Impairment of the Programmed Cell Death-1 Pathway Increases Atherosclerotic Lesion Development and Inflammation

De-xiu Bu, Margarite Tarrio, Elena Maganto-Garcia, George Stavrakis, Goro Tajima, James Lederer, Petr Jarolim, Gordon J. Freeman, Arlene H. Sharpe, Andrew H. Lichtman

Objective—Programmed cell death-1 (PD-1) is a member of the CD28 superfamily that delivers negative signals on interaction with its 2 ligands, PD-L1 and PD-L2. We studied the contribution of the PD-1 pathway to regulation of T cells that promote atherosclerotic lesion formation and inflammation.

Methods and Results—We show that compared with Ldlr⁻/⁻ control mice, Pdl1⁻/⁻Ldlr⁻/⁻ mice developed larger lesions with more abundant CD4⁺ and CD8⁺ T cells and macrophages, accompanied by higher levels of serum tumor necrosis factor-α. Iliac lymph node T cells from Pdl1⁻/⁻Ldlr⁻/⁻ mice proliferated more to αCD3 or oxidized low-density lipoprotein stimulation compared with controls. CD8⁺ T cells from Pdl1⁻/⁻Ldlr⁻/⁻ mice displayed more cytotoxic activity compared with controls in vivo and in vitro. Administration of a blocking anti-PD-1 antibody increased lesional inflammation in hypercholesterolemic Ldlr⁻/⁻ mice with more lesional T cells and more activated T cells in paraaortic lymph nodes. The changes in lesional T-cell content when PD-1 was absent or blocked were also observed in bone marrow chimeric Ldlr⁻/⁻ mice lacking PD-L1 and PD-L2 on hematopoietic cells.

Conclusion—PD-1 has an important role in downregulating proatherogenic T-cell responses, and blockade of this molecule for treatment of viral infections or cancer may increase risk of cardiovascular complications. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: atherosclerosis ■ cytokines ■ immune system ■ T cells ■ costimulation

A D4⁺ Th1 cells contribute significantly to the progression of atherosclerosis. Previous work from our laboratory has demonstrated the importance of the B7/CD28 costimulatory pathway in the priming of proatherogenic Th1 responses. Negative regulatory (“coinhibitory”) members of the B7/CD28 family are also important modulators of T-cell responses in a variety of disease conditions. The programmed cell death–1 (PD-1) receptor binds programmed death–ligand 1 (PD-L1) and PD-L2 (also known as B7-H1 and B7-DC, respectively). PD-1 is inducibly expressed on T cells, B cells, macrophages, and some types of dendritic cells. PD-L1 is expressed on both hematopoietic and nonhematopoietic cells types. In contrast, PD-L2 expression is restricted mainly to dendritic cells and macrophages. PD-1 and PD-L1 interactions have been implicated in controlling immune tolerance, autoimmunity, and immune responses to viral infections and tumors. Persistent antigenic stimulation in the setting of chronic viral infection leads to sustained upregulation of PD-1 expression on viral-specific CD8⁺ cytotoxic T lymphocyte (CTL) and contributes to T-cell exhaustion; therefore, antibody-mediated blockade of PD-1 or PD-L1 is being studied as a new therapeutic approach for chronic viral infections and cancer.

We have shown that PD-L1 is expressed on both dendritic cells and macrophages in aortic lesions in hypercholesterolemic low-density lipoprotein receptor knockout (Ldlr⁻/⁻) mice, and hypercholesterolemic Ldlr⁻/⁻ mice that lack PD-L1 and PD-L2 develop significantly increased atherosclerosis with more lesional T cells compared with Ldlr⁻/⁻ controls. However, the role of PD-1 in regulating proatherogenic T-cell responses has not been directly examined. PD-1 is known to inhibit T-cell activation by binding to B7-1, as well as to PD-L1 and PD-L2, and it is possible that other receptors for PD-L1 exist. Because PD-1, and not PD-L1 or PD-L2, is the target of new therapies being developed for cancer and chronic viral infections, it is important to know whether PD-1 is required to regulate proatherogenic T-cell responses in the arterial wall. Here we describe in vivo studies that directly address this question.

Materials and Methods

An expanded Supplemental Methods section is available online at http://atvb.ahajournals.org.

Received on: December 2, 2010; final version accepted on: February 25, 2011.

From the Departments of Pathology (D.-x.B., M.T., E.M.-G., G.S., P.J., A.H.S., A.H.L.) and Surgery (G.T., J.L.), Brigham and Women’s Hospital, Department of Medical Oncology, Dana-Farber Cancer Institute (G.J.F.) and Department of Medicine (G.J.F.), Harvard Medical School, Boston, MA.

