 1k). The results of the HE staining have shown that the aortic AS lesions were the most severe in mice in the ApoE-/- / P\(^{-/-}\) + DCs group, followed by those in the ApoE-/- / P\(^{-/-}\) + P-sel group; lesions in mice in the ApoE-/- / P\(^{-/-}\) + NS and ApoE-/- / P\(^{-/-}\) + NS groups were the lightest (Fig. 1i). The Masson’s trichrome staining results have
discovered that fibrosis of the aortic lesions in the ApoE-/- /PSGL-/- + DCs group was the most severe, followed by that in the ApoE-/- /P-/- + P-sel group; fibrosis in the ApoE-/- /P-/- + NS and ApoE-/- /PSGL-/- + NS groups was the lightest (Fig. 1j). The oil red O staining results indicated that aortic atherosclerosis was the most severe in the ApoE-/- /PSGL-/- + DCs group, followed by the ApoE-/- /P-/- + P-sel group; the lesions in the ApoE-/- /P-/- + NS and ApoE-/- /PSGL-/- + NS groups were the lightest (Fig. 1k).

Maturation of DCs with transfer into mice
The contribution of exogenous DCs to the increase of mature DCs in the peripheral blood of the recipients was studied through a FACS analysis, and the results are shown in Fig. 2. Mice treated with DCs demonstrated a higher expression of surface markers of the DCs in comparison with mice treated with NS. The above results are consistent with those obtained in a previous study (Ye et al., 2017).

Normality among the four treatment groups
The tested immunologic variables follow a normal distribution, with no statistical significance for any variable in any treatment group (Tab. 1). A comparison of the tested immunologic variables among the four treatment groups, however, yielded significant differences for these variables (Tab. 2). The highest expression of all surface markers was noted in the ApoE-/- /P-/- + P and ApoE-/- /PSGL-/- + DC groups (P < 0.0001).

Flow cytometry was applied to detect the proportion of CD11c+/CD40+, CD11c+/CD80+, CD11c+/CD83+, CD11c+/CD86+, and CD11c+/MHCII double-positive cells (Fig. 2). The results are as following. ① ApoE-/- / PSGL-/- + DCs group surface markers CD11c+/CD40+, CD11c+/CD80+, CD11c+/CD83+, CD11c+/CD86+, and CD11c+/MHCII double-positive cells had the highest ratios of 3.84%, 7.49%, 3.68%, 2.05%, and 8.98%. ② The proportion of CD11c+/CD40+, CD11c+/CD80+, CD11c+/CD83+, and CD11c+/MHCII double-positive cells in the ApoE-/- / P-/- + P-sel group had generally lower ratios at 3.94%, 7.24%, 1.79%, 3.03%, and 5.44%. ③ The surface markers CD11c+ / CD40+ and CD11c+ / CD80+ for the ApoE-/- / PSGL-/- + NS group and ApoE-/- / P-/- + NS group surface markers CD11c+/CD83+, CD11c+/CD86+, and CD11c+/MHCII double-positive cells had the lowest ratios.

Pairwise group comparisons with the surface markers have produced several significant results, as shown in Tab. 3 (p-values are adjusted). By comparing the treatment groups, the findings are as following. ① The ApoE-/- / P-/- + NS
group and ApoE-/-/PSGL-/- + NS group had significantly reduced double-positive cell levels. There was no statistical difference between these two groups for CD40 and CD83. ② Comparing the ApoE-/- / P-/- + NS group and the ApoE-/- / P-/- + P-sel group, the proportion of double-positive cells decreased significantly (P < 0.05). ③ Compared with the

![Flow cytometric dot plots](image)

**FIGURE 2.** Representative flow cytometric dot plots demonstrating the proportion of CD40, CD80, CD83, CD86 and MHC II cells expressing double-positive CD11C cells in portal cells in each group.

