Deficiency of the Long Pentraxin PTX3 Promotes Vascular Inflammation and Atherosclerosis

Giuseppe Danilo Norata, PhD; Patrizia Marchesi, MSc; Vivek Krishna Pulakazhi Venu, MSc; Fabio Pasqualini, BS; Achille Anselmo, PhD; Federica Moalli, MSc; Irene Pizzitola, MSc; Cecilia Garlanda, VetScD; Alberto Mantovani, MD, PhD; Alberico Luigi Catapano, PhD

Background—Immune responses participate in several phases of atherosclerosis; there is, in fact, increasing evidence that both adaptive immunity and innate immunity tightly regulate atherogenesis. Pentraxins, a superfamily of acute-phase proteins that includes short pentraxins such as C-reactive protein or long pentraxins such as PTX3, a molecule acting as the humoral arm of innate immunity. To address the potential role of PTX3 in atherogenesis, we first investigated the expression of PTX3 during atherogenesis, generated double-knockout mice lacking PTX3 and apolipoprotein E, and then studied the effect of murine PTX3 deficiency on plasma lipids, atherosclerosis development, and gene expression pattern in the vascular wall.

Methods and Results—PTX3 expression increases in the vascular wall of apolipoprotein E–knockout mice from 3 up to 18 months of age. Double-knockout mice lacking PTX3 and apolipoprotein E were fed an atherogenic diet for 16 weeks. Aortic lesions were significantly increased in double-knockout mice and mice heterozygous for PTX3 compared with apolipoprotein E–knockout mice. Mice lacking PTX3 showed a more pronounced inflammatory profile in the vascular wall as detected by cDNA microarray and quantitative polymerase chain reaction analysis and an increased macrophage accumulation within the plaque. Finally, lesion size correlated with the number of bone marrow monocytes.

Conclusion—PTX3 has atheroprotective effects in mice, which, in light of the cardioprotective effects recently reported, suggests a cardiovascular protective function of the long pentraxin 3 through the modulation of the immunoinflammatory balance in the cardiovascular system. (Circulation. 2009;120:699-708.)

Key Words: genes ■ immunology ■ inflammation ■ vasculature

Immune responses participate in several phases of atherosclerosis; and both adaptive immunity and innate immunity are believed to tightly regulate atherogenesis.1 Pentraxins, an essential component of the humoral arm of innate immunity, are a superfamily of acute-phase proteins highly conserved during evolution and characterized by a multimeric, usually pentameric, structure.2 The pentraxin family is composed of the short pentraxins such as C-reactive protein (CRP) and serum amyloid P component (SAP) and by long pentraxins such as PTX3, which shows similarities to CRP and SAP but has an unrelated, long N-terminal domain coupled to the C-terminal pentraxin domain and differs in gene organization, cellular source, and ligands recognized.3

Clinical Perspective on p 708

Experimental evidence on the role of CRP in atherogenesis and vascular disease is mixed.4,5 A proatherogenic role for CRP in vivo when overexpressed in apolipoprotein E–knockout (ApoE<sup>−/−</sup>) mice has been shown; however, subsequent work failed to confirm this observation in either human CRP<sup>+</sup> or rabbit CRP transgenic mice on ApoE<sup>−/−</sup> or ApoE<sup>3-Leiden</sup> background.9 The possibility that pentameric versus monomeric CRP affects atherogenesis development differently may explain these discrepancies.10 In addition, the evidence for a role of CRP in other vascular phenotypes is mixed; although Danenberg et al<sup>11</sup> observed a prothrombotic effect of CRP in mice, Tennent et al<sup>12</sup> failed to confirm these findings. Furthermore, CRP has been shown to adversely affect vascular remodeling and blood pressure.13,14

CRP sequence and regulation have not been conserved in evolution from mouse to human. Indeed, mouse CRP does not behave as an acute-phase protein, and human CRP does not bind mouse C1q,15 thus hampering assessment of its in vivo function through genetic and pharmacological approaches to determine its role in human pathology. On the contrary, the conservation of sequence, gene organization,
and regulation of PTX3 in evolution allows us to address questions about the pathophysiological roles of PTX3 using gene-modified mice.16

PTX3 is rapidly produced and released by several cell types, particularly mononuclear phagocytes, dendritic cells, fibroblasts, and endothelial cells in response to either inflammatory or atheroprotective signals.3,17–20 Recent studies in gene-modified mice showed that PTX3 has complex, nonredundant functions in vivo, ranging from the assembly of a hyaluronic acid–rich extracellular matrix and female fertility to innate immunity against diverse microorganisms.3,21–23 PTX3 was initially described as an early marker for primary local activation of innate immunity and inflammatory responses.24,25; is highly expressed in the heart during inflammatory reactions, in atherosclerotic lesions, and in vascular cells in response to inflammatory stimuli; and is increased in plasma in patients with arterial inflammation, particularly unstable angina pectoris.26–29

