Macrophage-Specific Expression of Paraoxonase-1 via Stem Cell Replacement Reduces Atherosclerosis

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One theory predicts that atherosclerosis, the major cause of death in industrialized societies[1,2], is caused by an inflammatory response of arterial wall macrophages to oxidized low density lipoproteins (ox-LDL)[3-6]. Paraoxonase-1 (PON1), a plasma phosphoesterase, decreases the formation of ox-LDL and its ability to induce arterial wall inflammation[7,8]. Multiple case-controlled studies show associations between PON1 gene polymorphisms, enzymatic activity and myocardial infarction[9,10]. Gene-targeted mice deficient in PON1[11] had increased atherosclerosis while transgenic mice with elevated PON1 had reduced atherosclerosis[12]. We now report a method providing therapeutic expression of PON1 by macrophages as a means to ameliorate atherosclerosis in homozygous familial hypercholesterolemic (HFH) mice for which the only effective treatment in humans is liver-transplantation[13]. Hematopoietic stems cells expressing a PON1 transgene (PON1-tg) whose expression is confined to macrophages were engrafted into atherosclerosis-susceptible LDL receptor-/- mice. Recipient mice expressed PON1 at levels sufficient to reduce the atherosclerosis without affecting plasma levels of lipoproteins or circulating, fully functional monocyte/macrophages. These studies suggest that transgenic expression of PON1 by macrophages may provide an effective means to ameliorate lesion formation in the face of intractable hyperlipidemia.

PON1 gene expression is confined to liver parenchymal cells, which inefficiently secrete the active enzyme as a component of HDL[14]. In order to express PON1 in arterial wall macrophages, the progenitor of atherosclerotic “foam cells”[4-6], we constructed a transgene using the proximal promoter of the human acetyl-LDL receptor (SRA) gene. This promoter element has been demonstrated to confer macrophage-specific expression[15]. Peritoneal macrophages obtained from progeny of three separate lines of founder C57BL/6 mice having genomic DNA containing the PON1 transgene showed varying levels of PON1 mRNA expression (Fig. 1a). Further analysis showed that the highest level of expression of PON1 mRNA by peritoneal macrophages from PON1-tg mice was similar to the level expressed in the livers of non-transgenic mice (data not shown). No PON1 mRNA was detected in peritoneal macrophages obtained from non-transgenic littermates (lanes 2-7, 8 and 9-Fig. 1a). In non-transgenic mice, liver exclusively expressed PON1 mRNA, while none was detected in spleen, lung, kidney or brain (Figure 1b). In contrast, transgenic expression of PON1 using the macrophages-specific SRA promoter[15] caused more PON1 mRNA to be expressed in tissues enriched with resident macrophages (e.g., liver, spleen and lung -Fig. 1b).

PON1 has the unusual characteristic of retaining its N-terminal signal sequence[16]. Thus, when expressed in CHO cells, PON1 is retained on the surface membrane; its secretion requires the addition of HDL to the medium[14]. Our analysis of PON1 expression and enzyme activity in peritoneal macrophages supports these findings. Macrophages from PON1-tg mice displayed ~3-fold greater phenyl acetate esterase activity compared to macrophages obtained from non-transgenic littermates (Fig. 1c). All of the PON1 enzyme activity was in the cell pellet; none was detected in the culture medium. Immunofluorescence of peritoneal macrophages obtained from PON1-tg mice shows the presence of PON1 antigens on the surface membrane, whereas none was detected in non-transgenic macrophages (Fig. 1d).

We examined if transplantation of bone marrow from PON1-tg mice would allow recipient LDL receptor-/- mice to express PON1 in a long-term manner sufficient to reduce atherosclerosis lesion formation. LDL receptor -/- mice were subjected to sub lethal levels of gamma radiation and then injected with bone marrow isolated from either PON1-tg mice or their non-transgenic

littermates. All animals in each group survived. Circulating white blood cells obtained from each recipient mouse one month after bone marrow transplantation were analyzed for the presence of PON1 mRNA using RT-PCR (Fig. 2a). While no PON1 mRNA was detected in white blood cells obtained from recipient mice that received bone marrow from non-transgenic littermates (lanes 1-8-Fig. 2a), PON1 mRNA was clearly present in plasma cells from recipient mice receiving bone marrow from PON1-tg mice (lanes 9-20-Fig. 2a). The recipient LDL receptor-/- mice were fed a high fat, cholesterol-enriched diet for 16 weeks in order to accelerate the formation of atherosclerosis[17]. Expression of PON1 by macrophages did not affect plasma levels of lipoproteins (Fig. 2b) and PON1 enzyme activity (Fig. 2c). In marked contrast, PON1 expression by macrophages significantly reduced the formation of atherosclerotic lesions in LDL receptor -/- mice by 40% $p<0.001$ (Fig. 2d and 2e).