| Variable | ApoE-/- /P -/- + NS | ApoE-/- /P -/- + P | ApoE -/- / PSGL1-/- + NS | ApoE -/- /PSGL1-/- + DC |
|----------|---------------------|-------------------|--------------------------|------------------------|
| Treg     | 0.4232              | 0.1109            | 0.5331                   | 0.1507                 |
| CD40     | 0.8371              | 0.6305            | 0.1312                   | 0.9076                 |
| CD80     | 0.4834              | 0.2343            | 0.7232                   | 0.7878                 |
| CD83     | 0.4207              | 0.2871            | 0.4248                   | 0.9759                 |
| CD86     | 0.4612              | 0.9845            | 0.6214                   | 0.9332                 |
| MHC II   | 0.9250              | 0.1988            | 0.2616                   | 0.8345                 |
| IL-6     | 0.5729              | 0.3648            | 0.1982                   | 0.4921                 |
| TNF-α    | 0.9322              | 0.3060            | 0.7188                   | 0.0896                 |
| HE       | 0.4099              | 0.9402            | 0.6335                   | 0.5578                 |
| Masson   | 0.9539              | 0.7509            | 0.6442                   | 0.7738                 |
| Oil red  | 0.6433              | 0.9102            | 0.1737                   | 0.9450                 |

Treg: Regulatory T cells; MHC II: Major histocompatibility complex II; IL: Interleukin; TNF: Tumor necrosis factor; HE: Hematoxylin and eosin; Masson: Masson’s trichrome; Oil red: Oil red O solution
ApoE-/- / PSGL-/- + DCs group, the ApoE-/- / PSGL-/- + NS group also showed a significant decrease in the proportion of double-positive cells, and the difference was statistically significant (P < 0.05). There were similarities between the ApoE-/- / PSGL-/- + DCs group and the ApoE-/- / P-/- + P-sel group, and the ApoE-/- / PSGL-/- + DCs group had higher level of double-positive cells for CD83, CD86, and MHC II. There was no meaningful difference between the two groups for CD40 and CD80 (P > 0.05). All these results have suggested that the expression of co-stimulatory factors of ApoE-/- / PSGL-/- and ApoE-/- / P-/--mice in mature peripheral blood DCs increases after the injection of exogenous wild-type mouse-derived DCs.

Comparison of atherosclerotic processes among the groups, as demonstrated with HE staining, Masson staining, and oil red staining

Fig. 3 shows several aspects of the atherosclerotic process of HE staining. We compared the HE staining of the aortic roots of mice in each group and found that the AS lesions were the mildest in the ApoE-/- / P-/- + NS and ApoE-/- / P-/- + NS groups. A small amount of medium-member smooth muscle cell proliferation was found, in addition to disordered endothelial cells, a small number of cholesterol crystals in the blood vessels, failing to form a fat nucleus, and a low overall intimal plaque volume. In the ApoE-/- / PSGL-/- + DCs group and the ApoE-/- / P-/- + P-sel group, AS lesions in the aortic root were the most

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**TABLE 2**

Comparison of variables among the four treatment groups

| Variable | ApoE-/- / P-/- + NS | ApoE-/- / P-/- + P | ApoE-/- /PSGL-/- + NS | ApoE-/- /PSGL -/- + DC | p-value |
|----------|---------------------|-------------------|----------------------|-----------------------|---------|
| Treg     | 20.3 ± 1.81         | 4.74 ± 0.95       | 23.55 ± 4.33         | 7.42 ± 0.94           | <0.0001 |
| CD40     | 1.63 ± 0.24         | 3.94 ± 0.3        | 1.25 ± 0.24          | 3.84 ± 0.32           | <0.0001 |
| CD80     | 3.76 ± 0.37         | 7.24 ± 0.38       | 5.92 ± 0.62          | 7.49 ± 0.36           | <0.0001 |
| CD83     | 0.38 ± 0.12         | 1.79 ± 0.31       | 0.62 ± 0.16          | 3.68 ± 0.25           | <0.0001 |
| CD86     | 1.67 ± 0.23         | 3.03 ± 0.27       | 1.19 ± 0.22          | 2.05 ± 0.24           | <0.0001 |
| MHC II   | 3.59 ± 0.2          | 5.44 ± 0.57       | 4.71 ± 0.23          | 8.98 ± 0.41           | <0.0001 |
| IL-6     | 276.11 ± 105.73     | 723.02 ± 121.71   | 256.01 ± 65.91       | 814.26 ± 241.93       | <0.0001 |
| TNF-α    | 752.12 ± 452.62     | 2227.02 ± 503.33  | 1048.13 ± 333.43     | 2631.27 ± 683.63      | <0.0001 |
| HE       | 25.33 ± 4.68        | 42.83 ± 5.04      | 27.67 ± 4.84         | 48.83 ± 7.57          | <0.0001 |
| Masson   | 18 ± 3.58           | 32.17 ± 4.26      | 18 ± 4.34            | 40.5 ± 4.85           | <0.0001 |
| Oil red  | 16.67 ± 2.8         | 36.83 ± 4.22      | 13.33 ± 3.56         | 52.67 ± 5.24          | <0.0001 |