Whether PTX3 plays a role in the pathogenesis of vascular diseases, however, is unclear. PTX3 is a potent inhibitor of the autocrine and paracrine stimulation exerted by fibroblast growth factor-2.30 In a model of lipopolysaccharide toxicity and in cecal ligation and puncture, PTX3 overexpression resulted in increased resistance.24 Furthermore, in a model of acute myocardial infarction caused by coronary artery ligation, PTX3-deficient mice showed increased heart damage associated with a greater no-reflow area and increased inflammatory response, a phenotype reversed by exogenous PTX3.16 Together, these data support a cardioprotective function of PTX3.

To address the potential role of PTX3 in atherogenesis, we generated double-knockout mice lacking PTX3 and ApoE and studied the effect of murine PTX3 deficiency on lipid metabolism, atherosclerosis development, and gene expression patterns in the vascular wall.

Methods
Detailed descriptions of the generation of double-transgenic animals and treatment, plasma lipid analysis and atherosclerosis quantification, cDNA microarray and quantitative polymerase chain reaction analyses, immunohistochemistry, and statistics are presented in the online-only Data Supplement. All experimental procedures were in accordance with the institutional guidelines for animal research. The cDNA microarray experiments were performed according to the Minimum Information About a Microarray Experiment guidelines. The raw data for each hybridization and the final processed files are accessible at the following address: http://www.sisalombardia.it/dati3/dati3.htm.

Results
PTX3 Expression in ApoE−/− Mouse Aorta
To evaluate the expression of PTX3 during atherogenesis, we performed a time-course experiment in ApoE−/− mice fed a chow diet and investigated PTX3 mRNA and protein expression in the aortas. Compared with 3 months of age, PTX3 mRNA expression at 12 and 18 months of age was significantly increased (3.11±0.24- and 2.55±0.32-fold versus 3-month-old mice, respectively; P<0.01; Figure 1A). This effect was specific for PTX3 because neither the CRP nor SAP mRNA level was changed throughout the experiment (Figure 1A). In agreement, PTX3 protein expression increased in the atherosclerotic plaque (Figure I of the online-only Data Supplement), localizing in endothelial cells, polymorphonuclear cells, and macrophages within the atherosclerotic plaque (Figure 1B through 1E).

Quantitative Morphometry of Atherosclerosis in Double-Knockout Mice
After 16 weeks on a Western-type diet, the double-knockout mice showed no significant differences in body weight or plasma lipids (the Table). Mice lacking PTX3 had an increased atherosclerotic lesion area in the aortic sinus and ascendant aorta (the Table, Figures 2 and 3, and Figures IIA and IIB of the online-only Data Supplement). Specifically, female PTX3+/−/ApoE−/− and PTX3−/−/ApoE−/− mice showed a 36% and a 55% increase and male PTX3+/−/ApoE−/− and PTX3−/−/ApoE−/− mice showed a 37% and 47%
increase in atherosclerotic lesion area in the aortic sinus (P<0.05 for all) compared with PTX3+/−/ApoE−/− mice. Similar results were reported when the ascendant aorta tract was investigated (the Table, Figure 3, and Figure IIB of the online-only Data Supplement). Overall, female mice developed larger atherosclerotic lesions compared with male mice. This is probably due to the lack of ApoE, which has been reported to affect atherosclerotic lesion formation differently in female and male mice. Indeed, no difference in the vessel was observed in female compared with male PTX3−/− mice (data not shown).

The presence of atherosclerosis in the aorta was then investigated at the thoracic and abdominal levels. Except for 1 double-knockout female, no lesions were observed at the thoracic level (Figure IIIA of the online-only Data Supplement) or the abdominal level (Figure IIIB of the online-only Data Supplement). Similarly, no atherosclerotic lesions were detectable in the thoracic and abdominal aortas from males (data not shown). Next, we investigated the effect of PTX3 deficiency in ApoE−/− mice on a chow diet. The extent of atherosclerotic lesions was less compared with mice fed a Western-type diet; indeed, only in 18-month-old female mice, which have a degree of atherosclerosis similar to that reported in mice on a chow diet (Figure IV of the online-only Data Supplement). We therefore focused the next analysis on only mice on an ApoE−/− background.

