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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, 2016; 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 responses, 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 reduces 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

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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-Oufella *et al*., 2011; Boshuizen 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 administered 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 (Miltenyi 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 9th week, 200 uL of NS was injected into the tail vein of the mice, and another 200 uL of NS was subsequently injected subcutaneously into the inner thigh of the mice every week till the 16th week.

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

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

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

The expression of surface molecules on the DCs in peripheral blood was checked via flow cytometry analysis. Notably, 1×10^6 cells were stained at each step of the process with specific antibodies (Abs) at 4°C for 1 hour in 100 μL of PBS containing 2% of bovine serum albumin. Fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-labeled monoclonal Abs (all purchased at BioLegend (San Diego, CA, USA)) were used to stain MHC class II (FITC), CD40 (FITC), CD80 (FITC), CD83 (FITC), CD86 (FITC) and CD11c (PE). Additionally, FITC-or PE-labeled IgG was substituted for controls matched by isotype.

Histological analysis

Aortic samples were opened longitudinally and stained with Oil red O solution for the purpose of assaying the burden of lipid in the aorta. Aortic roots were embedded in 10% buffered formalin in preparation for cutting serial sections, each of 5 μm thickness. Besides, hematoxylin and eosin (H & E) staining was used to analyze the lesions, and Masson's trichrome was used to demarcate the fibrous area.

Cytokine analysis

Plasma levels of mouse interleukin (IL)-6 and tumor necrosis factor (TNF)-α 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°C in advance. After the microplate wells were coated with purified rat anti-mouse IL-6 or TNF-α, 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-α 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.

Real-time RT-PCR analysis

As previously outlined by Ye *et al.* (2017), RNA in the aorta was extracted using the RNAiso Reagent Plus (Takara Bio Inc., Shiga, Japan), and complementary DNA (cDNA) was constructed using an RT-PCR kit (Takara Bio Inc.), according to the manufacturer's recommendations. The primer sequences and expected sizes of the products are shown in Tab. I. Changes in mRNA expression were normalized with GAPDH and calculated using the 2-ΔΔCq method. In addition, the expression level of IL-6 and TNF-mRNA in the aortas of the mice was assessed by real-time PCR.

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 and the lowest in ApoE -/- / PSGL1-/- + NS group (Figs. 1b and 1e); the expression of CD80, CD83, MHC II, TNF- α, HE and Masson is the highest in ApoE -/- / PSGL1 -/- + DC group and the lowest 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 -/- / PSGL1 -/- + DC group and the lowest in ApoE -/- / PSGL1-/- + NS group (Figs. 1e and 1k). The results of the HE staining have shown that the aortic AS lesions were the most severe in mice in the ApoE-/- / PSGL-/- + DCs group, followed by those in the ApoE-/- /P-/- + P-sel group; lesions in mice in the ApoE-/- /P-/- + NS and ApoE-/- / PSGL-/- + NS groups were the lightest (Fig. 1i). The Masson's trichome staining results have

