Impairment of the PD-1 pathway increases atherosclerotic lesion development and inflammation

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SUPPLEMENTAL MATERIALS AND METHODS

Mice
Mice were fed a clinton/cybulsky high fat rodent diet with 1.25% added cholesterol \(^1\) for 5 and 10 weeks. Wild type C57BL/6 mice, used as bone marrow donors, were purchased from Jackson Laboratories.

Bone marrow transplantation (BMT) protocol
Briefly, at 6 weeks of age, recipient male \(Ldlr^{-/-}\) mice were lethally irradiated using a cesium source (total dose 1300 Rads) split in two doses 4 hours apart. Marrow was harvested from donor male C57BL/6 or male \(Pd-l1/2^{-/-}\) mouse femurs after the second irradiation; 10 million marrow cells were injected into the retro-orbital venous plexus of recipient mice. Recipient mice were fed normal chow and were treated with Septrin for 4 weeks while hematopoietic reconstitution took place. Assessment for successful reconstitution was made by flow cytometric analysis of blood leukocytes, PCR for the wildtype \(Ldlr\) allele in blood DNA, and flow cytometry for PD-L1 expression on the chimeric white blood cells stimulated with IFN\(\gamma\) for 24 hours in vitro. Starting at 4 weeks after marrow injection, chimeric mice were fed cholesterol-containing diet for 10 weeks.

Serum lipid analysis
After overnight fasting, blood was collected for lipid profiles. Briefly, total cholesterol and triglycerides were measured by enzymatic colorimetric assays using cholesterol esterase/oxidase
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and lipoprotein lipase, respectively. The low and high density lipoprotein cholesterol (LDL and HDL) were measured using homogeneous enzymatic colorimetric assays.

Multiplexed Cytokine assays
Sera taken from mice at the end of the experiment and culture supernatants were analyzed for cytokine concentrations using luminex bead-based multiplex assays specific for IL-4, 5, 6, 10, 12p40, 12p70, IL17a, IFNγ, TNFα, and MCP-1. Recombinant cytokine standards (Bio-Rad, Hercules, CA) were used to calculate cytokine concentrations and data were analyzed using StarStation 2.3 software (Applied Cytometry, Sheffield UK).

Atherosclerotic Lesion Assessment
Briefly, aortic roots cryosections (7 μm thick) throughout the aortic sinus (total distance covered, approximately 300 μm) were taken for analyses. From the area in which 3 aortic valve cusps are clearly seen, 6 to 8 sections at 35 μM interval were collected for Oil Red O (ORO) staining for lesional size quantification. Other sections were reserved for other analyses. The area from the aortic arch to iliac aortas was formalin fixed, pinned-out and en face stained with ORO. Images of sections were captured digitally and quantified using IMAGEPROPLUS software (Media Cybernetics). For aortic root sections, the plaque lesion area was quantified, and the results were expressed as the mean of 6 to 8 sections per mouse. Quantification of the ORO stained en face lesions of aortic arch and abdominal aortas was performed as described 2, 3. Measurements and evaluation of the atherosclerotic lesions were performed in a blinded fashion.
Immunohistochemistry and immunofluorescent staining of aortic lesions