### Gene Expression Analysis in Aorta Atherosclerotic Lesion

Next, we investigated the gene expression patterns in the aorta focusing on the tract between the aortic sinus and the ascending aorta in mice fed a Western-type diet for 16 weeks. A mouse inflammation–focused cDNA microarray was used; the data are reported for each group of animals and according to gender (Figure 4). Several genes were modulated in both male and female mice and some were selectively modulated in only 1 gender. Of note, a cluster of proinflammatory molecules such as chemokines (Ccl2, Ccl3, Ccl5, and receptors as CCR2, CCR5, and Ccl8), cytokines (interleukin [IL]-4, IL-6, tumor necrosis factor-α), and TRAF3 was upregulated in both PTX3−/+/ApoE−/− and PTX3−/−/ApoE−/− mice compared with PTX3+/+/ApoE−/− mice. Furthermore, several transcription factors involved in intracellular proinflammatory signaling such as nuclear factor-kB and the related protein Irak1, Fos, Jun, GATA3, GATA4, Egr2, and Egr3 were upregulated in both PTX3−/+/ApoE−/− or PTX3−/−/ApoE−/− mice compared with PTX3+/+/ApoE−/− mice. Finally, key mediators in the inflammatory response as toll-like receptor 2 and 4 or Cxcl-2 were also upregulated in mice lacking PTX3.

Modulation of the expression of genes involved in inflammatory vascular responses was confirmed by quantitative polymerase chain reaction (Figure 5). The expression of chemokines such as Ccl2, Ccl3, and Ccl5 and receptors as CCR2, CCR5, and CX3CR1 was upregulated in PTX3−/+/ApoE−/− and PTX3−/−/ApoE−/− mice compared with PTX3+/+/ApoE−/− mice, with some minor differences depending on gender (Figure 5A and 5B). In addition, other key factors such as matrix metalloproteinase-9, IL-6, and insulin-like growth factor were upregulated in mice lacking PTX3. Analysis of the gene expression patterns therefore suggests that the lack of PTX3 in a proatherogenic background may be associated with an increased inflammatory status in the vascular wall, which, in turn, contributes to the atherogenic process observed.
To further address this issue, we investigated the mRNA expression of adhesion molecules (key markers of endothelial dysfunction). Increased expression of intracellular adhesion molecule, vascular cell adhesion molecule-1, endothelial leukocyte adhesion molecule-1, and platelet/endothelial cell adhesion molecule mRNA expression was observed in female PTX3+/+ mice, PTX3+/− mice, and PTX3−/− mice compared with PTX3−/− mice (Figure 5C, left); except for intracellular adhesion molecule-1, a similar pattern was observed in male mice (Figure 5C, right). These findings confirm the presence of a vascular dysfunction associated with the lack of functional PTX3. Furthermore, these expression patterns appeared to be specific for the vascular wall; indeed, when investigated in peripheral blood mononuclear cells, no significant differences were observed in the absence of PTX3 were observed (Figure VI of the online-only Data Supplement).

Characterization of the Atherosclerotic Lesions
The presence of macrophages (Figure 6B, 6G, and 6N), smooth muscle cells (Figure 6D, 6I, and 6P), and T lymphocytes (Figure 6E, 6L, and 6Q) also was investigated in the atherosclerotic plaque from PTX3+/+ mice (Figure 6A through 6E), PTX3+/−/ApoE−/− mice (Figure 6F through 6J), and PTX3−/−/ApoE−/− mice (Figure 6K through 6O) fed a Western-type diet for 16 weeks. Atherosclerotic plaques contained several macrophages and smooth muscle cells, whereas the amount of T lymphocytes was limited. The macrophage-positive areas in the atherosclerotic lesion in PTX3−/−/ApoE−/− mice (Figure 6A through 6E), PTX3+/−/ApoE−/− mice (Figure 6F through 6J), and PTX3−/−/ApoE−/− mice (Figure 6K through 6O) for aortic sinus (n=8 for each group) expressed as mean±SD (*P<0.05 vs PTX3+/+/ApoE−/− mice).

Figure 2. Aortic sinus atherosclerosis in PTX3+/+/ApoE−/−, PTX3+/−/ApoE−/−, and PTX3−/−/ApoE−/− mice. A, Representative sections of the aortic sinus stained with hematoxylin and eosin for PTX3+/+ mice, PTX3+/− mice, and PTX3−/− mice. Scale bar=100 μm. Data are shown for female mice; for male mice, see Figure II of the online-only Data Supplement. B and C, The extent of the atherosclerotic lesion in PTX3+/+/ApoE−/−, PTX3+/−/ApoE−/−, and PTX3−/−/ApoE−/− female mice for aortic sinus (n=8 for each group) expressed as mean±SD (*P<0.05 vs PTX3+/+/ApoE−/− mice).
sinus (Figure VII and VIII of the online-only Data Supplement) and ascending aorta (data not shown). These observations suggest that PTX3 deficiency is not associated with altered stability of the atherosclerotic plaque.