Flow cytology analysis of blood show that the presence of PON1 in macrophages did not alter plasma cell type or number (Fig. 3a and b). Similarly, bone marrow from both groups showed similar ability to differentiate into macrophages that were similarly responsive to LPS (Fig. 3c-h). The combined data indicate that expression of PON1 in macrophages block atherogenesis without impairing the appropriate development of monocytes from HSC or their response to bacterial signals (e.g., LPS).

Our data show that PON1 expression by macrophages mitigates atherosclerotic lesion formation associated with hyperlipidemia caused by loss of LDL receptor expression. Since neither plasma levels of lipoproteins or PON1 enzymatic activity were altered by the expression of PON1 in macrophages, PON1 acted on or within macrophages. A parsimonious interpretation of the combined data is that the expression of the PON1 transgene by arterial wall macrophages reduced the formation of atherosclerosis by decreasing the formation and/or activity of proatherogenic signals that act on or within macrophages. These putative signals may be similar to the oxidized phospholipids, shown to be responsible for the activation of endothelial cells[8,18]. Our finding are consistent with recent findings by others showing that factors that operate distal to hyperlipidemia can act in a dominant manner in regard to atherogenesis [19,20]. These studies suggest that transgenic expression of PON1 by macrophages, the most distal cellular site of atherogenesis,

may provide an effective means to ameliorate lesion formation in the face of intractable hyperlipidemia.

METHODS

PON1 transgene

The coding region of human PON-1 (a generous gift from Dr. C. Furlong, University of Washington) was inserted into the HindIII and EcoRV sites of the polylinker in the plasmid Fxba-A1, which contains the macrophages-specific human acetyl-LDL receptor promoter and the human growth hormone polyadenylation signal[15]. The sequence of the resulting plasmid (pMacPON) was verified by sequencing both strands over the region of the PON-1 cDNA. The transgenic vector was isolated from plasmid sequence by restriction digestion with XhoI and NotI. The resulting 7.4 kb fragment containing the transgenic vector was isolated following separation using low melting agarose and purified 2X using Qiagen DNA columns.

PON1-tg mice

PON1-tg mice were made by injecting the excised PON1 transgene into C57BL/6 embryos. The resulting pups were screened for the presence of the PON1 transgene using genomic DNA obtained from the tail[18]. Positive mice were bred with C57BL/6J mice (Jackson labs). The progeny were screen for the presence of the PON1 transgene in genomic DNA. Some of the pups were treated with thioglycolate 48 h prior to isolating peritoneal macrophages[15]. The relative abundance of PON1 mRNA was determined by northern blotting .

Bone Marrow transplantation

C57BL/6J, LDL receptor -/- mice (male, 6-8 weeks old) were subjected to sub lethal radiation and were injected with the bone marrow derived from PON1-tg mice and non-transgenic littermates, as described [21]. Mice were fed a chow diet for one month after which they were fed a "Western" diet (TD96335; containing 1.25% cholesterol, 6% fat obtained from Harlan Teklad Labs). After 16 weeks, mice were sacrificed and atherosclerosis lesions in the proximal aortas were quantitated[18].

Plasma lipids and PON1 activity

Blood was collected by retro-orbital puncture from mice that were fasted for 14 h. The lipid content of lipoprotein fractions was quantitated[22]. Paraoxonase activity determined using paraoxon and phenylacetic acid[14,18].