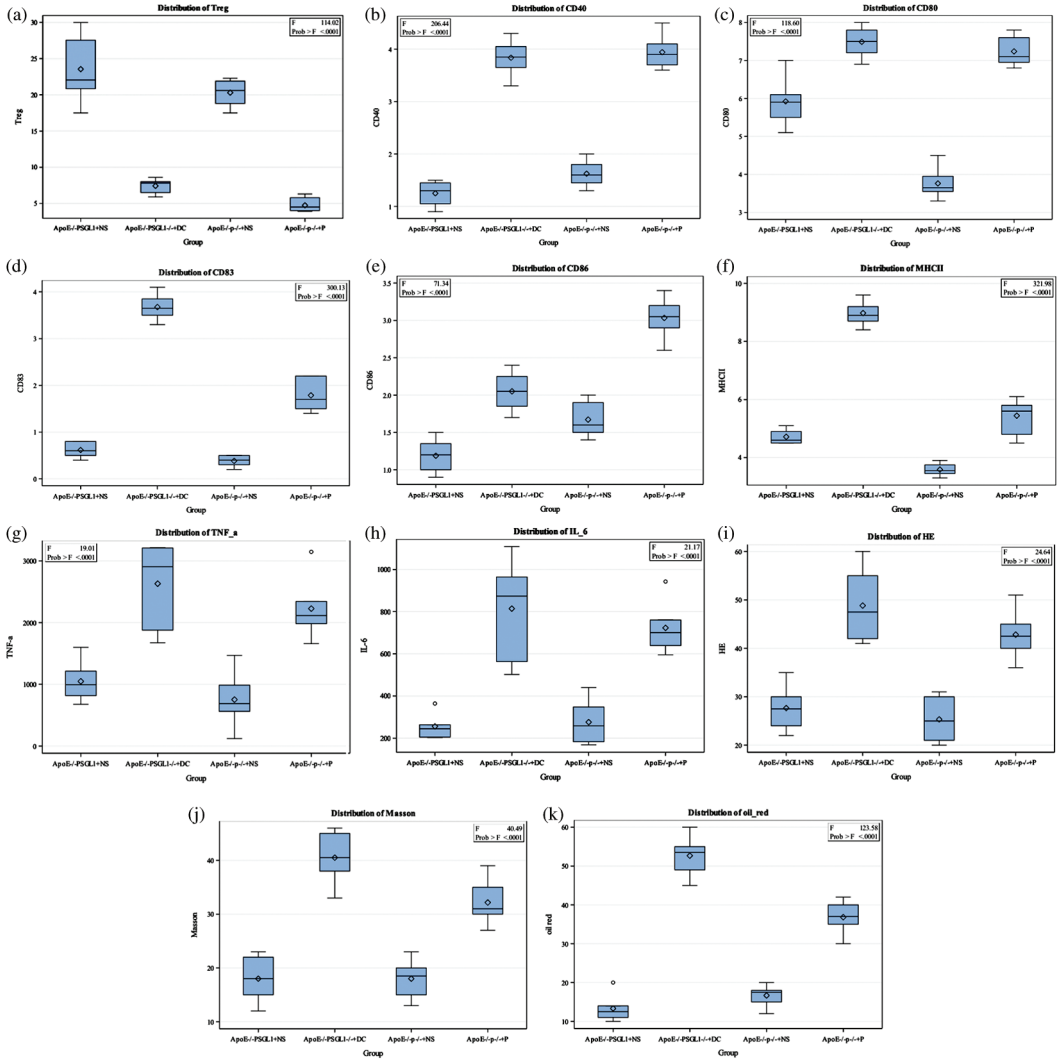

FIGURE 1. FACS plots of DC surface marker expressions of (a) Treg, (b) CD40, (c) CD80, (d) CD83, (e) CD86, (f) MHC II, (g) TNF-α, (h) IL-6, (i) HE, (j) Masson's trichome staining, and (k) oil red O staining for the four treatment groups.

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-/- /PSGL1-/- + 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+, CD11c +/CD86+, 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