Finally, to investigate possible mechanisms related to the increased presence of macrophages in the plaques, a series of additional experiments were performed. First, peritoneal macrophages isolated from either wild-type mice or mice lacking PTX3 were stimulated with oxidized low-density lipoprotein, but similar proinflammatory gene expression patterns and oxidative stress responses were observed (data not shown). Next, leukocyte subpopulations were investigated in blood and bone marrow in a subgroup of mice. In agreement with recent findings, we observed increased bone marrow and blood monocytosis in ApoE/H/H mice (data not shown). Furthermore, preliminary findings showed that in ApoE/H/H female mice, PTX3 deficiency was associated with a more pronounced bone marrow monocytosis that correlated with the extent of atherosclerotic lesions (Figure 7), in agreement with Combadiere et al using a different mouse model.

Discussion

Although PTX3 has been described as an early marker for primary local activation of innate immunity and inflammatory response in the vascular wall, there is no genetic evidence in humans of its role in the pathogenesis of vascular disease. In contrast to other pentraxins such as CRP and SAP, the long pentraxin PTX3 is well conserved at the primary sequence, gene organization, and regulation levels from mouse to human. It is thus possible to address the pathophysiological role of PTX3 using gene-modified mice. To this aim, we investigated the role of PTX3 on atherosclerosis in mice lacking PTX3 on an ApoE/H/H background. PTX3/H/H mice develop larger atherosclerotic lesions, which are associated with an increased proinflammatory gene expression pattern in the vascular wall, increased bone marrow monocytosis, and macrophage accumulation within the plaque. Of note, in ApoE/H/H mice, increased PTX3 expression was observed in advanced atherosclerotic lesions, confirming previous observations in human atherosclerotic lesions; and
Figure 4. cDNA microarray analysis for genes involved in inflammatory response of the aortic arch from PTX3/H11001/H11001/ApoE mice. The gene expression patterns in the aorta were investigated in the tract between the aortic sinus and ascending aorta. A mouse inflammation-focused cDNA microarray was used; data are presented for each group of animals and are expressed as fold of induction of PTX3/H11001/H11001/ApoE or PTX3/H11001/H11001/ApoE mice vs PTX3/H11001/H11001/ApoE mice. See the online-only Data Supplement for details.
was associated with macrophages and polymorphonuclear and endothelial cells. Whether this increase reflects a proatherogenic role of PTX3 or is a marker of a modulation of the immunoinflammatory response in the vascular wall is debated. PTX3 plasma levels predict 3-month mortality in patients with myocardial infarction, even after adjustment for major risk factors and other acute-phase prognostic markers. These results, coupled with those showing the upregulation of endothelial cell tissue factor in response to PTX3, suggested a potential pathogenic role for PTX3 in cardiovascular disease. However, there are at least some exceptions to the modulation of PTX3 expression by inflammatory mediators. The Th1-related and proatherogenic cytokine interferon-γ seems to be inhibitory, whereas the immunoregulatory and antiatherogenic IL-10 stimulates PTX3 expression in dendritic cells and monocytes, thus suggesting a potential antiatherogenic role for PTX3. Furthermore, several studies have clearly shown that efficient apoptotic cell clearance inhibits the inflammatory response and reduces the development of atherosclerosis and the size of the necrotic core. PTX3 binds to apoptotic cells, enhancing C1q binding and complement-mediated clearance of apoptotic cells, and may therefore contribute to limiting the size and complexity of atherosclerotic lesions. In a different setting, such as small-vessel vasculitis, PTX3 inhibits phagocytosis of late apoptotic neutrophils, thus promoting their accumulation in the vessel wall. The in vivo role of PTX3 in inflammatory conditions has been investigated through the use of PTX3-overexpressing and -deficient mice. PTX3 inhibits fibroblast growth factor–dependent activation of smooth muscle cells in vitro and neointimal formation in vivo. PTX3 overexpression resulted in increased resistance to sepsis in the cecal ligation and puncture model. Furthermore, in a model of acute myocardial infarction caused by coronary artery ligation, PTX3-deficient mice showed exacerbated heart damage with a
greater no-reflow area and increased inflammatory response; this phenotype was reversed by exogenous PTX3, supporting the concept that PTX3 possesses a cardioprotective function.

