Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice

Rima Elhage[a,b], Jacek Jawien[a], Mats Rudling[c], Hans-Gustaf Ljunggren[d], Kiyoshi Takeda[e], Shizuo Akira[e], Francis Bayard[b], Göran K. Hansson[a,*]

[a]*Department of Medicine at Karolinska Hospital and Center for Molecular Medicine L8:03, Karolinska Institute, SE-17176 Stockholm, Sweden*
[b]*INSERM U397, Institut Louis Bugnard, Toulouse, France*
[c]*Center for Metabolism and Endocrinology, Department of Medicine, and Center for Nutrition and Toxicology, Novum, Karolinska Institute at Huddinge University Hospital, Stockholm, Sweden*
[d]*Center for Infectious Medicine, Department of Medicine, Karolinska Institute at Huddinge University Hospital, Stockholm, Sweden*
[e]*Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*

Abstract

Objective: Atherosclerosis is an inflammatory disease in which T helper 1 (Th1) immunity has been proposed to play an important role. Naïve CD4+ T cells differentiate into interferon-γ (IFN-γ) producing Th1 effector cells when stimulated by interleukin-18 (IL-18) and IL-12. We wanted to directly test whether the Th1 pathway is proatherogenic. **Methods:** We bred IL-18$^{-/-}$ mice with apolipoprotein E$^{-/-}$ (apoE$^{-/-}$) mice and assessed atherosclerosis in the aortic root of the offspring. **Results:** 24-week-old IL-18 deficient apoE$^{-/-}$ mice exhibited substantially reduced lesion size (93 866±11 273 vs. 144 019±9667 μm^2 in IL-18$^{+/+}$×apoE$^{-/-}$ mice, $P=0.005$). Lesion cells in compound knockout mice displayed reduced I-A^b expression, implying reduced local IFN-γ stimulation. These mice also had an increased proportion of α-SM-actin+ smooth muscle cells, compatible with a more stable lesion phenotype. Immunoglobulin G (IgG) subclass analysis of antibodies to malondialdehyde-modified low density lipoprotein indicated increased Th2 and reduced Th1 helper to B cell antibody production. Surprisingly, serum cholesterol and triglyceride levels were significantly higher in IL-18$^{-/-}$×apoE$^{-/-}$ mice in spite of their reduced atherosclerosis. However, no changes in lipoprotein cholesterol patterns were registered. **Conclusion:** These data show reduced atherosclerosis and Th1 activity in spite of increased serum cholesterol in IL-18 deficient apoE$^{-/-}$ mice. They support a proatherogenic role for IL-18.
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Keywords: Atherosclerosis; Interleukin-18; T cells; Inflammation; Mouse models

1. Introduction

Inflammation plays an important role in the development of atherosclerosis and its complications [1–5]. Inflammatory cells including monocyte/macrophages and T cells infiltrate atherosclerotic lesions and markers of inflammation are elevated in individuals at risk as well as in patients with atherosclerotic cardiovascular disease [6]. In addition, titers of antibodies to the atherosclerosis-associated antigens, oxidized low density lipoprotein (oxLDL) [7,8], and heat shock protein 60 (hsp60) [9,10] correlate with disease development [3].

Ample evidence points to an important role for both innate and adaptive immune mechanisms in the disease process, however, the precise mediators involved in its pathogenesis are not fully elucidated. The T helper 1 cells (Th1) cytokine, interferon-γ (IFN-γ) and the immune activating cell surface receptor pair CD40/CD154 (CD40 ligand) are important proatherogenic factors, genetic deficiencies of which reduce atherosclerosis. On the other hand, immunity carried by B cells appears to play an atheroprotective role since transfer of B cells from atheros-

*Corresponding author. Tel.: +46-8-5177-3227; fax: +46-8-313-147.
E-mail address:* goran.hansson@cmm.ki.se (G.K. Hansson).

clerotic mice to disease-prone ones, protects the latter from developing severe disease. Interestingly, relative protection can also be obtained by immunization with candidate antigens such as oxidized low-density lipoprotein and heat shock protein 60.