FACS Analysis

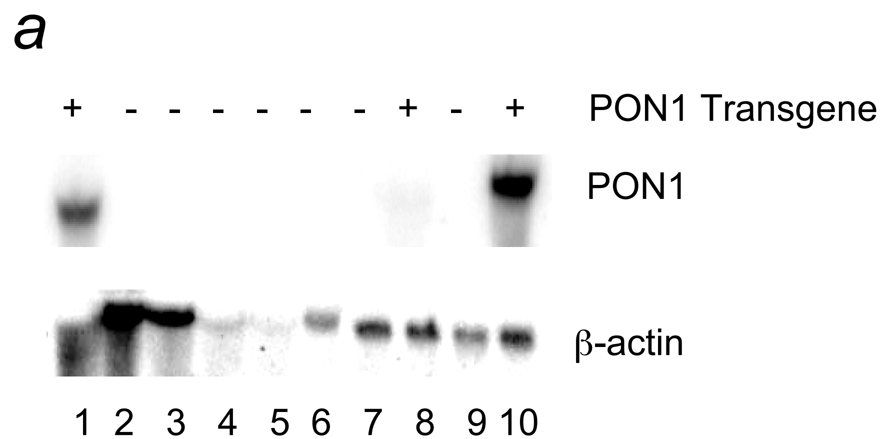
Fluorescence activated cell sorting (FACS) was performed according to the protocol described by Pharmigen. FcBlock (Pharmigen # 01241D), anti-Gr-1(CD-11b) (Pharmigen # 553129), and anti-MacIII (Pharmigen # 553324) were added to blood, mixed, and incubated on ice in the dark for thirty minutes. The mixture was then washed with 2% FBS in PBS and the pellet was incubated with Pharmlyse Ammonium Chloride Lysing Reagent (Pharmigen #555899) in the dark for 30 minutes. The resuspended pellet was washed and subjected to FACS analysis.

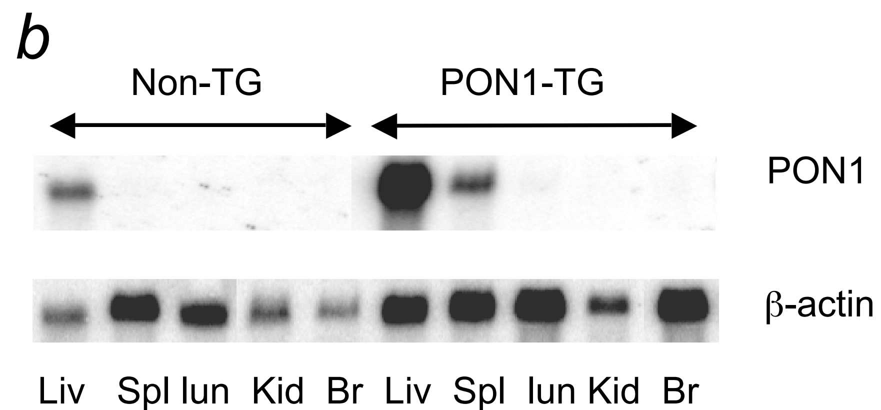
Real Time PCR Experiments

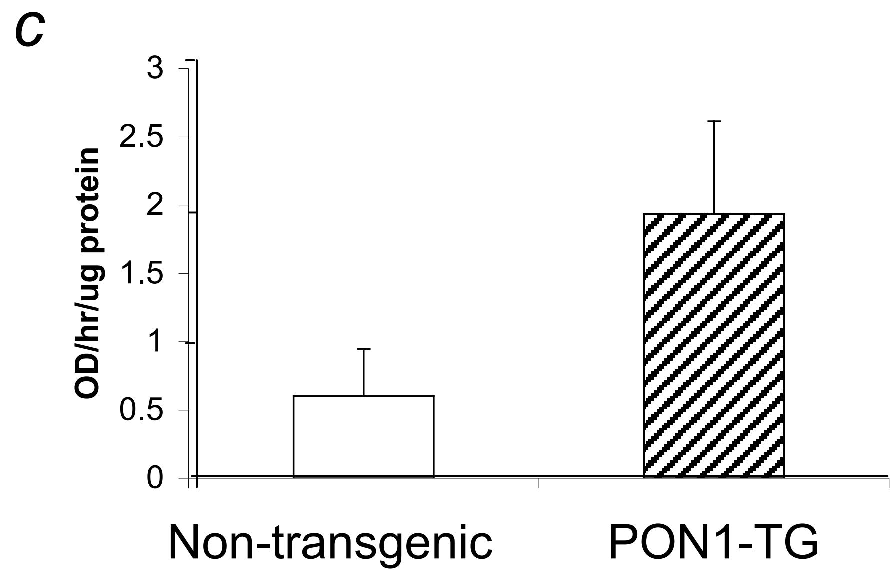
Bone marrow cells were extracted from the femurs and tibias of male mice 4-6 weeks of age. Cells were plated overnight in RPMI 1640 with L glutamine, 10%FBS, and 30% L-cell conditioned media [23]. Non-adherent cells were removed at 24hr, counted and plated at a density of 500,000 cells/ml with 10ml/100 mm plate. After five days of culture in RPMI 1640 with L glutamine, 10%FBS, and 30% L-cell conditioned media, adherent cells were harvested with a cell lifter and replated in a 6 well plate at a density of 500,000 cells/ml, 1.5 ml/well. L cell media was removed 24 h later and the cells were put in RPMI 1640 with L glutamine and 10% FBS for 24 h. After an additional 24 h, media was removed and replaced with 1.5 ml of RPMI 1640 with L glutamine and 10% FBS, or 1.5 ml RPMI 1640 with L glutamine, 10% FBS and 2 μg/ml LPS. Cells were harvested 24 h later and the total RNA isolated using a Qiagen Rneasy Mini Kit. RT PCR was performed on the total RNA using random hexamers and the cDNA was used for real time Taqman PCR. PCR primers for TNFα, SRA, CD-36, GAPDH and macrosialin have been described [24]. PCR primers for IL-6 were (5' to 3'): CTT CAC AAG TCG GAG GCT TAA TTA C (forward), AGA ATT GCC ATT GCA CAA CTC TT (reverse) and CAT GTT CTC TGG GAA ATC GTG GAA ATG AGA (probe); PCR primers for IL-1β were (5' to 3'): AGG CAG GCA