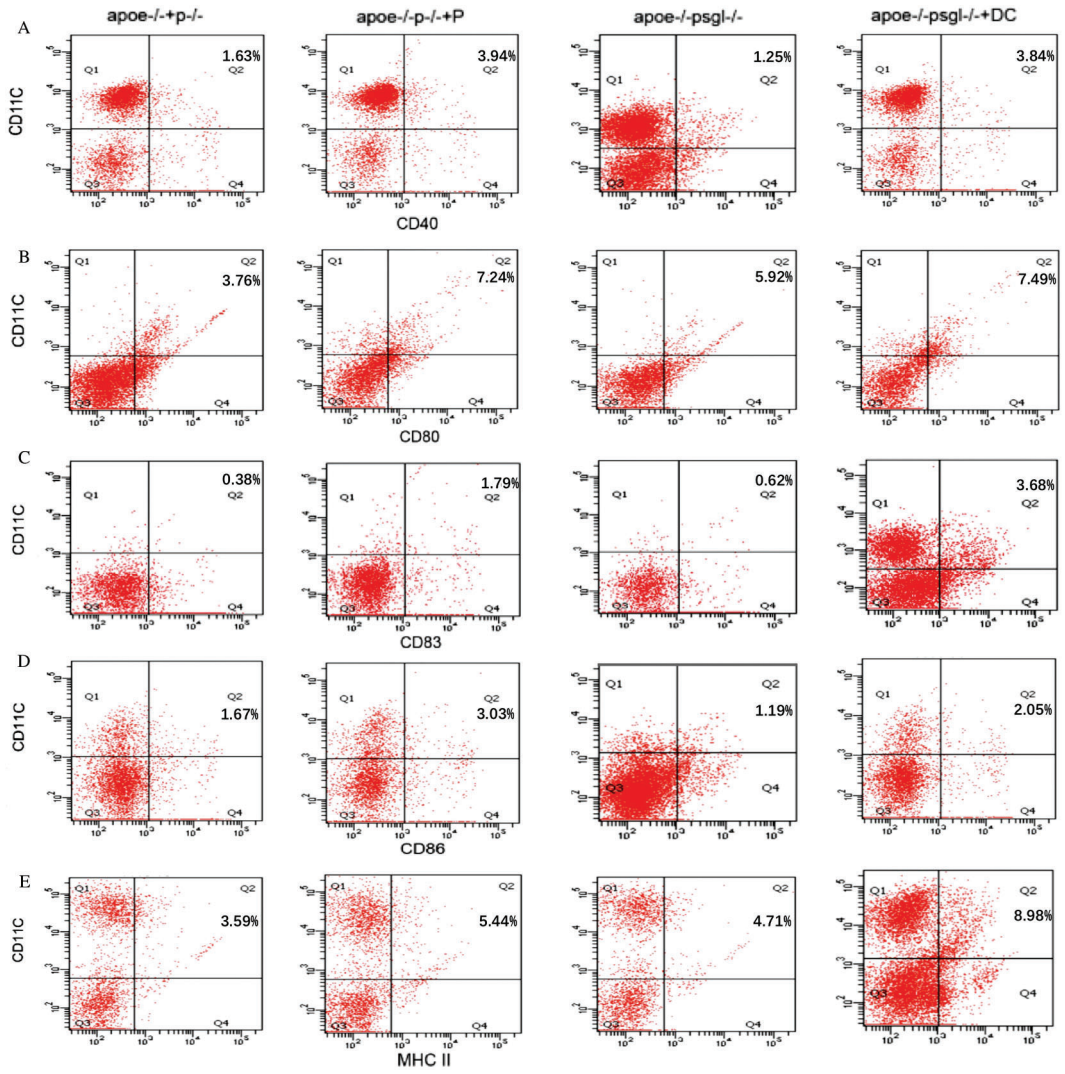

FIGURE 2. Representative flow cytometric dot plots demonstrating the proportion of CD40, CD80, CD83, CD86 and MHC II cells expressing double-positive CD11C cells in portal cells in each group.

TABLE 1

Evaluation of normality among the variables in the four treatment groups

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

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

TABLE 2

Comparison of variables among the four treatment groups

Variable	ApoE-/- /P -/- + NS	ApoE-/- /P -/- + P	ApoE -/- /PSGL1-/- + NS	ApoE -/- /PSGL1 -/- + 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 ± 503.53	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