Th1 differentiation is induced when naïve CD4+ T cells recognize antigen under conditions when interleukin-12 (IL-12) and interleukin-18 (IL-18) are present in the microenvironment. This initiates a program that involves T-cell activation, cell divisions, and the development of a Th1 effector phenotype. The latter may include expression of certain cell surface receptors and is manifested as a tendency to secrete interferon-γ, tumor necrosis factor-α (TNF-α), and certain other cytokines when antigens are again encountered by the Th1 effector cell [11].

Human atherosclerotic lesions express Th1 cytokines as well as the Th1 permissive cytokines IL-12 and IL-18. Receptors for the latter cytokine are also abundant in lesion cells and it has been speculated that IL-18 signalling causes plaque destabilization. Interestingly, administration of a plasmid encoding a soluble, recombinant IL-18 binding protein to apolipoprotein E-knockout (apoE$^{-/-}$) reduced atherosclerosis after a 9-week treatment period [12]. In another study, treatment of atherosclerotic apoE$^{-/-}$ mice with recombinant IL-18 led to an increased size of atherosclerotic lesions [13]. These findings suggest that IL-18 aggravates existing atherosclerosis. However, the effects of IL-18 on early atherosclerosis remained partly unclear since these studies were limited by the time frame during which the plasmid or recombinant protein could be administered.

To test the hypothesis that IL-18 accelerates atherogenesis, we therefore crossed apoE$^{-/-}$ mice with interleukin-18$^{-/-}$ mice. Our data show that this Th1 inducing cytokine exerts an important, accelerating effect on lesion growth and inflammatory activity.

2. Methods

2.1. Generation of double-deficient mice

Male IL-18$^{-/-}$ mice were obtained from Research Institute for Microbial Diseases, Osaka University, Japan. They were bred with female apoE$^{-/-}$ mice that were originally obtained from The Jackson Laboratory, ME, USA and which were routinely maintained in the Animal Department of Center for Molecular Medicine, Karolinska Institute, Stockholm, Sweden. Both strains were back-crossed for more than 10 generations to the C57BL/6J background. F1 heterozygotes were mated to obtain an F2 generation, which was genotyped to select male IL-18$^{-/-}$×apoE$^{-/-}$ and IL-18$^{+/+}$×apoE$^{-/-}$ littermates that were used for experiments. Genetic screening was carried out by PCR on tail DNA using the apoE gene primers: 5′-GCCTAGCCGAGGGAGAGCCG-3′ (oIMR180), 5′-TGTGACTTGGGAGCTCTGCAGC-3′ (oIMR181) and

5′-GCCGCCCCGACTGCATCT-3′ (oIMR182) recommended by the Jackson Laboratory, and the following IL-18 gene primers: 5′-TAATGGGTGGTCTTCTCATCTCTGTGT-3′ (specific for the targeted gene), 5′-TTGCTGCACCTAGAGGTAT-GTACTGAC-3′ (specific for the native IL-18 gene upstream of the targeting construct) and 5′-ATCGCCTTCTATCGCCTTCTTGACGAG-3′ (specific for the *neo* resistance gene of the targeting construct) (Fig. 1). Ten IL-18$^{-/-}$×apoE$^{-/-}$ and 10 IL-18$^{+/+}$×apoE$^{-/-}$ mice were sacrificed with an overdose of Ketalar® at 24 weeks of age. Sera were harvested, citrated, and frozen, and the heart and proximal aorta dissected out and snap-frozen in liquid nitrogen. All animal procedures were approved by the Stockholm North Ethical Committee on Animal Experiments.

2.2. Plasma lipids and lipoproteins

Total cholesterol and triglycerides were assayed using commercially available kits (Roche Molecular Biochemicals, Indianapolis, IN, USA). Size fractionation of lipoproteins was carried out by fast protein liquid chromatography (FPLC) using a micro-FPLC column (30×0.32 cm Superose6B, Amersham Pharmacia, Uppsala, Sweden) coupled to a system for on-line separation and subsequent detection of cholesterol [14]; 10 μl of plasma was injected into the FPLC column from every animal, and the cholesterol content in lipoproteins was determined on-line using the commercially available MPR 2 1 442 350 cholesterol assay kit (Roche), which was continuously mixed with the separated lipoproteins at a flow rate of 40 μl/min. Absorbance was measured at 500 nm, and the data were collected every 20th second using EZChrom software (Scientific Software, SanRamon, CA, USA).