Treg: Regulatory T cells; MHC II: Major histocompatibility complex II; IL: Interleukin; TNF: Tumor necrosis factor; HE: Hematoxylin and eosin; Masson: Masson’s trichrome; Oil red: Oil red O solution

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FIGURE 3. HE staining of mouse aorta specimens. A represents the ApoE-/- / P-/- group; B represents the ApoE-/- / P-/- + P-sel group; C represents the ApoE-/- / PSGL-/- group; and D represents the ApoE-/- /PSGL-/- + DCs group. HE staining microscopy: the larger images are 40×, and the smaller, inset images are the two random fields of view at 100×. Image Pro Plus 6.0 software was used to analyze the optical density of the HE images, and the plaque area ratio (plaque area/aortic lumen area) histogram was constructed.

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severe, particularly in the former group. Endothelial cells were locally seen in the aortic vascular endothelium. Also noted was a disordered arrangement, obvious proliferation of vascular smooth muscle, atherosclerotic plaque deposition, and many white needle-shaped internal cholesterol crystals. Large necrotic lipid pool formation was detected in the plaque, and the plaque surface was covered with a thin layer of dense fibrous caps; rupture of the fibrous caps was noted in some areas, and the overall observation led to the conclusion that the area of vascular plaques was large. Moreover, inflammatory complex lesions such as bleeding and thrombus formed on the surface of the plaques.

Masson’s trichrome staining was performed on the aortic root lesions in mice, as shown in Fig. 4. The findings are as follows. 

### TABLE 3

Pairwise group comparisons with variables

| Variable | Group 1 vs. 2 | Group 1 vs. 3 | Group 1 vs. 4 | Group 2 vs. 3 | Group 2 vs. 4 | Group 3 vs. 4 | p-value |
|----------|---------------|---------------|---------------|---------------|---------------|---------------|---------|
| Treg     | <0.0001       | 0.0578        | <0.0001       | <0.0001       | 0.1538        | <0.0001       | <0.0001 |
| CD40     | <0.0001       | 0.0517        | <0.0001       | <0.0001       | 0.8806        | <0.0001       | <0.0001 |
| CD80     | <0.0001       | <0.0001       | <0.0001       | <0.0001       | 0.6604        | <0.0001       | <0.0001 |
| CD83     | <0.0001       | 0.3267        | <0.0001       | <0.0001       | <0.0001       | <0.0001       | <0.0001 |
| CD86     | <0.0001       | 0.0034        | 0.0257        | <0.0001       | <0.0001       | <0.0001       | <0.0001 |
| MHC II   | <0.0001       | <0.0001       | <0.0001       | <0.0001       | <0.0001       | <0.0001       | <0.0001 |
| IL-6     | 0.0004        | 0.9962        | <0.0001       | 0.0004        | 0.7295        | <0.0001       | <0.0001 |
| TNF-α    | 0.0004        | 0.7472        | <0.0001       | 0.0035        | 0.5285        | <0.0001       | <0.0001 |
| HE       | 0.0002        | 0.8903        | <0.0001       | 0.0008        | 0.2862        | <0.0001       | <0.0001 |
| Masson   | <0.0001       | 1.0000        | <0.0001       | <0.0001       | 0.0148        | <0.0001       | <0.0001 |
| Oil red  | <0.0001       | 0.4998        | <0.0001       | <0.0001       | <0.0001       | <0.0001       | <0.0001 |

Group 1: ApoE-/- / P-/- + NS; Group 2: ApoE-/- / P-/- + P; Group 3: ApoE-/- / PSGL1-/- + NS; Group 4: ApoE-/- / PSGL1-/- + DC.