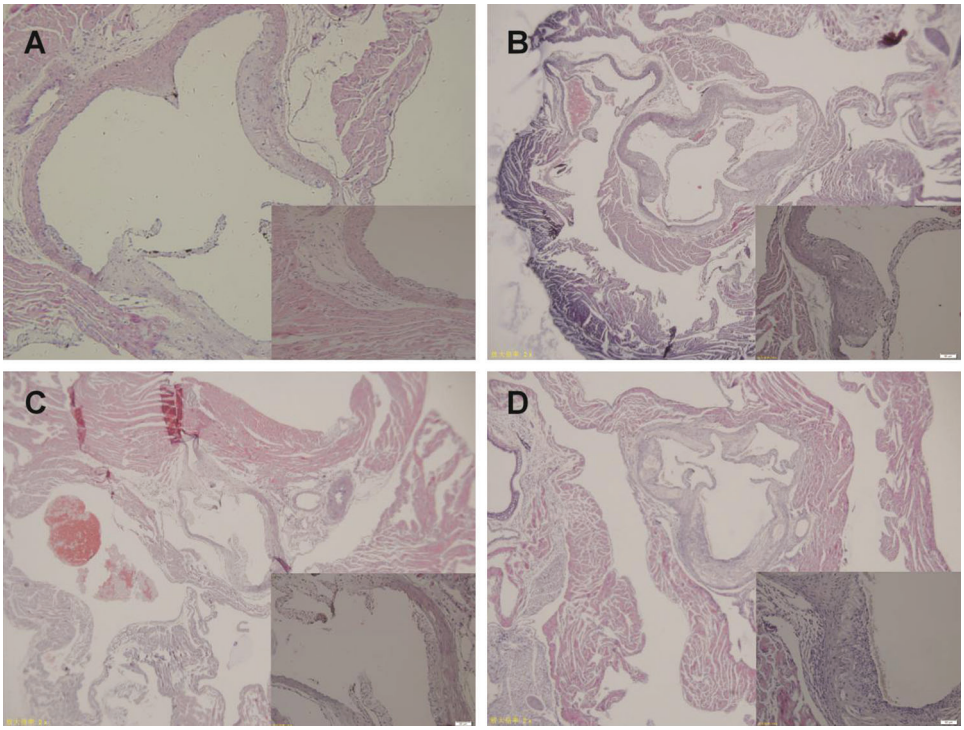

FIGURE 3. HE staining of mouse aorta specimens. A represents the ApoE-/- /P-/- group; B represents the ApoE-/- /P-/- + P-sel group; C represents the ApoE-/- /PSGL-/- group; and D represents the ApoE-/- /PSGL-/- + DCs group. HE staining microscopy: the larger images are 40×, and the smaller, inset images 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.

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 ($P < 0.05$). ④ 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-/- / PSGL-/- + 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

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	0.0067	<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	0.0002	<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

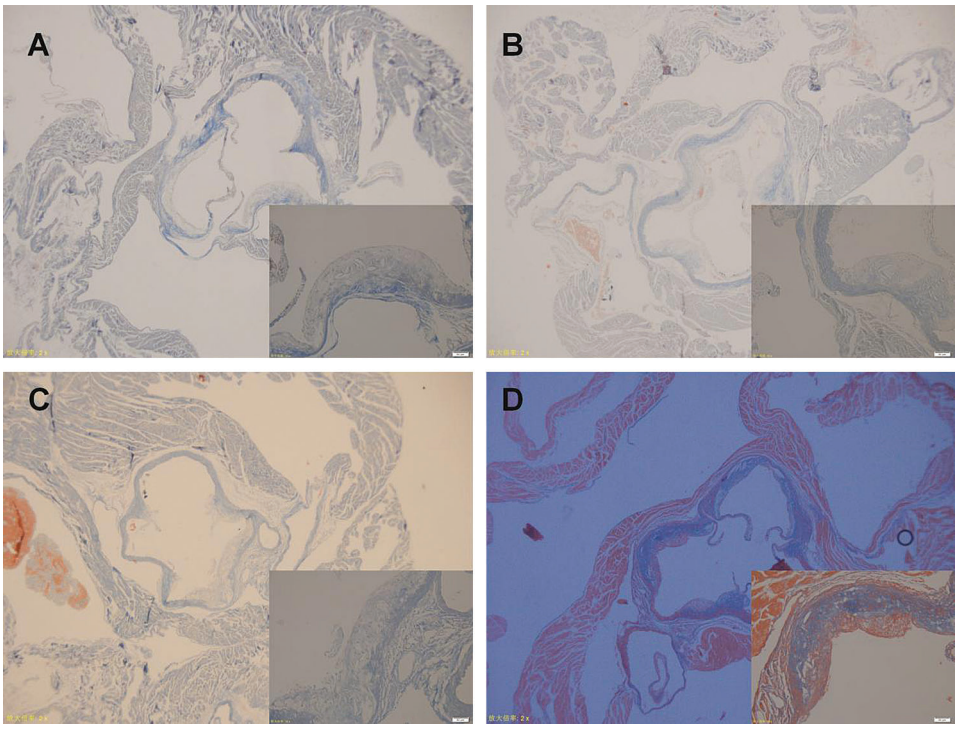

FIGURE 4. Masson's trichrome staining of mouse aortic root specimens. A: ApoE-/- /P-/-group; B: ApoE-/- /P-/- + P-sel group; C: ApoE-/- / PSGL-/- group; and D: ApoE-/- / PSGL-/- + DCs group. Masson's trichrome staining (the larger images are 40×, and the smaller, inset images represent the two random 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).