2.3. Tissue preparation and lesion analysis

Serial cryostat sections were cut from the proximal 1 mm of the aortic root. Hematoxylin-Oil red O-stained
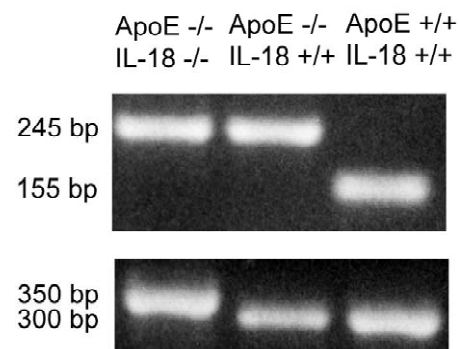

Fig. 1. Genotyping of mice. Upper band shows PCR products characteristic for ApoE$^{-/-}$ (245 bp) and wild-type (155 bp) mice. Lower band shows PCR products for IL-18$^{-/-}$ (350 bp) and wild-type (300 bp) mice.

sections were used for computer-assisted morphometry as described [15]. In brief, 10 sections were collected at every 100 μm over a 1-mm segment of the aortic root. For each section, images were captured in a computer and the surface area of the lesions were measured.

2.4. Immunohistochemistry

Cryostat sections from the proximal aorta were fixed in acetone, air-dried, incubated with normal rat serum, and reacted with monoclonal antibodies. A rat monoclonal was used to detect CD4, a biotinylated mouse monoclonal for I-A^b (both from PharMingen, San Diego, CA, USA), and an alkaline phosphatase labeled mouse monoclonal to stain anti-α-SM-actin of smooth muscle cells (Sigma, St Louis, MO, USA). Binding of rat monoclonals was revealed using biotinylated rabbit-anti-rat IgG (Vector Laboratories, Burlingame, CA, USA) and the binding of biotinylated antibodies staining CD4 and I-A^b (both from BD PharMingen, CA, USA) was visualized with an avidin DH-biotinylated peroxidase complex (Vectastain ABC kit, Vector Laboratories). The alkaline phosphatase-labeled monoclonals were visualized using the Fast Red-based Alkaline phosphatase substrate kit I (Vector Laboratories). Immunohistochemical data were obtained by manual counting of total cells in all lesions of one section per mouse. The analysis was validated when another investigator recounted the slides. Both investigators were blinded with regard to the origin of the slides (group, treatment) and inter-investigator difference was less than 15%.

2.5. Anti-LDL antibodies

LDL ($d=1.019–1.063$ g/ml) was obtained under sterile conditions by ultracentrifugation of human plasma collected from 10 donors. MDA modification was carried out as described [5] and the extent of modification assessed by spectrophotometry at 400 and 470 nm. The titers of specific anti-MDA-LDL and anti-LDL antibodies were measured by ELISA, using alkaline phosphatase (for IgG2a) or peroxidase (for IgG1) -labeled anti-mouse-IgG [16].

2.6. Statistical analysis

Results are expressed as mean±S.E.M. The data were analyzed by Student's t-test. $P<0.05$ was considered as statistically significant.

3. Results

3.1. Generation and characterization of IL-18 deficient apoE$^{-/-}$ mice

IL-18$^{-/-}$ mice were crossed with apoE$^{-/-}$ mice and littermate F1 animals crossed to obtain homozygous IL-

Table 1
Serum lipids in interleukin-18 (IL-18) competent and IL-18 deficient apolipoprotein E knockout mice

IL-18 genotype	Serum cholesterol (mmol/l[a])	Serum triglycerides (mmol/l[a])
IL-18$^{+/+}$	20±1.7	1.39±0.12
IL-18$^{-/-}$	30±2.4*	2.8±0.23**

Values are means±S.E.M. *Significantly different from IL-18$^{+/+}$: $P=0.0004$; **Significantly different from IL-18$^{+/+}$: $P=0.007$.
[a] For cholesterol, 1 mmol/l equals 25.91 g/dl; for trigycerides, 1 mmol/l is 88.0 g/dl.