We have extended these findings by demonstrating that PTX3 exerts also atheroprotective functions in a mice model. The lack of PTX3 is associated with increased macrophage accumulation in the atherosclerotic lesions and increased bone marrow monocytosis. Of note, the extent of the lesion size correlates with the number of bone marrow monocytes. Furthermore, mice lacking PTX3 show an increased expression of adhesion molecules, cytokines, and chemokines in the vascular wall, thus suggesting that PTX3 may modulate the vascular-associated inflammatory response. Although we cannot exclude that the change in gene expression profiles reflects the differences in lesion size, because increased bone marrow monocytosis and macrophage plaque accumulation were observed, it is reasonable to propose that the increased inflammatory response in the vascular wall may reflect the presence and activation of these cells in PTX3-deficient animals.

Despite initial findings suggesting a potential pathogenic role for PTX3 in cardiovascular disease, recent observations, including our findings, support the possibility that PTX3 may act as a molecule at the crossway between proinflammatory and antiinflammatory stimuli, perhaps balancing the overactivation of a proinflammatory, proatherogenic cascade. Therefore, the increased levels of PTX3 in cardiovascular disease could reflect a protective physiological response, thus correlating with the severity of the disease.

Indeed, vascular wall cells integrate signals generated by multiple factors to regulate the immunoinflammatory response, which is achieved by finely tuning vascular inflammation by antiinflammatory counterregulatory mechanisms that maintain the integrity and homeostasis of the vascular wall. Interestingly, PTX3 is modulated both by proinflammatory proatherogenic molecules and antiinflammatory atheroprotective molecules. Furthermore, although our results point to an atheroprotective function of PTX3, this molecule also possesses proinflammatory and prothrombotic potential; indeed, PTX3 has been reported to induce tissue factor in monocytes and endothelial cells. Therefore, the molecule may have different functions in different settings or temporal windows of vascular pathology.
Data are presented as mean \( \pm \) SD (\( r^2 = 0.46, P < 0.01 \)).

Similar to adaptive immunity in which different players such as Th2 and T-regulatory cells could limit atherosclerosis progression, it is possible that effectors of the humoral arm of the innate immunity such as PTX3 might finely tune the immunoinflammatory response during atherosclerosis. Whether this effect is the result of the interaction of PTX3 with molecules involved in leukocyte recruitment within the lesion and/or in monocyte generation in the bone marrow has to be addressed.

Our study has some limitations. First, we focused our analysis on a selective inflammatory gene expression profile, which implies that we could have missed the modulation of genes involved in different pathogenetic responses. Second, we used a mice model in which PTX3-deficient mice were crossed with ApoE-deficient mice, one of the most widely used models of atherosclerosis, which per se shows a lower basal immunoinflammatory activation in the vascular wall. We cannot exclude that PTX3 deficiency in other atherosclerosis-prone models such as low-density lipoprotein receptor–knockout mice might lead to different results.

Conclusions
We have shown that deficiency of the long pentraxin PTX3 in mice is associated with an increased burden of vascular lesions and inflammation in a mouse model of atherosclerosis. This suggests that PTX3 could play an atheroprotective role in addition to its recently reported cardio-protective functions, suggesting that components of the humoral arm of innate immunity such as PTX3 may exert beneficial effects on cardiovascular disease under conditions promoting atherosclerosis.

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Deficiency of the long pentraxin PTX3 promotes vascular inflammation and atherosclerosis.

Giuseppe Danilo Norata PhD 1,2, Patrizia Marchesi MSc 1, Vivek Krishna Pulakazhi Venu MSc 1, Fabio Pasqualini BS 3, Achille Anselmo PhD 3, Federica Moalli MSc 3, Irene Pizzitola MSc 1, Cecilia Garlanda VetScD 3, Alberto Mantovani MD, PhD 3,4, Alberico Luigi Catapano PhD 1,2.

1Department of Pharmacological Sciences, University of Milan, Italy.
2Center for the Study of Atherosclerosis, Società Italiana Studio Aterosclerosi, Ospedale Bassini, Cinisello Balsamo, Italy
3Istituto Clinico Humanitas, IRCCS, Rozzano, Milan, Italy
4Department of Translational Medicine, University of Milan, Italy.

SUPPLEMENTAL MATERIAL

- Additional Material and Methods page 2-7
- Additional Figures page 8-15
Additional material and methods.