Correspondence to Andrew H. Lichtman, Brigham and Women’s Hospital, NRB Rm 752N, 77 Ave Louis Pasteur, Boston, MA 02115. E-mail alichtman@rics.bwh.harvard.edu

© 2011 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.111.224709
Animal Studies

Pdl1−/− mice on a C57BL/6 background, derived by targeted mutation in C57BL/6 ES cells, which results in deletion of the IgV domain, were crossed with Ldlr−/− mice on a C57BL/6 background (Jackson Laboratory) to establish a Pdl1−/−Ldlr−/− double-knockout mice. Pdl1−/−Ldlr−/− mice, derived as described, or C57BL/6 mice (Jackson laboratories) were used as bone marrow donors to produce Pdl1−/−Ldlr−/− or Pdl1−/−/−Ldlr−/− radiation chimeras, as described. Some Ldlr−/− mice were injected intra-peritoneally with anti-mouse PD-1 antibody (29F.1A12.D5, prepared in-house) or rat IgG2a (Bio X Cell, catalog no. BE0089), 200 μg/mouse, twice a week for 3 weeks. Pdl1−/− OT-1 transgenic mice were generated by backcrossing Pdl1−/− mice with OT-1 TCR transgenic mice. All the mice were given water ad libitum and were maintained on a 12/12-hour light/dark cycle under pathogen-free conditions in the Harvard New research building animal facility according to institutional and National Institutes of Health guidelines.

Serum Lipid Analysis

Mice lipid profiles were measured on the c501 module of the Cobas 6000 analyzer (Roche Diagnostics, Indianapolis, IN) using the assays developed for human use.

Multiplexed Cytokine Assays

Sera and culture supernatants were analyzed for cytokine concentrations using Luminex bead-based multiplex assays.

Atherosclerotic Lesion Assessment

Atherosclerotic lesions were analyzed in the aortic root, aortic arch, and descending aorta as previously described.

Immunohistochemistry and Double Immunofluorescence Staining of Aortic Lesions

Frozen aortic root sections were stained with antibodies specific for CD4, CD8, F4/80 for macrophages, and smooth muscle cell-α actin (SMC-α actin) for SMC, as described. Double immunofluorescence staining for CD3 and CD8 or CD4 or CDS was performed in the aortic sinus sections, as well as for SMC (SMC-α actin, fluorescein isothiocyanate, Sigma-Aldrich) and annexin V (Alexa Fluor 568, Invitrogen). Nuclei were stained with 4’,6-diamidino-2-phenylindole.

Cell Immunostaining and Flow Cytometry

Splenocytes, ieliac node lymphocytes, and aortic digests were stained for CD3, CD4, CD8, CD62L, CD25, CD44, and PD-1. Aortas from the ascending aorta to iliac bifurcation were cleaned of periadventitial connective tissue and subjected to enzymatic digestion, as described.

CTL Killing Assay

Mouse aortic SMC and mouse heart endothelial cells (EC) were prepared as previously described and cocultured with mouse Pdl1−/− OT-1 or Pdl1−/−/− CD8+ OT-1 CTL plus SIINFKL peptide antigen for 2 hours. Apoptotic cells were then quantified by annexin V/7-aminoactinomycin D (7-AAD) staining and flow cytometry.

CD4+ and CD8+ T-Cell Purifications and Quantitative Reverse Transcription–Polymerase Chain Reaction Analyses

Splenic CD4+ or CD8+ T cells were purified by immunomagnetic beads, and RNA was isolated, reverse transcribed, and subjected to quantitative reverse transcription–polymerase chain reaction analyses for the genes listed in Supplementary Table IV.

In Vitro Cell Proliferation Assay

CD4+ and CD8+ T cells were purified from spleen and lymph node of mice and stimulated with αCD3 or oxidized low-density lipoprotein (oxLDL) in vitro, and proliferation was measured by 3H-thymidine incorporation.

Statistics

All statistical analyses were performed using Prism software. Differences between 2 groups of mice were analyzed by Student t test and expressed as mean±SEM or by the Mann-Whitney test (for nonparametric data). For experiments with 3 or more groups, ANOVA with the Tukey multiple comparison test was used. A value of P<0.05 was considered significant.