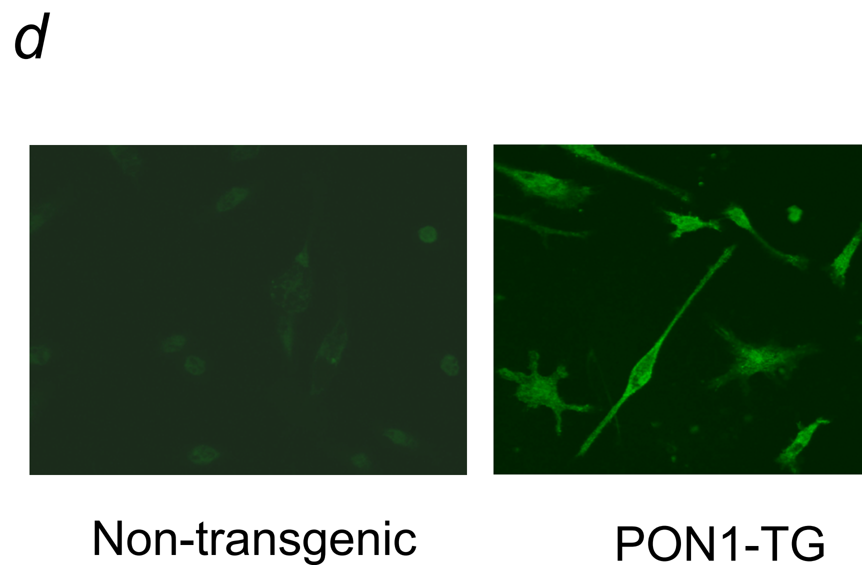
GTA TCA CTC ATT GT (forward), GGA AGG TCC ACG GGA AAG A (reverse) and TGT GGA GAA GCT GTG GCA GCT ACC TGT (probe); PCR primers for IL-12 were (5' to 3'): AAG GTG CGT TCC TCG TAG AGA A (forward), GAG CTT GCA CGC AGA CAT TC (reverse) and CAT CTA CCG AAG TCC AAT GCA AAG GCG (probe).

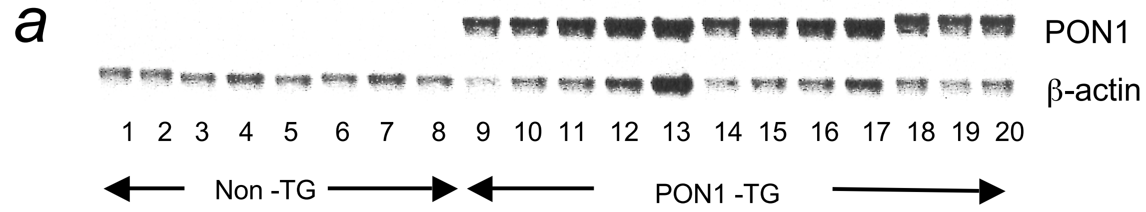
Results are given as mean ± S.D. Statistical significance was determined by Student's *t* test using double-tailed p values. Values of $p \leq 0.05$ were considered to be significant.