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. ① The ApoE-/- / PSGL-/- + DCs and ApoE-/- / P-/- + P-sel groups had the highest levels of vascular fibrosis, as shown by the largest blue staining area in the images. This result has demonstrated that the infiltration of collagen into the arterial intima of the plaque site in the lumen was of the largest scale, which directly confirms that the highest level of plaque fibrosis. Obvious cholesterol crystals were visible in the plaque, the lipid core was large, and the fiber cap was thin, but regarding visible plaque morphology, there was no plaque fiber cap rupture or bleeding. ② The fibrosis level of ApoE-/- / P-/- + NS group and ApoE-/- / PSGL-/- + NS group was minimal, suggesting that the collagen content of the vascular endometrium was significantly reduced, and the fibrosis level was the lowest. It's specifically manifested that

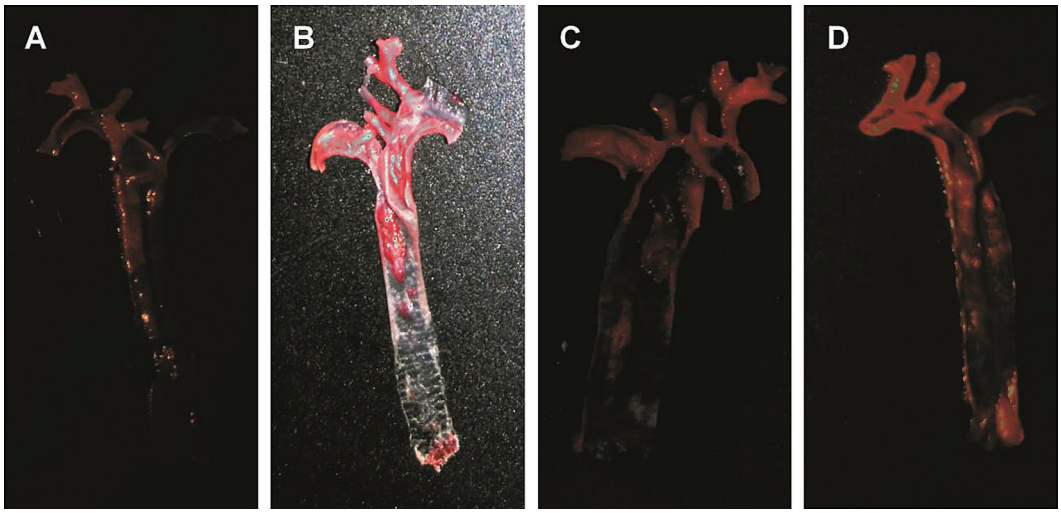

FIGURE 5. Oil red staining of mouse aorta specimens. The aorta specimens of mice were stained with oil red. A represents the ApoE-/- /P-/- group; B represents the ApoE-/- / P-/- + P-sel group; C represents the ApoE-/- /PSGL-/- group; and D represents the ApoE-/- / PSGL-/- + DCs group. Oil red stained 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.

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

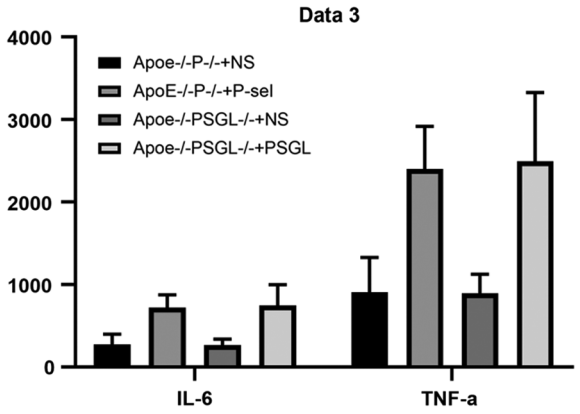

FIGURE 6. The levels of IL-6 and TNF-alpha in peripheral plasma of mice in each group were determined by ELISA. A represents the ApoE-/- /P-/- group; B represents the ApoE-/- /P-/- + P-sel group; C represents the ApoE-/- /PSGL-/- group; and D represents the ApoE-/- /PSGL-/- + DCs group.

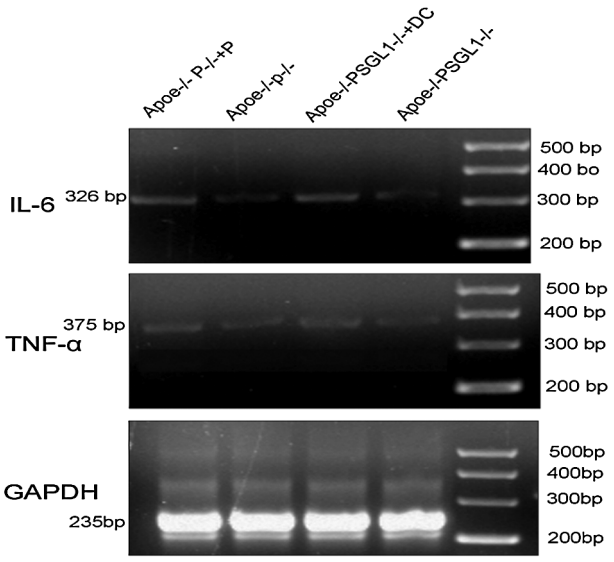

FIGURE 7. Real-time PCR was used to detect the expression of IL-6 and TNF-alpha in the aorta.