Frozen aortic root sections were fixed with acetone stained with antibodies specific for murine CD4, CD8, F4/80 (for macrophages) and smooth muscle cell α actin (SMC-α actin for SMC), as described 2,3. Briefly, acetone fixed cryostat sections were preincubated with 5% goat serum prior to incubation with primary rat anti mouse antibodies (BD Biosciences, Pharmingen) for 1h in room temperature, then were subsequently subjected to incubation with secondary biotinylated goat anti rat antibody followed by avidin-biotin peroxidase complex (Vector Laboratories) or Alkaline phosphatase substrate (Vector laboratories) for SMC-α actin, and developed with AEC solution (0.2mol/L Tris and 0.05 mol/L Tirs-HCl) or Levamisole (Vector laboratories). All the sections were counterstained with Hematoxylin. Quantification of F4/80 and SMC positive staining was performed by computer-assisted image analysis and expressed as percentage of intimal area, in order to normalize for differences related to wall remodeling between the study groups. Quantification of CD4+ and CD8+ T cells was performed by counting individual positively stained lesional cells. Two-color immunofluorescence staining was performed to identify CD3+CD4+ or CD3+CD8+ T cells in aortic lesions. After preincubation with 5% normal serum for 30 minutes, aortic roots sections were incubated with primary the above primary antibodies CD4 or CD8 for 1h in room temperature, followed by goat anti-rat biotin Alexa Fluor 555 10 µg/mL (Molecular Probes). Subsequently, 1 µg/mL anti-mouse CD3 (Alexa Fluor 488, BD Biosciences, Pharmingen) was incubated for 30 minutes at room temperature. Two color immunofluorescence was also performed to quantify apoptotic SMC in lesions. Slides were double stained with anti-SMC-α actin (FITC, Sigma) and anti-annexin V (Alexa Fluor® 568, Invitrogen). After washing with distilled water, slides were coversliped with Fluorsave mounting
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medium (with DAPI) (Vector Laboratories). Fluorescent images were acquired with a LSM510 META (Zeiss) confocal microscope using a 40x or 63x oil-immersion objective and were analyzed using LSM Image Browser software and quantified by MetaMorph software.

Aortic digestions and cell recovery
Briefly, dissected aortas were digested with 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s, 60 U/ml DNase1, and 450 U/ml collagenase type I (all enzymes were obtained from Sigma-Aldrich) in PBS containing 20 mM Hepes at 37°C for 1 h. Cell suspension was obtained by passing the digest through a cell strainer, and the cells were then immunofluorescently stained and analyzed by flow cytometry.

Immunostaining and flow cytometric analysis of cell suspensions from lymphoid tissues and aorta
Splenocytes, iliac node lymphocytes and aortic digests were stained for CD3, CD4, CD8, CD62L, CD25, CD44 and PD-1. Blood from chimeras was stained for PD-L1. All antibodies were purchased from Biolegend. For intracellular IFNγ (XMG 1.2, Biolegend), the staining buffer set from eBioscience was used according to the manufacturer recommended protocol. In brief, after extracellular staining with other antibodies, the cells were paraformaldehyde-fixed, permeabilized with detergent, and stained with PE-labeled 0.2 µg anti-mouse/rat IFNγ. All analyses were performed on a FACS caliber flow cytometer (BD Biosciences) with FlowJo 7.2.5 software (BD Biosciences).

CTL killing Assay

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Mouse heart endothelial cells (EC) were prepared from juvenile mouse hearts by collagenase I digestion (Worthington), followed by sequential magnetic bead sorting (Dynal), using beads coated with antibodies to CD31 and CD102 (BD Pharmingen). Mouse aortic smooth muscle cells (SMC) were prepared from juvenile mouse aortas by digestion, first with 1mg/mL collagenase II (Gibco), and then with 1mg/mL Elastase III (Sigma) and 2.5mg/mL collagenase I (Gibco). EC or SMC were plated on fibronectin-coated 12 well plates and grown to confluence. Monolayers were pretreated with 100U/ml IFNγ (Peprotech), and 300ng SIINFEKL peptide for 2 hours, washed twice with PBS, and then incubated with the activated, rested CD8 T cells from either Pd1− OT-1 and Pd1+/+ OT-1 for one hour. Plates were then washed twice in PBS, and detached from the plate using Trypsin-Versene (Lonza).Cells were surface stained using CD90.1-APC (Biolegend), in order to identify and exclude T cells from the analysis. Cells were washed twice more in DPBS and then stained with annexinV-PE and 7-AAD in annexin binding buffer (BD Pharmingen), and analyzed by flow cytometry (BD FacsCalibur). Analysis was performed using FloJo (Tree Star).