Animals and treatment

Double knock out mice (PTX3 -/-; Apo E -/- mice) were generated by crossbreeding mice deficient in PTX3 \(^1\) with mice lacking apolipoprotein E (purchased from the Jackson Laboratories, Bar Harbour, Maine, USA). Both animals were on C57Bl/6J background; after two generations, three groups were selected: PTX3 -/-; Apo E -/- mice, PTX3+/--; Apo E -/- mice and PTX3 +/+; Apo E -/- mice used for the control group. Each genotype was evaluated following PCR amplification for specific DNA sequences, for PTX3 as previously described \(^1\) and for Apo E as indicated here:


At 9 weeks of age, animals were fed ad libitum with western type diet (21% fat, 0.15% cholesterol and 19.5% casein, Piccioni Italy) for 16 weeks. All experimental procedures were in accordance with the institutional guidelines for animal research. The mice were sacrificed with an injection of Avertin 2.5% (Aldrich Chemical Co.USA). The heart and the arterial tree were perfused with saline solution under physiological pressure. Then the aortas and the hearts were isolated, placed in a storing solution (RNA laterICE\textregistered, Ambion, Germany) at \(-20^\circ\text{C}\) or paraffin embedded \(^2\). For RNA isolation the samples were homogenized in a dismembrator (B. Braun, Melsungen AG, Germany) then processed as described \(^3\).

Plasma lipid analysis

Blood samples were collected in EDTA tubes immediately before death by retro-orbital bleeding and plasma was separated by low-speed centrifugation at 4°C. The measurement of plasma lipids
was performed by standard enzymatic techniques (ABX for Cobas Mira Plus, Montpellier, France); HDL-C was determined after precipitation of apoB-containing lipoproteins.

Quantification of atherosclerosis

Cross sequential sections 5 µm thick were prepared from the aortic root up to 150µm after the valve leaflets were no longer detectable. For the aortic sinus and the ascending aorta the slides were stained with hematoxilin-eosin, for each tract of the aorta 15 sections were analyzed. Images of the aortas were captured with Axiovert 200 microscope and the atherosclerotic lesion area was quantified by computer image analysis, using Axiovision LE rel 4.4 software by two blinded observer. Data are expressed as µm² and are calculated as the differences between the area of the media + intima subtracted of the media area. The quantification of the collagen content was performed following picro-sirius red staining, images of the aortas were captured with Axiovert 200 microscope (ZEISS, Milan, Italy) under regular and polarized light, then fibrillar collagen, detected by yellow birefringence, was quantified using Image J analysis software, capable of colours segmentation and automation by programmable macros, by two blinded observer. Data are expressed as the percentage of the total atherosclerotic lesion area covered by fibrillar collagen ± standard error.

cDNA microrray analysis

A comprehensive gene expression analysis was performed in the tract of the aorta between the aortic sinus and the ascending aorta where the morphometric analysis was performed. For this purpose 10µm section were prepared. Non aortic tissue was removed from each section under the
microscope for a total of 150µm for each mouse. Aortas were removed from the glass and the samples from mice of the same group were pooled. RNA was extracted using the kit RNAeasy Formalin-fixed, paraffin-embedded (FFPE) tissue sections from Qiagen (Milan, Italy). The quality of the RNA was tested with standard procedures. Five hundred µg of RNA were then used for RNA amplification using the SuperScript RNA Amplification System (Invitrogen, Italy) according to the manufacturer instructions. Two µg of amplified RNA were then processed for microarray experiments while the remaining was used for validation with Q-PCR as described below. For microarray experiments Dual Chip Mouse Inflammation microarray was used (Eppendorf, Italy). Biotin 11-dATP and biotin 11-cCTP cDNA was synthesized according to the protocol from the manufacturer and hybridized to the microarrays. For these experiments the gene expression pattern in male and female were investigated separately. The arrays were developed with the Silverquant Detection Kit (Eppendorf, Italy), the images were acquired with the Silverquant Detection Software and analyzed with the Silverquant Analysis Software (Eppendorf, Italy). Data are expressed as fold of induction of PTX3+/ApoE−; or PTX3+/−/ApoE− mice vs PTX3+/+/ApoE− mice. For each group, two replicates, each resulting from pooling the mRNA of four animals were analyzed. (The cDNA microarray experiments were performed according to the MIAME guidelines, the raw data for each hybridization and the final processed files are accessible at the following address: http://www.sisalombardia.it/dati3/dati3.htm).

Real time quantitative polymerase chain reaction

Total RNA was reverse transcripted as described 5, 6. Three µL of cDNA were amplified by real-time quantitative PCR with 1X Syber green universal PCR mastermix (BioRad, Italy). The specificity of the Syber green fluorescence was tested as described 5. The primers used have been
described previously. Each sample was analyzed in duplicate using the IQ\textsuperscript{TM}-Cycler (BioRad). The PCR amplification was related to a standard curve ranging from 10-11 mol/L to 10-14 mol/L and data were normalized for the housekeeping gene ribosomal protein L13a (RLP13a) (NM_009438).