TNF-alpha in the ApoE-/- / PSGL-/- + DCs group and the ApoE-/- / P-/- + P-sel group was the highest, although it was relatively higher in the ApoE-/- / PSGL-/- + 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-/- + NS and the ApoE-/- /PSGL-/- + 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-/-, 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-α, 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 -/- / PSGL1-/- + NS group. The least robust response was seen in the ApoE -/- / PSGL1 -/- + 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-/- / PSGL1-/- + DCs group have presented the highest expression of the two cytokines. Despite that, the results obtained by ELISA are different from those 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 PSGL, 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 8^{th}, 12^{th}, 15^{th}, and 20^{th} 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-α have been proven to be involved in atherosclerosis in knockout mice. Branen *et al.* (2004) studied mice deficient in both ApoE and TNF-α, also compared the extent of atherosclerosis produced, with the finding that inhibition of TNF-α 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 (Urzainqui *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-/- 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-/- + 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-/- + DCs mice, after adding exogenous mature DCs to the ApoE-/-/PSGL-/- + 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-/-/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 atherosclerosis 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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References

Ait-Oufella H, Taleb S, Mallat Z, Tedgui A (2011). Recent advances on the role of cytokines in atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **31**: 969–979. DOI 10.1161/ATVBAHA.110.207415.

Angiari S, Rossi B, Piccio L, Zinselmeyer BH, Budui S, Zenaro E, Della Bianca V, Bach SD, Scarpini E, Bolomini-Vittori M, Piacentino G, Dusi S, Laudanna C, Cross AH, Miller MJ, Constantin G (2013). Regulatory T cells suppress the late phase of the immune response in lymph nodes through P-selectin glycoprotein ligand-1. *Journal of Immunology* **191**: 5489–5500. DOI 10.4049/jimmunol.1301235.

Banchereau J, Steinman RM (1998). Dendritic cells and the control of immunity. *Nature* **392**: 245–252. DOI 10.1038/32588.

Bauriedel G, Skowasch D, Welsch U, Luderitz B (2006). Role of dendritic cells in specific atherosclerosis types. *European Heart Journal* **27**: 116; author reply 116–117.

Bobryshev YV (2005). Dendritic cells in atherosclerosis: current state of the problem and clinical relevance. *European Heart Journal* **26**: 1700–1704. DOI 10.1093/eurheartj/ehi282.

Bobryshev YV (2010). Dendritic cells and their role in atherogenesis. *Laboratory Investigation* **90**: 970–984. DOI 10.1038/labinvest.2010.94.

Bobryshev YV, Watanabe T (1997). Subset of vascular dendritic cells transforming into foam cells in human atherosclerotic lesions. *Cardiovascular Pathology* **6**: 321–331. DOI 10.1016/S1054-8807(97)00022-7.

Boshuizen MC, de Winther MP (2015). Interferons as essential modulators of atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **35**: 579–588. DOI 10.1161/ATVBAHA.115.305464.

Branen L, Hovgaard L, Nitulescu M, Bengtsson E, Jovinge S (2004). Inhibition of tumor necrosis factor-alpha reduces atherosclerosis in apolipoprotein E knockout mice. *Arteriosclerosis, Thrombosis, and Vascular Biology* **24**: 2137–2142. DOI 10.1161/01.ATV.0000143933.20616.1b.

Chen L (2004). Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nature Reviews Immunology* **4**: 336–347. DOI 10.1038/nri1349.

Chistiakov DA, Sobenin IA, Orekhov AN, Bobryshev YV (2014). Dendritic cells in atherosclerotic inflammation: the complexity of functions and the peculiarities of pathophysiological effects. *Frontiers in Physiology* **5**: 1961–1969.