Results

Atherosclerotic Lesion Development and Phenotype in Pdl1−/−/−Ldlr−/− Mice

We generated Pdl1−/−Ldlr−/− mice by crossbreeding the relevant parent strains, and we compared lesion development in Pdl1−/−Ldlr−/− and Ldlr−/− mice fed a cholesterol diet for 5 and 10 weeks. Serum lipids did not differ significantly between the 2 groups of mice after either 5 or 10 weeks of this diet (Supplemental Table I). At 5 weeks, lesion size was similar in both groups (Figure 1a and 1c), but the lesions of the PD-1-deficient mice had markedly more inflammatory cells, including CD4+ and CD8+ T cells and macrophages (Figure 2a and 2b). After 10 weeks of the diet, the Pdl1−/−Ldlr−/− mice had increased lesion size compared with Ldlr−/− controls (Figure 1a and 1c). The percentage of the total cross-sectional vessel wall area was increased, and there was an outward remodeling, resulting in an increase in total aortic root cross-sectional area in Pdl1−/−Ldlr−/− mice compared with Ldlr−/− controls (Figure 1a). Additionally, endothelial lesion analysis of the entire aorta distal to the aortic root revealed more lesions in the Pdl1−/−Ldlr−/− mice than in the Ldlr−/− controls (Figure 1b and 1d). Immunohistochemical staining revealed more abundant CD4+ and CD8+ T cells and macrophages in the lesions of Pdl1−/−Ldlr−/− mice compared with Ldlr−/− mice (Figure 2a and 2b). SMC content was similar in the lesions of both groups of mice (Figure 2a and 2b, bottom panels). All these data indicate that PD-1 acts to limit proatherogenic immune responses.

Effects of PD-1 Deficiency on Lesional Cell Death and CTL Cytotoxicity

The increased number of CD8+ T cells in lesions of Pdl1−/− mice and the lack of increased SMC content despite an overall increase in lesion size suggested that PD-1 deficiency may cause more CD8+ CTL cytotoxicity and SMC death in the lesions. We therefore performed double immunofluorescence staining of aortic sinus of the Pdl1−/−Ldlr−/− and Ldlr−/− mice for annexin V (to detect apoptotic cells) and SMC-α actin (to detect SMC). We found more apoptotic cells, including SMC and non-SMC (Figure 3a and 3b), in the lesions of PD-1-deficient mice after 10 weeks of diet, compared with the lesions from control mice. We did not detect any annexin V–positive lesional SMC at 5 weeks of diet (data not shown). We also performed an in vitro CTL killing assay by coculturing mouse aortic SMC or heart EC with Pdl1−/− or control CD8+ CTL from OT-1 TCR transgenic mice. Fluorescence-activated cell sorting (FACS) analyses of annexin V/7-AAD–stained cells demonstrated that...
effector Pdl−/− OT-1 cells kill more SMC or ECs compared with Pdl+/+ OT-1 (Figure 3c).

**Effects of PD-1 Deficiency on T-Cell Gene Expression and Immune Responses in Ldlr−/− Mice**

To determine why CD4+ and CD8+ T-cell infiltration was greater in the lesions of hypercholesterolemic Pdl−/− Ldlr−/− mice, we analyzed expression of selected genes by quantitative reverse transcription–polymerase chain reaction of RNA from purified splenic CD4+ and CD8+ T cells, taken after 10 weeks of cholesterol diet. The results demonstrated that both PD-1-deficient CD4+ and CD8+ T cells expressed higher levels of proinflammatory cytokine genes Ifng and Tnf, as well as increased levels of chemokine receptor genes Ccr5, Ccr6, and Cxcr3, compared with T cells from Ldlr−/− mice (Figure 4a and 4b). These data suggested that PD-1-deficient T cells may be more competent at migrating to inflammatory sites compared with wild-type T cells and that they produce more inflammatory cytokines.