\textit{Immunohistochemistry}

Eight-mm consecutive frozen sections were cut, mounted on Superfrost slides (Bio-Optica, Milan, Italy) and fixed for 15 min in 4\% PFA at room temperature. Sections were hydrated with PBS twice for 10 min, incubated for 5 minutes with PBS-0.03\% H2O2-1\% BSA and with PBS-1\% BSA for 10 minutes to block endogenous peroxidase and unspecific sites, respectively. Immunohistochemistry (IHC) was performed using a Rabbit IgG affinity purified against human PTX3; after 2 hours of incubation at room temperature, the slides were washed in PBS pH 7(3x5 minutes each).

The reactions were revealed by means of anti rabbit HRP-Envision System Labelled Polymer (Dako Cytomation). The chromogen was 3,3' diaminobenzidine free base (DAB). Double immunofluorescence was performed with rat anti mouse CD68 (Hycult Biotechnology, for macrophages) and anti human PTX3. PTX3 was developed in green (anti Rabbit 488 as secondary antibody), while CD68 in red (anti Rat 594 as secondary antibody). Nuclei were stained with DAPI (Invitrogen). The slides were mounted with FluorSave (Calbiochem) and analysed with fluorescence microscopy.

For macrophage, smooth muscle cell and T-lymphocyte detection, rat anti mouse F4/80 (antigen, AbD Serotec, Oxford, UK, dilution 1:100), rabbit polyclonal to CD3 and rabbit polyclonal to smooth muscle actin, (Abcam, Cambridge UK, dilution 1:50 and 1:100 respectively), or non-
immune IgG as a control were used (4°C overnight) followed by incubation with a secondary biotinylated antibody (anti-rabbit IgG or anti-rat IgG, Vector Laboratories, Burlingame, CA, USA, diluted 1:200 in 1% normal goat serum and PBS) 1h at room temperature. Immunoreactivity was visualized using avidin-biotin-peroxidase method (Vectastain ABC kit from Vector Laboratories, Burlingame, CA, USA) with 3,3′-diaminobenzidine substrate (Sigma, Italy) as the chromogen. Dehydrated sections were observed using Zeiss Axioskop microscope (Zeiss) and analysed with color-video image analysis system linked to the microscope, for the quantification of the immunoreactive area the Image J analysis software, capable of colours deconvolution and segmentation by programmable macros, was used.

Statistics

Data were analyzed using SPSS 16.0 for Windows (SPSS, Chicago, IL). The effects of PTX3 deficiency were analyzed in the all group and for female and male separately. Data in Figure 1 were analyzed using one-way ANOVA. Data in table 1, figure 2, 3, 5 and 6 were analyzed with two way ANOVA followed by Sheffe contrast. When histological data were compared between groups, parametric t-tests setting the significance level at p<0.05 were used and to compensate for a potential lack of normal distribution, nonparametric Wilcoxon tests were applied. Data in figure 7 were analysed following bivariate correlation test.

References


Figure I on line supplement:
PTX3 expression in aging ApoE −/− mice.
Serial sections of ascending aorta from ApoE −/− mice were immunostained with an anti PTX3 specific antibody followed by alexa555 conjugated secondary antibody (red signal), and counterstained with Hoechst 33258 (blue) the autofluorescence of the laminas is shown in green. Control slides were immunostained with non-immune IgG. A representative panel is shown. Scale bar: 20μm.

For immunofluorescence studies, mice aorta specimens (5μm thick) were incubated with rabbit-polyclonal anti-PTX3 (1:20) overnight at 4°C, followed by incubation with anti-rabbit IgG Alexa 555 or 633-conjugated or (1:100, Invitrogen, Molecular Probes) for 1h then propidium iodide or Hoescht 55 was added for 20’ to stain the nuclei. The coverslips were analyzed with confocal microscope as described 28.
Figure II on line supplement:
Aortic sinus and ascending aorta atherosclerosis in male PTX3 -/-;ApoE -/-, PTX3+/-;ApoE -/- and PTX3 +/+;ApoE -/- mice fed with the western type diet.
A representative panel of the aortic sinus of thoracic aorta (upper panels) and of abdominal aorta (lower panels) of PTX3 -/-;ApoE -/-, PTX3+/-;ApoE -/- and PTX3 +/+;ApoE -/- mice is shown. At two months of age, mice were fed a western type diet for 4 months than animals were sacrificed and aortic sinus analysed as described in the material and methods section.
Female thoracic aorta

PTX3 +/- / APOE +/-
PTX3 +/- / APOE +/-
PTX3 +/- / APOE +/-

BPTX3 +/- / APOE +/-
PTX3 +/- / APOE +/-
PTX3 +/- / APOE +/-

Figure III on line supplement:
Thoracic and abdominal atherosclerosis in male PTX3 +/-;ApoE +/-, PTX3+/-;Apo E +/- and PTX3 +/-;ApoE/- mice fed with the western type diet.