Choi JH, Do Y, Cheong C, Koh H, Boscardin SB, Oh YS, Bozzacco L, Trumpfheller C, Park CG, Steinman RM (2009). Identification of antigen-presenting dendritic cells in mouse aorta and cardiac valves. *Journal of Experimental Medicine* **206**: 497–505. DOI 10.1084/jem.20082129.

Ding Z, Liu S, Wang X, Dai Y, Khaidakov M, Romeo F, Mehta JL (2014). LOX-1, oxidant stress, mtDNA damage, autophagy, and immune response in atherosclerosis. *Canadian Journal of Physiology and Pharmacology* **92**: 524–530. DOI 10.1139/cjpp-2013-0420.

Gao S, Liu J (2017). Association between circulating oxidized low-density lipoprotein and atherosclerotic cardiovascular disease. *Chronic Diseases and Translational Medicine* **3**: 89–94. DOI 10.1016/j.cdtm.2017.02.008.

Gistera A, Hansson GK (2017). The immunology of atherosclerosis. *Nature Reviews Nephrology* **13**: 368–380. DOI 10.1038/nrneph.2017.51.

Hansson GK (2009). Atherosclerosis-an immune disease: the Anitschkov lecture 2007. *Atherosclerosis* **202**: 2–10. DOI 10.1016/j.atherosclerosis.2008.08.039.

Hansson GK, Libby P (2006). The immune response in atherosclerosis: a double-edged sword. *Nature Reviews Immunology* **6**: 508–519. DOI 10.1038/nri1882.

Hogg N, Landis RC (1993). Adhesion molecules in cell interactions. *Current Opinion in Immunology* **5**: 383–390. DOI 10.1016/0952-7915(93)90057-Y.

Hoshino K, Kaisho T, Iwabe T, Takeuchi O, Akira S (2002). Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation. *International Immunology* **14**: 1225–1231. DOI 10.1093/intimm/dxf089.

Hristov M, Weber C (2011). Differential role of monocyte subsets in atherosclerosis. *Thrombosis and Haemostasis* **106**: 757–762. DOI 10.1160/TH11-07-0500.

Iwasaki A, Medzhitov R (2004). Toll-like receptor control of the adaptive immune responses. *Nature Immunology* **5**: 987–995. DOI 10.1038/ni1112.

Joffre O, Nolte MA, Sporri R, Reis e Sousa C (2009). Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. *Immunological Reviews* **227**: 234–247. DOI 10.1111/j.1600-065X.2008.00718.x.

Kleemann R, Zadelaar S, Kooistra T (2008). Cytokines and atherosclerosis: a comprehensive review of studies in mice. *Cardiovascular Research* **79**: 360–376. DOI 10.1093/cvr/cvn120.

Koltsova EK, Garcia Z, Chodaczek G, Landau M, McArdle S, Scott SR, von Vietinghoff S, Galkina E, Miller YI, Acton ST, Ley K (2012). Dynamic T cell-APC interactions sustain chronic inflammation in atherosclerosis. *Journal of Clinical Investigation* **122**: 3114–3126. DOI 10.1172/JCI61758.

Koulis C, Chen YC, Hausding C, Ahrens I, Kyaw TS, Tay C, Allen T, Jandeleit-Dahm K, Sweet MJ, Akira S, Bobik A, Peter K, Agrotis A (2014). Protective role for Toll-like receptor-9 in the development of atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* **34**: 516–525. DOI 10.1172/JCI61758.

Kruth HS (2011). Receptor-independent fluid-phase pinocytosis mechanisms for induction of foam cell formation with native low-density lipoprotein particles. *Current Opinion in Lipidology* **22**: 386–393. DOI 10.1097/MOL.0b013e32834adadb.

Lahoute C, Herbin O, Mallat Z, Tedgui A (2011). Adaptive immunity in atherosclerosis: mechanisms and future therapeutic targets. *Nature Reviews Cardiology* **8**: 348–358. DOI 10.1038/nrcardio.2011.62.

Libby P (2012a). Inflammation in atherosclerosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **32**: 2045–2051. DOI 10.1161/ATVBAHA.108.179705.

Libby P (2012b). Inflammation in atherosclerosis. *Journal of the Association of Physicians of India* **48**: 265–266.

Lien E, Means TK, Heine H, Yoshimura A, Kusumoto S, Fukase K, Fenton MJ, Oikawa M, Qureshi N, Monks B, Finberg RW, Ingalls RR, Golenbock DT (2000). Toll-like receptor 4 imparts ligand-specific recognition of bacterial lipopolysaccharide. *Journal of Clinical Investigation* **105**: 497–504. DOI 10.1172/JCI8541.