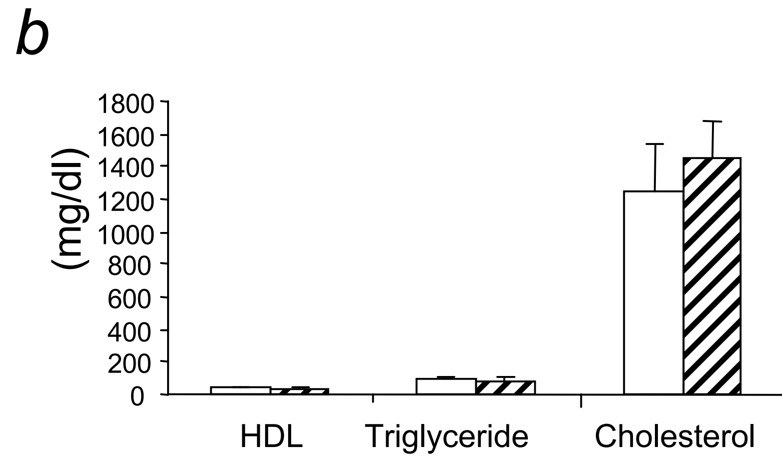

a
+ - - - - - - + - +
PON1 Transgene
PON1
β-actin
1 2 3 4 5 6 7 8 9 10

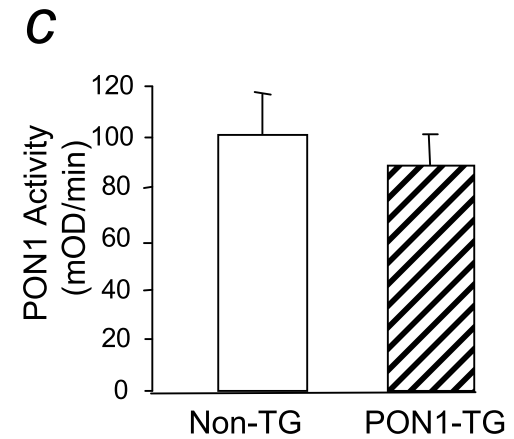

b
Non-TG
PON1-TG
PON1
β-actin
Liv Spl lun Kid Br Liv Spl lun Kid Br

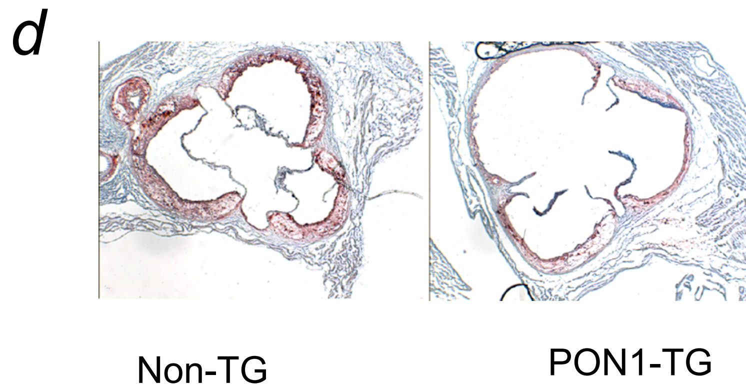

c
OD/hr/ug protein
3
2.5
2
1.5
1
0.5
0
Non-transgenic
PON1-TG


d
Non-transgenic
PON1-TG


a
PON1
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Non -TG
PON1 -TG


b
(mg/dl)
1800
1600
1400
1200
1000
800
600
400
200
0
HDL
Triglyceride
Cholesterol