**CD4+ and CD8+ T cell purifications and quantitative RT-PCR (qRT-PCR) analyses**

Splenic CD4+ or CD8+ T cells were purified by MACS beads (Miltenyi Biotec). Total RNA was extracted by RNeasy kit (QIAGEN), and reverse-transcribed using the ThermoScript RT-PCR system and random hexamer primers according to the manufacturer’s instructions (Invitrogen), and amplified by real-time PCR with SYBR Green PCR mix (Applied Biosystem) and Step-One Detection System (Applied Biosystem) according the manufacturer’s instructions. Levels of specific gene expression in the samples are presented relative to endogenous levels of Actb gene expression in the same sample. The sequences of the primers are listed in Table SIV.
In vitro cell proliferation assay

Purified splenic CD4⁺ or CD8⁺ T cells were stimulated in 96-well cultures with αCD3 (5µg/ml) or human copper-oxidized LDL (oxLDL, 10 µg/ml, Biomedical Technologies Inc.) plus irradiated T cell–depleted spleen cells (1 × 10⁶ cells/well) for 72h. In some experiments, iliac node lymphocytes were stimulated with αCD3 (5µg/ml) or oxLDL (10 µg/ml) and cultured in 96-well plate for 72h. Culture supernatants were removed at 48 hours and analyzed by Luminex cytokine assays. During the last 16 h, cells were pulsed with 1 μCi of [³H] thymidine (PerkinElmer) followed by harvesting and analysis of incorporated [³H] thymidine in a β-counter (1450 Microbeta, Trilux, PerkinElmer).
Figure I. Effects of PD-1 deficiency on T cell activation of the atherosclerotic mice. a. Cell counts on iliac lymph nodes from Pd1−/− Ldlr−/− and Ldlr−/− mice after 5 week of cholesterol diet. b to e. FACS analyses for numbers of total CD4+ (b) and CD8+ (d) T cells and a fraction of activated T cells (CD25+) in the subset of CD4+ (c) and CD8+ (e) T cells of iliac lymph nodes from Pd1−/− Ldlr−/− and Ldlr−/− mice after 5 week cholesterol diet. n=5 to 6 from each group. Data shown are mean ± SEM. Differences between two groups of mice were analyzed by the Mann-Whitney test.
Figure II. Characterization of hematopoietic reconstitution with donor cells in bone marrow chimeras. 

a. PCR detection of the wildtype *Ldlr* DNA (*Ldlr wt*) gene product (383bp) in PBLs 4 weeks after transplantation. The upper bands are *Ldlr* knockout (*Ldlr ko*) gene products (800bp). “WT chimeras” represents control *Pd-l1/2*+/+*Ldlr*+/+ → *Ldlr*−/− bone marrow chimeras while “*Pd-l1/2* chimeras” represents *Pd-l1/2*+/+*Ldlr*+/+ → *Ldlr*−/− chimeras. Numbers C1-C8 refers to different chimeric mice; three controls were used here: “KO” indicates a non-irradiated *Ldlr*−/− mouse, “Het” is a “heterozygous non-irradiated *Ldlr*+/+ mouse” and “Wt” is a non-irradiated C57Bl/6 *Ldlr*+/+ mouse.