To evaluate the changes in T-cell activation in the absence of PD-1 in the context of atherosclerosis, we examined iliac lymph nodes and spleens from hypercholesterolemic Pdl−/− Ldlr−/− mice. After 5 weeks of diet,
there was no difference in the number of iliac lymph node cells between these 2 groups (Supplemental Figure 1a). However, FACS analyses showed that there were more total CD4+ T cells and a higher fraction of activated CD4+ and CD8+ T cells (CD25+ or CD69+) compared with T cells from control mice. (Supplemental Figure Ib to Ie), although the number of splenic CD4+ and CD8+ T cells and the fraction of activated T cells in the spleens were similar between these 2 groups after 5 or 10 weeks of diet (data not shown). Therefore, we asked whether the iliac lymphocyte response to TCR stimulation might be different when PD-1 signaling is absent in the putative athero-antigen oxLDL in vitro. a, Representative images of double immunofluorescence staining for annexin V (red) and SMC-α actin (green) and mounted with 4',6-diamidino-2-phenylindole mounting medium (blue) in aortic sinus section from Pdl1−/− Ldlr−/− and Ldlr−/− mice after 10 weeks of cholesterol diet. Arrows indicate triple-positive stained cells (yellow and orange) in the top panels and annexin V (red)-positive but SMC-negative cells in the bottom panels. Scale bars=20 μm. b, Quantitative analysis of double-positive staining for annexin V and SMC-α actin (upper panel) and of annexin-positive but SMC-α actin-negative staining (lower panel) for each experimental group. Each data point represents the mean value determined from 3 aortic sinus sections from each mouse; horizontal bars represent the mean value for all the mice in each group. *P<0.05 (ANOVA with the Tukey multiple comparison post test). c, FACS analyses for CTL killing of mouse aortic SMC (left panel) and mouse heart EC (right panel). Mouse effector CD8+ T cells prepared from Pdl1−/− OT-1 and Pdl1−/− OT-1 transgenic mouse (see details in Supplemental Materials) were cocultured with mouse aortic SMC or mouse heart EC, respectively, for 1 hour before being stained for CD90 and annexin V and 7-AAD. Neither splenic CD8+ nor CD4+ T cells were characterized as being in late apoptosis, and cells that were positive for 7-AAD but not annexin V were categorized as dead. Data shown are mean±SD and are representative of 2 different sets of experiments with similar results. Differences between 2 groups of mice were analyzed by the Mann-Whitney test. *P<0.05 indicates significant difference compared with the counterpart Pdl1−/− T-cell group.

Effects of PD-1 Blocking Antibody Administration on Atherosclerotic Lesions in Ldlr−/− Mice

We next evaluated the impact of an anti-PD-1 blocking antibody on atherosclerosis. Six-week-old Ldlr−/− mice were fed a cholesterol diet for 5 weeks, and starting from the third week of the diet, mice received intraperitoneal injections of PD-1 antibody or rat IgG, 200 μg/mouse, twice a week for 3 weeks before all the tissue collections. Serum lipids did not differ significantly between the 2 groups at time of euthanization (Supplemental Table II).

PD-1 antibody treatment did not change the lesion size development, but it significantly increased lesional CD4+ and CD8+ T cells compared with mice receiving control IgG at 5 weeks of diet (Figure 5a and 5b). The two groups had similar lesional macrophage and SMC contents (Figure 5a and 5b, bottom panel).

Evaluation of iliac lymph nodes showed that mice receiving anti-PD-1 antibody treatment developed larger nodes with more CD4+ T cells and CD8+ T cells (Figure 6a, 6b, and 6e), FACS analyses of activation markers on T cells revealed more CD4+ and IFN-γ producing CD4+ and CD8+ T cells (Figure 6c, 6d, 6f, and 6g) in the anti-PD-1-treated group. Moreover, the percentage of splenic IFN-γ producing CD4+ T cells and CD8+ T cells was significantly higher in the mice treated with anti-PD-1 antibody compared with the control IgG treated mice (data not shown), although the total percentages of splenic CD4+ T cells and CD8+ T cells were similar in both groups of mice.
Lesion size did not differ between recipients of marrow chimeras were euthanized, and aortic root lesions were analyzed. Serum lipids did not differ significantly between the 2 groups at that time (Supplemental Table III). To further characterize the lesional infiltrating CD4 cells, we performed double immunofluorescence stainings on splenic CD8 T cells in response to cCD3 for 48 hours. Splenic CD8 T cells were purified from Pdl1−/− Ldlr−/− and Ldlr−/− mice after 5 weeks of cholesterol diet. d and e, Iliac lymphocyte proliferation measured by 3H-thymidine incorporation during the final 16 hours of a 72-hour culture with cCD3 (f) or oxLDL (g). f and g, TNF-α secretion measured by Luminex cytokine assays (see Materials and Methods) from supernatants of iliac lymphocytes cultured with cCD3 for 48 hours from Pdl1−/− Ldlr−/− and Ldlr−/− mice after 5 weeks of cholesterol diet (f) and from sera of Pdl1−/− Ldlr−/− and Ldlr−/− mice after 10 weeks of cholesterol diet (g). c to f, n = 6 from each group; g, n = 10 from each group. Data shown are mean ± SEM. Differences between 2 groups of mice were analyzed by the Mann-Whitney test.

Figure 4. Effects of PD-1 deficiency on T-cell gene expression and immune responses of the atherosclerotic mice. a and b, Quantitative reverse transcription–polymerase chain reaction analyses of Il7r, Tnf, Ccr5, Ccr6, and Cxcr3 were performed on unstimulated splenic CD8 (a) and CD4 T-cell (b) RNA isolated from Pdl1−/− Ldlr−/− and Ldlr−/− mice after 10 weeks of cholesterol diet. n = 8 from each group. Data shown are mean ± SEM. c, FACS analyses for intracellular IFN-γ expression by immunofluorescence staining on splenic CD8 T cells in response to cCD3 for 48 hours. Splenic CD8 T cells were purified from Pdl1−/− Ldlr−/− and Ldlr−/− mice after 5 weeks of cholesterol diet.