Liuzzo G (2001). Atherosclerosis: an inflammatory disease. *Rays* **26**: 221–230.

Mayerl C, Lukasser M, Sedivy R, Niederegger H, Seiler R, Wick G (2006). Atherosclerosis research from past to present–on the track of two pathologists with opposing views, Carl von Rokitansky and Rudolf Virchow. *Virchows Archiv* **449**: 96–103. DOI 10.1007/s00428-006-0176-7.

McLaren JE, Michael DR, Ashlin TG, Ramji DP (2011). Cytokines, macrophage lipid metabolism and foam cells: implications for cardiovascular disease therapy. *Progress in Lipid Research* **50**: 331–347. DOI 10.1016/j.plipres.2011.04.002.

Mellman I, Steinman RM (2001). Dendritic cells: specialized and regulated antigen processing machines. *Cell* **106**: 255–258. DOI 10.1016/S0092-8674(01)00449-4.

Moore KJ, Sheedy FJ, Fisher EA (2013). Macrophages in atherosclerosis: a dynamic balance. *Nature Reviews Immunology* **13**: 709–721. DOI 10.1038/nri3520.

Murphy AJ, Woollard KJ, Hoang A, Mukhamedova N, Stirzaker RA, McCormick SPA, Remaley AT, Sviridov D, Chin-Dusting J (2008). High-density lipoprotein reduces the human monocyte inflammatory response. *Arteriosclerosis, Thrombosis, and Vascular Biology* **28**: 2071–2077. DOI 10.1161/ATVBAHA.108.168690.

Napoli C (1997). Low density lipoprotein oxidation and atherogenesis: from experimental models to clinical studies. *Giornale Italiano di Cardiologia* **27**: 1302–1314.

Pulendran B, Tang H, Manicassamy S (2010). Programming dendritic cells to induce T(H)2 and tolerogenic responses. *Nature Immunology* **11**: 647–655. DOI 10.1038/ni.1894.

Ridker PM, Buring JE, Rifai N (2001). Soluble P-selectin and the risk of future cardiovascular events. *Circulation* **103**: 491–495. DOI 10.1161/01.CIR.103.4.491.

Ross R (1999). Atherosclerosis—an inflammatory disease. *New England Journal of Medicine* **340**: 115–126. DOI 10.1056/NEJM199901143400207.

Schaftenaar F, Frodermann V, Kuiper J, Lutgens E (2016). Atherosclerosis: the interplay between lipids and immune cells. *Current Opinion in Lipidology* **27**: 209–215. DOI 10.1097/MOL.0000000000000302.

Son YI, Egawa S, Tatsumi T, Redlinger RE Jr, Kalinski P, Kanto T (2002). A novel bulk-culture method for generating mature dendritic cells from mouse bone marrow cells. *Journal of*

Immunological Methods **262**: 145–157. DOI 10.1016/S0022-1759(02)00013-3.

Tabas I, Bornfeldt KE (2016). Macrophage phenotype and function in different stages of atherosclerosis. *Circulation Research* **118**: 653–667. DOI 10.1161/CIRCRESAHA.115.306256.

Urzainqui A, Martínez del Hoyo G, Lamana A, de la Fuente H, Barreiro O, Olazabal IM, Martin P, Wild MK, Vestweber D, González-Amaro R, Sánchez-Madrid F (2007). Functional role of P-selectin glycoprotein ligand 1/P-selectin interaction in the generation of tolerogenic dendritic cells. *Journal of Immunology* **179**: 7457–7465. DOI 10.4049/jimmunol.179.11.7457.

Webb NR, Moore KJ (2007). Macrophage-derived foam cells in atherosclerosis: lessons from murine models and implications for therapy. *Current Drug Targets* **8**: 1249–1263. DOI 10.2174/138945007783220597.

Weber C, Zernecke A, Libby P (2008). The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nature Reviews Immunology* **8**: 802–815. DOI 10.1038/nri2415.

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Ye Z, Jin M, Wang S, Zhang J, Song X, Huang R (2017). Subcutaneous injection of dendritic cells aggravates atherosclerosis in ApoE knockout mice by activation of TLR4. *Molecular Medicine Reports* **16**: 6041–6049. DOI 10.3892/mmr.2017.7339.