b. FACS analyses for PD-L1 expression in PBLs 4 weeks after transplantation. Blood leukocytes from non-irradiated *Ldlr*−/− mouse and chimeras were treated with or without 100 unit/ml IFNγ for 24h, stained for PD-L1, and analyzed by flow cytometry. Data is representative from 8 mice from each group of chimeras.
Figure III. Effects of haematopoietic PD-L1/2 deficiency on atherosclerotic lesion development. a. Representative cross sections of Oil Red O (ORO) stained aortic roots (original magnification x40) and of immunohistochemistry for CD4, CD8, macrophage (F4/80) and smooth muscle cell (SMC-α-actin) on aortic sinus from $Pd-l1/2^{-/-}$ and WT bone marrow chimeras after 10 weeks of cholesterol diet (original magnification x200). b. Quantitative
analysis of immunohistochemical staining of lesions from the same mice. Each data point represents the mean value determined for each mouse; horizontal bars represent the mean value for each group. Differences between two groups of mice were analyzed the Mann-Whitney test, NS indicates not statistically significant.
Figure IV. Marked increases in CD4⁺ and CD8⁺ T cells are consistently seen in atherosclerotic lesions of Ldlr⁻/⁻ mice with impaired PD-L/PD-1 signaling. Double immuno-fluorescent staining for CD3 (Green) and CD4/CD8 (red) were performed in the aortic sinus sections. a to c are representative of a projection of stacked sections stained with CD3 and
CD4 or CD8 antibody and mounted with DAPI mounting medium. Aortic sinus sections are selected from the adjacent sections which were underwent immnohistochemistry for CD4, CD8 and macrophages (shown in above figures) of $Pd1^{-/-} Ldlr^{-/-}$ and $Ldlr^{-/-}$ mice after 5 or 10 weeks of cholesterol diet (a); of $Ldlr^{-/-}$ mice after 5 weeks of cholesterol diet underwent PD-1 antibody or rat IgG treatment (b); of $Pd-l1/2^{-/-} Ldlr^{+/+} \rightarrow Ldlr^{-/-}$ chimeras, $Pd-l1/2^{+/+} Ldlr^{+/+} \rightarrow Ldlr^{-/-}$ chimeras after 10 weeks of cholesterol diet (c). Arrow bars indicate triple positive stained cells (yellow and orange); scale bars: 20µm.
Figure V. Hypercholesterolemia induces increased PD-1 expression on aortic T cells. Total number of CD3⁺, CD3⁺ CD8⁻, and CD3⁺CD8⁺ T cells was evaluated by FACS staining of pooled cell suspensions prepared from the enzymatically digested aortas of three Ldlr⁻/⁻ mice either fed with cholesterol or control diet for 8 weeks (a – c). Number of PD-1⁺ cells in the subset of CD3⁺ CD8⁻, and CD3⁺CD8⁺ T cells evaluated by FACS from the above samples (d, e). n=6 per group. Data shown are mean ± SD. Data are from 1 of 2 experiments with similar results.
Table I. Mice Serum Lipid profiles in *Pd1<sup>−/−</sup>Ldlr<sup>−/−</sup>* and control mice

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<td><strong>mg/dl</strong></td>
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<td><strong>Total cholesterol</strong></td>
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<td><strong>Triglyceride</strong></td>
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<td>169.5±5.2</td>
<td>195.3±6.3</td>
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**Table I to III.** Mouse blood was collected from heart at time of euthanasia; total cholesterol and triglycerides were measured by enzymatic colorimetric assays using cholesterol esterase/oxidase and lipoprotein lipase, respectively. The low and high density lipoprotein cholesterol (LDL and HDL) were measured using homogeneous enzymatic colorimetric assays. n=8 to 10 per group. Data shown are mean ± SEM. * indicates p<0.05 when compared to control diet group. Differences between two groups of mice were analyzed by Student’s *t* test.
Table II. Serum lipid profiles in anti-PD-1 treated and control mice after 5 w cholesterol diet

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<td>HDL cholesterol</td>
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<td>Triglyceride</td>
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Table III. Mice Serum lipid profiles in bone marrow chimeras after 10 w cholesterol diet

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<th>Control chimeras</th>
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<tr>
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<td>LDL cholesterol</td>
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<td>HDL cholesterol</td>
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<td>Triglyceride</td>
<td>328.5±65.8</td>
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Table IV: Mouse Oligonucleotide primers used for qRT-PCR

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Online Supplement References