c
PON1 Activity
(mOD/min)
120
100
80
60
40
20
0
Non-TG
PON1-TG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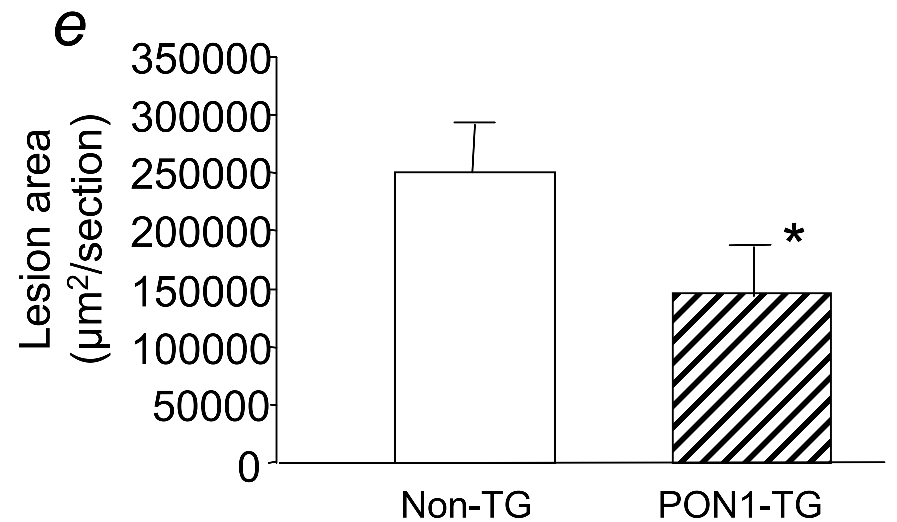
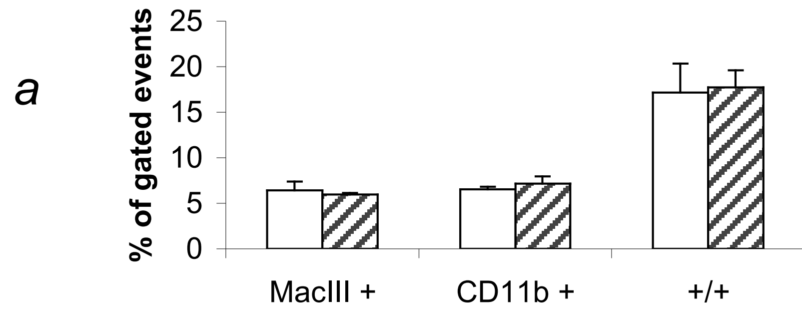
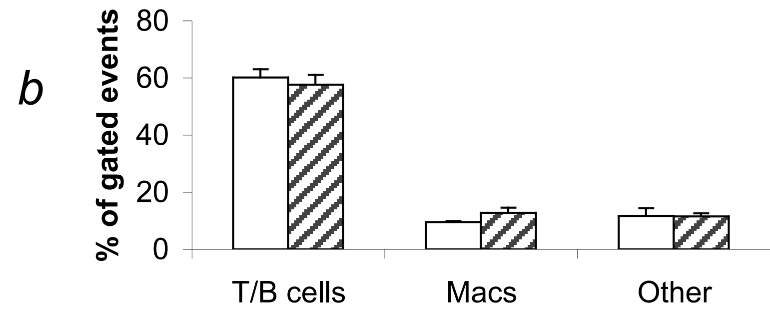
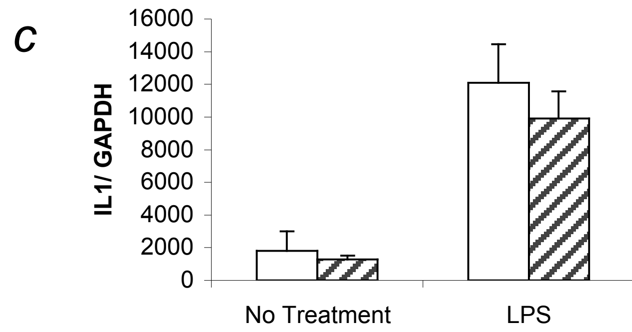
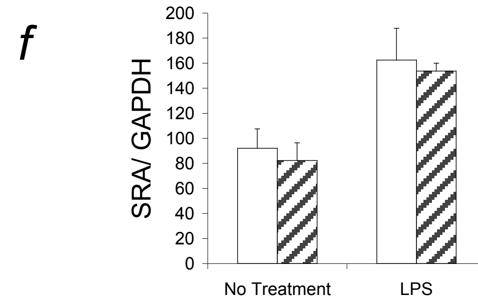
Non-TG
PON1-TG