PD-L1/2 Deficiency in Bone Marrow–Derived Cells Results in Enhanced Atherosclerotic Lesion Inflammation

We have previously reported that cholesterol diet–fed Pdl1/2−/− Ldlr−/− mice had more lesion development and more lesion inflammation than Ldlr−/− controls.11 To assess whether PD-L1 and PD-L2 expression on bone marrow–derived cells was responsible for regulation of proatherogenic T-cell responses, we prepared Pdl1−/− Ldlr−/− → Ldlr−/− and control Pdl1−/2−/− Ldlr−/− → Ldlr−/− bone marrow chimeras. Hematopoietic reconstitution with donor cells was confirmed by polymerase chain reaction detection of the wild-type Ldlr allele in an aliquot of blood collected 4 weeks after bone marrow transplantation (Supplemental Figure IIA). The rest of the blood was lysed and cultured with or without IFN-γ stimulation overnight to induce PD-L1 expression. FACS analysis showed that PD-L1 was expressed to similar levels in all the wild-type to Ldlr−/− chimeras after IFN-γ treatment, whereas no PD-L1 was detected in all Pdl1/2−/− → Ldlr−/− chimeras (Pdl1-Ldlr−/− chimeras) (Supplemental Figure IIB). All these data indicated a successful reconstitution. Therefore, we started feeding the chimeras with the cholesterol diet at this point.

After 10 weeks of cholesterol diet feeding, the bone marrow chimeras were euthanized, and aortic root lesions were analyzed. Serum lipids did not differ significantly between the 2 groups at that time (Supplemental Table III). Lesion size did not differ between recipients of Pdl1/2−/− or Pdl1/2−/− marrow (Supplemental Figure IIIA and IIIB, top panels). However, there were significantly more CD4+ T cells, CD8+ T cells, and macrophages in the lesions of the Pdl1/2−/− marrow recipients (Supplemental Figure IIIA and IIIB), whereas SMC content was equivalent in both group of chimeras (Supplemental Figure IIIA and IIIB, bottom panel). Strikingly, CD8+ T cells were abundant in the lesions of wild-type to Ldlr−/− chimeras. This result is consistent with our previous findings in the hypercholesterolemic Pdl1/2−/− Ldlr−/− mice.11 There was no difference in the expression of activation markers on splenic or iliac node T cells between the 2 groups, and the T cells from spleen and nodes of both groups proliferated the same amount and secreted comparable amounts of cytokines in response to in vitro reactivation by cCD3 or oxLDL. Furthermore, there was no evidence of increased systemic inflammation in either group, because plasma cytokines were mostly undetectable (data not shown).

Marked Increases in Both CD4+ and CD8+ T Cells Are Consistently Seen in Atherosclerotic Lesions of Ldlr−/− Mice With Impaired PD-L/PD-1 Signaling

To further characterize the lesional infiltrating CD4+ cells and CD8+ cells, we performed double immunofluorescence staining for CD3 and CD4 or CD8 in aortic sinus lesions from Pdl1−/− Ldlr−/− mice, anti-PD-1-treated mice, and Pdl1/2−/− bone marrow chimeras, as well as appropriate controls. Confocal analyses of these stained lesions showed marked increases in both CD4+ and CD8+ T cells in mice with deficient or blocked PD-1 pathway (Supplemental Figure IV).
PD-1 Is Upregulated on Aortic T Cells in Hypercholesterolemic Ldlr−/− Mice

We examined whether diet-induced hypercholesterolemia could induce increased PD-1 expression in T cells from aortic lesions. Pooled aortas from Ldlr−/− mice fed either a cholesterol diet or a control diet for 8 weeks were enzymatically digested, and the recovered leukocytes were stained for CD3, CD8, and PD-1. The enzymatic digestion rendered CD4 undetectable, and we therefore judged that a large majority of the CD3+ CD8− cells were CD4+ T cells. FACS analyses indicated that the cholesterol diet induced an increase in aortic wall T cells, including CD3+ CD8− T cells and CD3+ CD8− T cells (Supplemental Figure Va to Vc). More T cells from hypercholesterolemic aortas expressed PD-1 than T cells from aortas of Ldlr−/− mice fed the control diet (Supplemental Figure Vd and Vc).