Treg: Regulatory T cells; MHC II: Major histocompatibility complex II; IL: Interleukin; TNF: Tumor necrosis factor; HE: Hematoxylin and eosin; Masson: Masson’s trichrome; Oil red: Oil red O solution

FIGURE 4. Masson’s trichrome staining of mouse aortic root specimens. A: ApoE-/- / P-/- group; B: ApoE-/- / P-/- + P-sel group; C: ApoE-/- / PSGL1-/- group; and D: ApoE-/- / PSGL1-/- + DCs group. Masson’s trichrome staining (the larger images are 40×, and the smaller, inset images represent the two random fields of view at 100×). The Masson images were analyzed for optical density using Image Pro Plus 6.0 software to produce a histogram of collagen composition ratio (collagen fiber area / aortic root vessel wall area).
the overall vascular lumen plaque area was the smallest, and only a few blue infiltrated plaques could be found with the microscope. The cap was thicker, there was no rupture on the plaque surface or bleeding and thrombus manifestations, indicating that the plaque is stable in nature.

We performed oil red O staining on the aorta of each group (Fig. 5). The results are shown as below. ① Both the ApoE-/- / PSGL-/- + DCs group and ApoE-/- / P-/- + P-sel group demonstrated the highest degree (i.e., the largest area) of staining of the aortic specimens, representing lipid spots. The block area ratio (plaque area/endometrial area) had the highest ratio, which was the most severe erosion of lipid components in the lumen. ② The ApoE-/- / P-/- + NS and ApoE-/- / PSGL-/- + NS groups showed the least degree of oil red staining, suggesting the lowest ratio (plaque area/total endometrial area) of the lipid plaque area, and the overall aortic oil red staining area was the smallest, indicating that the lowest degree of plaque lipid.

Inflammatory activation in recipients of DCs
DC maturation can lead to the activation of the inflammatory system through the use of T cells, given previous analysis. ELISA was used to determine the levels of IL-6 and TNF-alpha in the peripheral plasma of mice in each group (Fig. 6). The level of TNF-alpha was generally higher in all four treatment groups than that of IL-6 produced. The highest levels of IL-6 and TNF-alpha were produced by the Apo -/- / PSGL -/- + DCs group, followed closely by the ApoE -/- / P -/- + P-sel group.

Levels of IL-6 and TNF-alpha measured in the mice plasma
Fig. 7 illustrates the results of cytokine expression detected by applying real-time PCR (RT-PCR), with IL-6 and TNF-alpha in all four groups included. The expression levels of IL-6 and TNF-alpha mRNA were measured, with the results showing that the expression concentration of both IL-6 and

**FIGURE 5.** Oil red staining of mouse aorta specimens. The aorta specimens of mice were stained with oil red. A represents the ApoE-/- / P-/- group; B represents the ApoE-/- / P-/- + P-sel group; C represents the ApoE-/- /PSGL-/- group; and D represents the ApoE-/- /PSGL-/- + DCs group. Oil red stained images were analyzed with Image Pro Plus 6.0 software for optical density, and the histogram of the area ratio of lipid plaque (plaque area/intima area) was constructed.

**FIGURE 6.** The levels of IL-6 and TNF-alpha in peripheral plasma of mice in each group were determined by ELISA. A represents the ApoE-/- / P-/- group; B represents the ApoE-/- / P-/- + P-sel group; C represents the ApoE-/- /PSGL-/- group; and D represents the ApoE-/- /PSGL-/- + DCs group.

**FIGURE 7.** Real-time PCR was used to detect the expression of IL-6 and TNF-alpha in the aorta.
TNF-alpha in the ApoE-/-/PSGL-1/- + DCs group and the ApoE-/-/P-/- + P-sel group was the highest, although it was relatively higher in the ApoE-/-/PSGL-1/- + DCs group. Notably, there was no statistically significant difference between the two groups. The ApoE-/-/P-/- + NS and ApoE-/-/P-/-+ NS groups had the lowest IL-6 and TNF-alpha expression levels also without statistically significant difference between the two groups. Comparing the ApoE-/-/P-/- + NS group and the ApoE-/-/P-/- + P-sel group, the IL-6 and TNF-alpha expression was largely decreased with statistical significance. Comparing the ApoE-/-/PSGL-1/- + NS and the ApoE-/-/PSGL-1/- + DCs groups, the expression of IL-6 and TNF-alpha was greatly reduced (P < 0.005).

Discussion

In this study, ApoE-/-/P-/- and ApoE-/-/PSGL-1/-, the two groups of double gene knockout mice, were established with hybridization technology, so to set up the experiment model. The experimental group was given P-selectin or exogenous DCs, while the control group was injected with NS. The two groups were matched based on different variables.