A representative panel of the aortic sinus of thoracic aorta (upper panels) and of abdominal aorta (lower panels) of PTX3 +/-;ApoE +/-, PTX3+/-;Apo E +/- and PTX3 +/-;ApoE/- mice is shown. At two months of age, mice were fed a western type diet for 4 months than animals were sacrificed and aortic sinus analysed as described in the material and methods section.
**Figure IV on line supplement:**

Aortic sinus atherosclerosis in PTX3 -/-; ApoE -/-; or PTX3 +/-; ApoE-/- mice and PTX3 +/+; ApoE/-/- mice fed chow diet.

The extent of the atherosclerotic lesion in PTX3 -/-;ApoE -/- mice, PTX3+/--;Apo E -/- mice and PTX3 +/+;ApoE/-/- mice for aortic sinus in female is shown for 6, 12 and 18 months old mice (n=6 for each group), and is expressed as mean ± S.D (* p<0.05 vs PTX3 +/-; ApoE -/-).
Female aortic sinus

PTX3 +/-

PTX3 +/+

PTX3 +/-

PTX3 -/-

Male aortic sinus

PTX3 +/-

PTX3 +/-

PTX3 +/-

PTX3 -/-

**Figure V on line supplement:**
Aortic sinus atherosclerosis in PTX3 -/-; PTX3 +/- and PTX3 +/-mice fed western type diet.

A representative panel of the aortic sinus of female (upper panels) and male (lower panels) PTX3 -/-, PTX3 +/- and PTX3 +/- mice is shown. At two months of age, mice were fed a western type diet for 4 months than animals were sacrificed and aortic sinus analysed as described in the material and methods section.
Supplemental Figure VI: Relative expression of inflammatory mediators and their receptors in peripheral blood mononuclear cells by quantitative real-time PCR.

Relative mRNA expression of CCL2, CCR2, CCR5, CX3CR1, IL6, MMP-9, is shown for PTX3 -/-; ApoE -/- mice, PTX3 +/-; Apo E -/- and for PTX3 +/+; ApoE -/- mice (male and female pooled, n=6 for each group). Results, normalised for the RLP13a expression, are shown as fold of induction vs. PTX3 +/+; ApoE -/- mice (data are expressed as mean ± S.D., * p<0,05 vs. PTX3 +/+; ApoE -/- mice).
Supplemental Figure VII:

Analysis of the fibrillar collagen content within the atherosclerotic lesion in female mice.

Panel A. Relative mRNA expression of collagen 1A1, 1A2 and 3A1 is shown for PTX3+/+/ApoE−/−, PTX3+/−/ApoE−/− and PTX3−/−/ApoE−/− mice. The results, normalised for the RLP13α expression, are shown as fold of induction vs. PTX3+/+/ApoE−/− (data are expressed as mean ± S.D.) (* p<0.05 vs. PTX3+/+/ApoE−/−). Panel B. Representative sections of aortic sinus stained with sirius red from female PTX3+/+/ApoE−/−, PTX3+/−/ApoE−/− and PTX3−/−/ApoE−/− mice are shown. The data are presented as the percentage of atherosclerotic lesion covered by Sirius Red positive fibrillar collagen, and are expressed as mean ± S.E, scale bar: 100μm. The percentage of the area positive for fibrillar collagen for aortic sinus is shown on the right part the panel (data are expressed as mean ± S.D).
Supplemental Figure VIII
Analysis of the fibrillar collagen content within the atherosclerotic lesion in male mice.

Panel A. Relative mRNA expression of collagen 1A1, 1A2 and 3A1 is shown for PTX3+/+/ApoE−/−, PTX3+/−/ApoE−/− and PTX3−/−/ApoE−/− mice. The results, normalised for the RLP13a expression, are shown as fold of induction vs. PTX3+/+/ApoE−/− (data are expressed as mean ± S.D.) (* p<0.05 vs. PTX3+/+/ApoE−/−). Panel B. Representative sections of aortic sinus stained with sirius red from female PTX3+/+/ApoE−/−, PTX3+/−/ApoE−/− and PTX3−/−/ApoE−/− mice are shown. The data are presented as the percentage of atherosclerotic lesion covered by Sirius Red positive fibrillar collagen, and are expressed as mean ± S.E, scale bar: 100μm. The percentage of the area positive for fibrillar collagen for aortic sinus is shown on the right part the panel (data are expressed as mean ± S.D).