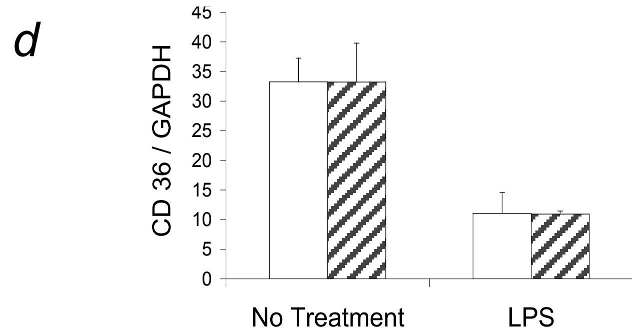

e
Lesion area
(μm2/section)
350000
300000
250000
200000
150000
100000
50000
0
*
Non-TG
PON1-TG

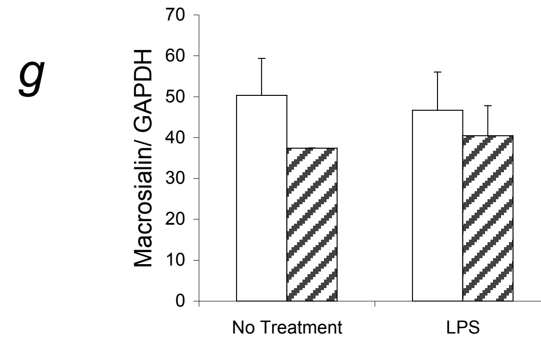

a
% of gated events
25
20
15
10
5
0
MacIII +
CD11b +
+/+

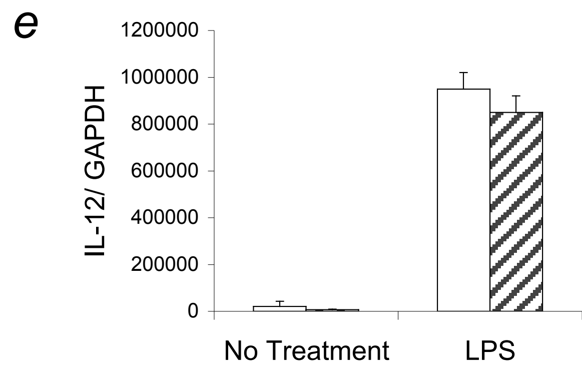

b
% of gated events
80
60
40
20
0
T/B cells
Macs
Other


c
IL1/ GAPDH
16000
14000
12000
10000
8000
6000
4000
2000
0
No Treatment
LPS


f
SRA/ GAPDH
200
180
160
140
120
100
80
60
40
20
0
No Treatment
LPS


d
CD 36 / GAPDH
45
40
35
30
25
20
15
10
5
0
No Treatment
LPS


g
Macrosialin/ GAPDH
70
60
50
40
30
20
10
0
No Treatment
LPS


e
IL-12/ GAPDH
1200000
1000000
800000
600000
400000
200000
0
No Treatment
LPS

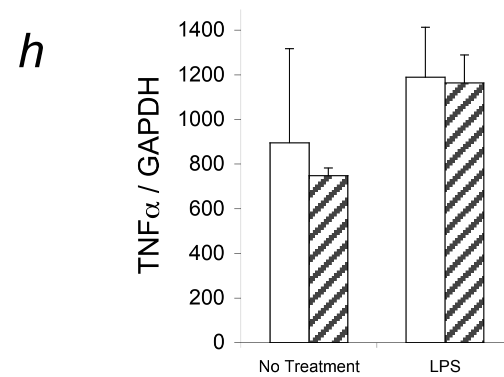

h
TNFα / GAPDH
1400
1200
1000
800
600
400
200
0
No Treatment
LPS

Figure Legends

Figure 1a. Expression of PON1 mRNA by peritoneal macrophages

Peritoneal macrophages were obtained from the mice 5 days after being treated with thioglycolate. Cells were cultured on plastic culture dishes, harvested and poly A-enriched RNA was northern blotted, probed for PON1 and β-actin and analyzed using a phosphoimager. Lane 1, founder group A1; lane 2; founder group A1, lane 3; founder group A1; lane 3, founder group A1, lane 4; founder group A2; lane 5, founder group A2; lane 6, founder group A2; lane 7, founder group A2; lane 8, founder group A2; lane 9, founder group B1; lane 10, founder group B1.

Figure 1b. Expression of PON1 mRNA in tissues from PON1-tg and non-transgenic mice

Poly-A RNA was isolated from the indicated tissues obtained from PON1-tg and non-transgenic littermates. The expression of PON1 mRNA relative to β-actin mRNA was determined, as described in Fig.1a. The following tissues were used: liver, (Liv) spleen (Spl), lung (lun), kidney (Kid), and brain (Br).