The results of this study were mostly pronounced in the ApoE-/-/P-/- + NS group of double knockout mice. The expression and subsequent levels of all the co-stimulatory factors, including Treg, CD40, CD80, CD83, CD86, MHC II, IL-6 and TNF-alpha, were significantly increased with the upregulation of mature DCs after injection of NS, thus exhibiting the factors for the formation of atherosclerosis. The levels of these factors were slightly less robust in the ApoE-/-/P-/- + P-selectin group, however higher than that of the ApoE-/-/PSGL-1/- + NS group. The least robust response was seen in the ApoE-/-/PSGL-1/- + DC group.

The expression of IL-6 and TNF-alpha in the peripheral plasma has shown that the TNF-alpha is expressed at a much higher level than IL-6, and ApoE-/-/P-/- + P-sel group and ApoE-/-/PSGL-1/- + DCs group have presented the highest expression of the two cytokines. Despite that, the results obtained by ELISA are different from those subsets in the spleen of mice were determined through flow cytometry to certain extent.

The apparent effect of exogenous DC injection into animal bodies after a double knockout was not as robust as that seen with P-selectin or even NS injection. The effect reported in ApoE-/- knockout mice after such injection, when mature DCs increased after subcutaneous injection of mature exogenous wild type DCs, was not regarded as the same in this experiment. This is either possibly related to the nature of the knockout status of the mice, or because the second gene knocked out other than ApoE is P-selectin or PSGL1, both of which are needed for the stimulation of DC maturation.

A similarly designed study was conducted by Koulis et al. (2014), in which researchers investigated double knockout ApoE-/-/TLR-9/-/- mice and control Apo-/-/-/ mice to see whether TLR-9 had a role in these ApoE-deficient animals. The study was observational, and the mice were fed a high fat diet for 8 weeks before being assessed in the 8th, 12th, 15th, and 20th week. Researchers found that the deletion of the TLR-9 gene exacerbated atherosclerosis in the deficient mice. In current study, the enhanced release of inflammatory co-factors has appeared to be relevant to the nature of the gene deficiency in the mice.

A majority of previous studies on the influence of exogenous DCs on vascular atherosclerosis were in vitro cell experiments. Pro-inflammatory cytokines such as TNF-alpha have been proven to be involved in atherosclerosis in knockout mice. Branen et al. (2004) studied mice deficient in both ApoE and TNF-alpha, also compared the extent of atherosclerosis produced, with the finding that inhibition of TNF-alpha reduced atherosclerosis in ApoE knockout mice. Current studies demonstrate a similar concept in an animal study using another immune factor, P-selectin. This cell-surface adhesion molecule that is involved in leukocyte rolling and attachment is associated with cardiovascular risk, as Ridker et al. (2001) demonstrated in a study introducing a positive correlation between soluble P-selectin levels and the risk of future vascular events in women.

The previous cytological experiments by our research group have also confirmed that P-selectin and the PSGL-1 receptor can be combined; they are ligands and receptors and can participate and play a significant role in the maturation of DCs (Ye et al., 2017). Both could increase the release of co-stimulators, antigen presentation, enhance regulation of the TLR-4 receptor, activate the downstream signaling pathway, and promote the waterfall effect of the inflammatory response (Iwasaki and Medzhitov, 2004). In vivo, exogenous DCs were used to observe the effect of the P-selectin/PSGL-1 pathway on atherosclerosis. PSGL-1 has been remarkably determined to demonstrate a mechanism for Treg-mediated suppression of an enduring immune response and induction of the autoimmune system (Angiari et al., 2013). The tolerogenic capacity of DCs when stimulated through PSGL-1 with P-selectin to produce specific Treg cells has been highlighted by other researchers (Urzaínqui et al., 2007).