Discussion

In this study, we established a clear role for the PD-1 in modulating experimental atherosclerosis in Ldlr−/− mice. The influence of the PD-1 pathway on atherosclerosis was examined directly using 2 different approaches, complete genetic loss of PD-1 receptor and treatment with blocking antibody to PD-1. In addition, we used bone marrow chimeras to study the effects of a lack of PD-1 receptors PD-L1/ PD-L2 only on hematopoietically derived cells. Our data from all 3 approaches indicate that the PD-1 pathway tightly controls lesional T-cell responses and restrains a potentially robust CD8+ T-cell response, which is usually minimal in mice when this coinhibitory pathway is intact.

PD-1 is a well-characterized receptor on T cells for PD-L1 and PD-L2. PD-L1 also has another receptor, B7-1,4 and a second receptor for PD-L2 has been proposed but not yet published.15 PD-1 is an activation antigen that is upregulated by T-cell activation and returns to basal levels following antigen clearance. In chronic infection and cancer, PD-1 remains high. Thus, the PD-1 molecule has been recognized as a hallmark of T-cell exhaustion, and PD-1-expressing antigen-specific T cells are dysfunctional in cytokine production and proliferation on antigen restimulation in a variety of viral infections.7,14,21 The role of PD-1 in atherosclerosis has not been studied previously.

Our data comparing hypercholesterolemic Pdl1−/−Ldlr−/− and Ldlr−/− mice establish that PD-1 exerts significant antiinflammatory and atheroprotective effects and is the likely relevant receptor for the antiinflammatory and atheroprotective effects of PD-L1 or PD-L2 that we have previously observed.11 The influence of PD-1 is manifest early in disease progression, because we observed marked increases in both CD4+ T cells and CD8+ T cells and macrophages in the aortic lesions in the PD-1-deficient mice after only 5 weeks of diet. Analyses of iliac node T-cell numbers, phenotype, proliferation, and cytokine secretion at 5 weeks indicate early dysregulation of systemic immune responses to the hypercholesterolemia in the Pdl1−/−Ldlr−/− mice. These data indicate that PD-1 deficiency results in an exaggerated T-cell–mediated immune response in the early stages of hypercholesterolemia and atherosclerosis. After 10 weeks of diet, the Pdl1−/−Ldlr−/− lesion phenotype was more striking, with larger atherosclerotic size, outward aortic root remodeling, and increased inflammatory cell infiltration, including a remarkable number of CD8+ T cells, as well as more apoptosis in the lesion. At the 10-week time point, splenic CD4+ and CD8+ T cells from Pdl1−/−Ldlr−/− mice expressed higher levels than the Ldlr−/− control mice of mRNAs indicative of functional activation, including Ifng, Tnf, Ccr5, Ccr6, and Cxcr3. Furthermore, the detection of elevated levels of serum TNF-α in the Pdl1−/−Ldlr−/− after 10 weeks of cholesterol diet is also consistent with continued profound dysregulation of the T cell–mediated immune response. Based on the cytokine profiles from either sera or supernatant of cultured cells, there was no obvious change in the balance of Th1 and Th2 cells when PD-1 deficient. We also did not detect any difference in numbers of CD4+ FOXP3+ cells between Pdl1−/−Ldlr−/− and Ldlr−/− mice either at 5 or 10 weeks, despite evidence suggesting PD-1 influences regulatory...
T-cell development and functions.\(^2\)

Overall, it appears that PD-1 plays a direct role in inhibiting activation of proatherogenic T cells.

The robust increase in lesion CD8\(^+\) T cells seen in lesions of the PD-1-deficient mice suggests that there may have been more CTL-mediated cytotoxicity in those lesions. Although at 10 weeks, lesion size was greater in the PD-1-deficient mice compared with Ldlr\(^{-/-}\) mice, there was not a concomitant increase in lesional SMC. We did observe more apoptotic lesional SMC in the PD-1-deficient mice compared with Ldlr\(^{-/-}\) controls, which may explain why SMC content did not increase, and this finding is consistent with more CTL-mediated cytotoxicity. In vitro assay also confirmed that PD-1-deficient CD8\(^+\) T cells are more potent killers of mouse aortic SMC.