Figure 1c. PON1 enzyme activity by peritoneal macrophages.

Peritoneal macrophages were analyzed for the presence of PON1 enzyme activity using phenyl acetate substrate,[14]. The data presented were obtained using the membrane fraction [14]. Each value represents the mean $\pm$ S.D. for 3 mice in each group. There was a significant difference between the two groups of mice, $p<0.05$. No enzyme activity was detected in the culture medium.

Figure 2a. PON1 mRNA in circulating white blood cells obtained from recipient LDL receptor -/- mice.

LDL receptor -/- mice were subjected to sub-lethal radiation and transplanted with bone marrow obtained from PON1-tg mice (PON1-TG) and from non-transgenic littermates (Non-TG). Circulating white blood cells obtained one month later were extracted for RNA and then analyzed by RT-PCR using primers specific for PON1 mRNA and β-actin mRNA.

Figure 2b. Plasma lipid levels in recipient LDL receptor-/- mice.

Plasma levels of cholesterol (TC), HDL cholesterol (HDL) and triglycerides (TG) in bone marrow recipient LDL receptor -/- mice fed a fat and cholesterol enriched diet for 16 weeks. Each value represents the mean $\pm$ S.D. There were 7 recipient LDL receptor-/- mice that received bone marrow from non-transgenic mice (open bars) and 12 recipient LDL receptor-/- mice that received bone marrow from PON1-tg mice (hatched bars). There were no significant differences between the two groups of mice in any of the indicated lipids and lipoproteins.

Figure 2c. PON1 enzyme activity in plasma of recipient LDL receptor-/- mice. Plasma obtained LDL receptor -/- mice that received bone marrow from PON1-tg mice (PON1-TG) and non-transgenic littermates (non-TG) was obtained and used to assay PON1 enzyme activity using paraoxon (Sigma, St Louis, MO) as the substrate. Each value represents the mean $\pm$ S.D. each group. There were 7 recipient LDL receptor-/- mice that received bone marrow from non-transgenic mice and 12 recipient LDL receptor-/- mice that received bone marrow from PON1-tg mice. There were no significant differences between the two groups of mice in the activity of PON1.

Figure 2d and 2e. Bone marrow engraftment of the PON1 transgene reduces the formation of atherosclerosis in recipient LDL receptor -/- mice.

Atherosclerosis lesion analysis was determined using oil red O stained frozen thin sections of aortas of recipient LDL receptor -/- mice that were fed a cholesterol-rich diet for 16 weeks. Fig 2.d shows representative micrographs of oil red O stained frozen sections. Fig. 2e shows the quantification of serial sections for mice receiving bone marrow cells obtained form either non-transgenic (non-TG) or PON1-transgenic (PON1-TG) mice. There were 7 recipient LDL receptor-/- mice that received bone marrow from non-transgenic mice and 12 recipient LDL receptor-/- mice that received bone marrow from PON1-tg mice. Each value represents the mean $\pm$ S.D. *Indicates a significant difference between groups, $p<0.001$.

Figure 3a and b. Fluorescence Activated Cell Sorting (FACS) analysis of plasma cells.

Blood was collected through retro-orbital eye bleed from four PON transgenic mice (hatched bars) and four non-transgenic littermates (open bars). Following removal of erythrocytes, plasma cells were incubated with marker antibodies and analyzed by fluorescent activated cell sorting (FACS) analysis. Data were derived from 20,000 gated events. (a) Analysis obtained from cells separated by fluorescent markers for: MacIII, CD11b antigen, and +/+ cells positive for both markers. (b) Separation based on size and granularity; "others" designates plasma cells other than T, B cells or macrophages.

Figure 3c-h. Response of bone marrow derived monocyte/macrophages to LPS.

Bone marrow cells were extracted from PON1-transgenic and non-transgenic littermates. Cells were cultured overnight in medium containing 10%FBS, and 30% L-cell conditioned media [23]. (There were equal numbers of differentiated monocyte/macrophages remaining on the culture dished obtained from both groups of mice.) Differentiated monocytes were re-plated in medium containing 10% FBS with and without 2 μg/ml LPS (as indicated). Cells were harvested 24 h later and the indicated mRNAs were quantified by real-time PCR. Each value represents the mean $\pm$ S.D of three separate analyses.

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