We have noticed that the expression of DC maturity in peripheral blood and the distribution of CD4+ T lymphocyte subsets in the spleen of mice were detected by flow cytometry. There has been observation on several other factors, as well. First, the maturity of DCs in the ApoE-/-/-/P-/- group and ApoE-/-/PSGL-1/-/- group decreased most significantly, so did the secretion levels of costimulatory factors (CD40, CD80, CD83, CD86 and MCH-II), indicating that both P-selectin and PSGL-1 receptors were involved in the maturation of DCs. Secondly, the maturity of DCs in the ApoE-/-/-/P-/- + P-selectin group was significantly higher than that in the ApoE-/-/-/P-/-/-/ control group. However, compared with the ApoE-/-/-/PSGL-1/-/- + DC mice, the maturity of the DCs was still decreased, indicating that there were other selectin pathways involved in the maturation of DCs besides P-selectin, in spite of the dominating role of P-selectin in the maturation of DCs compared with other types of selectins. Thirdly, in comparison with the ApoE-/-/-/PSGL-1/-/- + DCs mice, after adding exogenous mature DCs to the ApoE-/-/-/PSGL-1/-/- + NS mice, the expression of DCs tended to be common. The secretion levels of stimulating factors (CD40, CD80, CD83, CD86, and MCH-II) increased significantly, suggesting that in addition to the PSGL-1 receptors involved in the maturation of DCs, exogenous...
mature DCs can also stimulate the maturation of endogenous DCs. These results (Tab. 2) have shown that both P-selectin and PSGL-1 participate in the transformation of immature DCs. Fourthly, exogenous mature DCs can spontaneously stimulate the maturation of endogenous DCs under the same conditions. Last but not the least, even in the absence of P-selectin, other similar selectins still participate in the activation of immature DCs.

Similarly, after having compared the results of HE staining, Masson staining, and oil red staining of the aortic root of mice in each group, it's found that the AS lesions in the ApoE-/-/PSGL-/- + DCs group and ApoE-/-/P-/- + P-selectin group were the most substantial, and ApoE-/-/P-/- PSGL-/- + PSGL-selectin group were the most serious, regardless of the degree of local atherosclerosis, the level of intimal fibrosis, nor the area of aortic plaque. The levels of IL-6 and TNF-α in the peripheral blood of the mice in the -/- group and ApoE-/-/P-/- group were determined by ELISA (to indicate the intensity of inflammatory factors in peripheral blood). The results have indicated that the concentration of IL-6 and TNF-α in the peripheral plasma of the ApoE-/-/PSGL-/- + DCs group was the highest, followed by the ApoE-/-/P-/- + P-selectin group, then the ApoE-/-/P-/- group and lastly the ApoE-/-/PSGL-/- group. The pulp concentration level was the lowest. The results of HE staining, Masson staining and oil red staining were similar to those of the peripheral plasma inflammatory factors.

Real-time PCR was used to determine the distribution of IL-6, TNF-α mRNA, and the expression of CD11c, TLR-4, MyD88 and NF-KB in the root of the aorta of mice by immunofluorescence, generating consistent conclusions with the previous results, although with only the results for IL-6 and TNF-alpha presented in this paper.

Several limitations are available in this study. First of all, researches were conducted on only a few knock-out mice, nevertheless, the results should explain some of the problems encountered and lead to the exploration and study of the occurrence, development, and outcomes of inflammatory reactions. Secondly, there are many factors that influence the inflammatory response in addition to the classical immune inflammatory response pathway. In other well-known (and unknown) pathway processes, various internal environmental changes and interactions of various factors occur in the body, producing a range of results.

These results have suggested that exogenous mature DCs can stimulate immune inflammation through the P-selectin/PSGL-1 pathway, and ultimately promote the formation of atherosclerotic lesions in mice through the classical signal pathway. Exogenous mature DCs can stimulate the maturation of immature DCs and activate the expression of T cells through the P-selectin/PSGL-1 pathway. Exogenous mature DCs can activate the downstream TLR-4/MyD88-NF-kb signaling pathway through upregulation of the expression of the TLR-4 receptor, which activates the intensification of the cellular immune inflammatory response, and the occurrence and development of AS lesions in the body.

In vivo animal experiments have found that dendritic cells can stimulate the maturation of immature DCs in the body through the P-selectin/PSGL-1 pathway, thus triggering the expression of the effector T cells. If the receptor ligand binding in the reaction pathway can be inhibited, it is possible to inhibit the occurrence and development of AS, which indicates the direction of our future research.

Conclusions

Based on the expression of co-stimulatory factors in mature DCs, pathology, vascular fibrosis, inflammatory factor concentration, and mRNA expression level, we came to the conclusion that exogenous DCs can stimulate the maturation of DCs through the P-selectin/PSGL-1 pathway. This process may promote an immune inflammatory response, which would affect the occurrence and development of atherosclerotic lesions in mice.

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