Our finding that PD-1 expression is upregulated in aortic T cells in cholesterol diet-fed Ldlr\(^{-/-}\) mice compared with control diet-fed mice suggests that chronic exposure to atheroantigens may lead to a partial suppression of CD4\(^+\) and marked suppression of CD8\(^+\) T-cell responses to these antigens. Specific peptides that may drive proatherogenic T-cell response are not well characterized, and therefore we could not examine PD-1 expression or T-cell exhaustion on tetramer-identified T cells, as has been done for viral-specific T cells in mice and humans.\(^7,23\)

Given the phenotype of the PD-1-deficient Ldlr\(^{-/-}\) mice, we reasoned that antibody-mediated blockade of PD-1 may aggravate atherosclerotic lesion inflammation. Our results from short-term injection of rat anti-mouse PD-1 antibody into hypercholesterolemic Ldlr\(^{-/-}\) mice confirmed our prediction. The antibody-treated mice showed increases in lesional CD4\(^+\) and CD8\(^+\) T cells similar to those that we saw in the PD-1-deficient Ldlr\(^{-/-}\) mice. There was also a remarkable effect of this short-term PD-1 blockade on iliac lymph node T-cell activation. We do not consider the lack of increase lesion size in the mice treated with anti-PD-1 Ig a significant finding, given the limited time for which we could treat the mice because of development of mouse anti-rat Ig responses. Furthermore, plaque inflammation may be more clinically relevant than plaque growth in the clinical context of treatment of patients.

We found that Ldlr\(^{-/-}\) chimeras with Pd-l1/2\(^{-/-}\) bone marrow developed aortic lesions with more T cells and macrophages than did control chimeras with Pd-l1/2\(^{-/-}\) bone marrow but similar plaque sizes in the 2 groups. This is in contrast to our previous study\(^1\) in nonirradiated Pd-l1/2\(^{-/-}\) Ldlr\(^{-/-}\) mice, which showed both enhanced lesion inflammation and lesion size. This difference between enhanced lesion inflammation and comparable lesion size in the current study may reflect the influence of irradiation on lesion development, which we and others have found to be generally reduced compared with nonirradiated mice at relatively early time points.\(^2\) Because PD-L1 is also expressed on non–bone marrow–derived cells, including EC, it is possible that PD-L1 deficiency on both nonhematopoietic and hematopoietic cells is required to influence lesion size. Remarkably, there were abundant CD8\(^+\) T cells, almost as many as the CD4\(^+\) T cells in the lesions of the Pd-l1/2\(^{-/-}\) bone marrow chimeras. We believe this is the finding of greatest translational significance in this experiment, given that plaque inflammation is tightly associated with acute coronary syndromes. In this study, the lack of hematopoietic PD-L1 likely impaired regulatory functions of lesional antigen presenting cells interacting with PD-1\(^+\) T cells.

In summary, our data demonstrate that PD-1 plays a significant role in regulating both CD4\(^+\) and CD8\(^+\) T-cell responses in experimental atherosclerosis. Furthermore, CD8\(^+\) T cells, which are usually difficult to detect in mouse lesions and less common than CD4\(^+\) T cells, become prominent when PD-1 is absent or blocked. The data suggest that PD-1 agonists could have therapeutic benefit for exacerbations of atherosclerotic lesion inflammation, such as in acute coronary syndromes. Conversely, humanized function-blocking anti-PD-1 antibody therapies are being developed to enhance T-cell responses in patients with cancers or chronic viral infections, such as HIV and hepatitis C. Patients with HIV are at elevated risk for coronary artery disease and myocardial infarction compared with noninfected individuals, and highly active antiretroviral therapy leads to dyslipidemia.
and insulin resistance.25 It will therefore be important to consider the potential added risk of deregulating plaque-based T-cell responses by PD-1 blockade.

**Sources of Funding**
This work was supported by National Institutes of Health Grants HL087282 (to A.H.L.), R01-AI46414 and P01-AI56299 (to A.H.S.), and R01-AI056299 (to G.J.F.).

**Disclosures**
Dr Freeman has patents and receives patent royalties on the PD-1 pathway.

**References**
Impairment of the Programmed Cell Death-1 Pathway Increases Atherosclerotic Lesion Development and Inflammation
De-xiu Bu, Margarite Tarrio, Elena Maganto-Garcia, George Stavrakis, Goro Tajima, James Lederer, Petr Jarolim, Gordon J. Freeman, Arlene H. Sharpe and Andrew H. Lichtman

Arterioscler Thromb Vasc Biol. published online March 10, 2011;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2011/03/10/ATVBAHA.111.224709

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2011/03/09/ATVBAHA.111.224709.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Impairment of the PD-1 pathway increases atherosclerotic lesion development and inflammation

De-xiu Bu\textsuperscript{1}, Margarite Tarrio\textsuperscript{1}, Elena Maganto-Garcia\textsuperscript{1}, George Stavrakis\textsuperscript{1}, Goro Tajima\textsuperscript{2}, James Lederer\textsuperscript{2}, Petr Jarolim\textsuperscript{1}, Gordon J Freeman\textsuperscript{3}, Arlene H Sharpe\textsuperscript{1}, Andrew H Lichtman\textsuperscript{1}.

\textsuperscript{1}Department of Pathology, \textsuperscript{2}Department of Surgery, Brigham and Women's Hospital, \textsuperscript{3}Department of Medical Oncology, Dana-Farber Cancer Institute and Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA.
SUPPLEMENTAL MATERIALS AND METHODS

Mice
Mice were fed a clinton/cybulsky high fat rodent diet with 1.25% added cholesterol 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<sup>-/-</sup> 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<sup>-/-</sup> 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 IFNγ 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
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.
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 digests 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, permeablized 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
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
SUPPLEMENTAL MATERIAL-R1

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 immunofluorescent 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 $Pd1^{−/−}Ldlr^{−/−}$ and control mice

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6</th>
<th>$Pd1^{−/−}Ldlr^{−/−}$</th>
<th>$Ldlr^{−/−}$</th>
<th>$Pd1^{−/−}Ldlr^{−/−}$</th>
<th>$Ldlr^{−/−}$</th>
<th>$Pd1^{−/−}Ldlr^{−/−}$</th>
<th>$Ldlr^{−/−}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>mg/dl</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>72.5</td>
<td>369±18</td>
<td>374.8±8.8</td>
<td>885.5±117.7*</td>
<td>946.5±141.3*</td>
<td>1078±126.6*</td>
<td>988.5±27.3*</td>
</tr>
<tr>
<td>LDL</td>
<td>6</td>
<td>242.5±11.8</td>
<td>219.5±5.5</td>
<td>737±102.2*</td>
<td>787±131.1*</td>
<td>894±103.6*</td>
<td>807.5±30.6*</td>
</tr>
<tr>
<td>HDL</td>
<td>62.5</td>
<td>130±4.6</td>
<td>128.8±6.3</td>
<td>219.5±21.9*</td>
<td>201±14.8*</td>
<td>250±17.7*</td>
<td>209±4.2*</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>67</td>
<td>169.5±5.2</td>
<td>195.3±6.3</td>
<td>145±33.1</td>
<td>201±31.2</td>
<td>157.6±3</td>
<td>229.5±26.2</td>
</tr>
</tbody>
</table>

**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

<table>
<thead>
<tr>
<th>mg/dl</th>
<th>Anti-PD-1</th>
<th>Control IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>733.8±69.2</td>
<td>893.2±131.5</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>589.8±60.3</td>
<td>711.6±108.9</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>189.4±5</td>
<td>216.6±17.8</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>162.6±46.6</td>
<td>232.2±66.2</td>
</tr>
</tbody>
</table>
### Table III. Mice Serum lipid profiles in bone marrow chimeras after 10 w cholesterol diet

<table>
<thead>
<tr>
<th>mg/dl</th>
<th>Pd-Il2/−/− chimeras</th>
<th>Control chimeras</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>1046±77.4</td>
<td>1194±239.1</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>819±61.9</td>
<td>957.5±234.7</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>258±10.9</td>
<td>215.5±18.3</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>328.5±65.8</td>
<td>375±99.3</td>
</tr>
</tbody>
</table>
Table IV: Mouse Oligonucleotide primers used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actb</td>
<td>5'-TCC TTC GTT GCC GGT CCA-3'</td>
<td>5'-ACC AGC GCA GCG ATA TCG TC-3'</td>
</tr>
<tr>
<td>Ifng</td>
<td>5'-AAC GCT ACA CAC TGC ATC TTG-3'</td>
<td>5'-GCC GTG GCA GTA ACA GCC-3'</td>
</tr>
<tr>
<td>Tnf</td>
<td>5'-GCA CAG AAA GCA TGA CCC G-3'</td>
<td>5'-GCC CCC CAT CTT TTG GG-3'</td>
</tr>
<tr>
<td>Ccr6</td>
<td>5'-CCT CAC ATT CTT AGG ACT GGA GC-3'</td>
<td>5'-GGC AAT CAG AGC TCT CGG A-3'</td>
</tr>
<tr>
<td>Ccr5</td>
<td>5'-ACT GCT GCC TAA ACC CTG TCA TCT-3'</td>
<td>5'-TTC ATG TTC TCC TGT GGA TCG GGT-3'</td>
</tr>
<tr>
<td>Cxcr3</td>
<td>5'-AAC TCA GCC ATC CCT GTG TGA GAA-3'</td>
<td>5'-ATG GGC ACA TTC AGT GCT GAC AAC-3'</td>
</tr>
</tbody>
</table>
Online Supplement References