18$^{-/-}$×apoE$^{-/-}$ mice in the F2 generation. The latter were obtained at the expected Mendelian frequency and did not display any morphogenetic abnormalities. However, the average body weight in the apoE$^{-/-}$ IL-18$^{-/-}$ group was significantly higher than that of the apoE$^{-/-}$ IL-18$^{+/+}$ group (33.3±1.42 vs. 28.1±1.7 g, respectively, $P=0.035$).

3.2. Lipids and lipoproteins

When compared with IL-18$^{+/+}$×apoE$^{-/-}$ mice, IL-18 deficient ones had significantly higher serum cholesterol and triglyceride levels (Table 1). However, fast performance liquid chromatography of sera did not reveal any differences in lipoprotein distribution between groups (Fig. 2). Therefore, the 50% increase in serum cholesterol was due to increases in all lipoprotein fractions.

3.3. Quantitation of atherosclerotic lesions

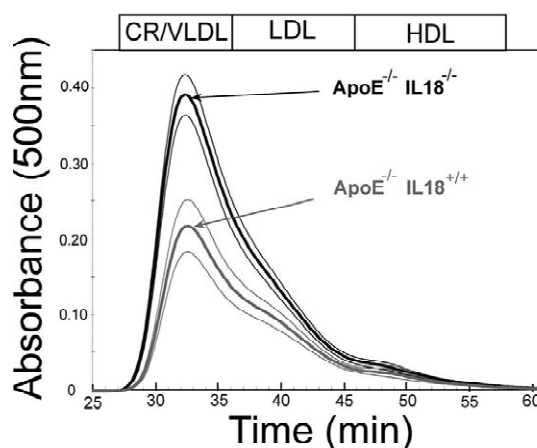Atherosclerotic lesions were analyzed in the aortic root

Fig. 2. Fast protein liquid chromatography (FPLC) analysis of lipoprotein–cholesterol profiles of mice sera. The absorbance, reflecting cholesterol concentration is plotted against retention time. From each of the indicated groups, five individual mice were analyzed. Black lines: mean and 1 S.D. of apoE$^{-/-}$ IL-18$^{-/-}$ mice; grey lines: mean and 1 S.D. of apoE$^{-/-}$ IL-18$^{+/+}$ mice.

of 24-week-old male IL-18$^{-/-}$×apoE$^{-/-}$ mice and their IL-18$^{+/+}$×apoE$^{-/-}$ littermates. The absence of IL-18 led to a reduction in lesion size, from 144 019±9667 μm^2 to 93 866±11 273 μm^2 (P=0.005). When the fraction area of lesions was calculated (i.e. lesion area/total vessel area), a similar reduction was registered (Fig. 3). Paradoxically, increased serum cholesterol was therefore associated with reduced atherosclerosis in IL-18 deficient apoE$^{-/-}$ mice.

3.4. Lesion composition

Immunohistochemical analysis of lesions revealed that expression of the interferon-γ inducible gene, I-A^b was significantly lower in IL-18$^{-/-}$×apoE$^{-/-}$ mice (Figs. 4 and 5c,d). The frequency of CD4+ cells also tended to be lower in IL-18$^{-/-}$×apoE$^{-/-}$ mice (Fig. 5a,b; 16±5 vs. 26±8 cells per 100 μm^2), however, this trend did not reach statistical significance. In parallel with the reduction of I-A^b expressing cells, there was an increase in α-SM-actin positive smooth muscle cells in the IL-18$^{-/-}$× apoE$^{-/-}$ mice (Figs. 5e,f and 6).

3.5. Anti-LDL antibodies

To estimate the extent to which IL-18 abrogation affected T helper cells in the humoral immune response to oxidized low density lipoproteins (LDL), we analyzed immunoglobulin G (IgG) isotypes of autoantibodies to modified LDL. As shown in Fig. 7, IL-18$^{-/-}$×apoE$^{-/-}$ mice displayed a significant, threefold increase in IgG1 antibodies to MDA-LDL. IgG2a antibodies showed a non-significant 1.4-fold increase in the IL-18 deficient mice. The IgG2a/IgG1 ratio was therefore reduced by 50%, from 2.6 to 1.3. This implies that the loss of IL-18 signalling led
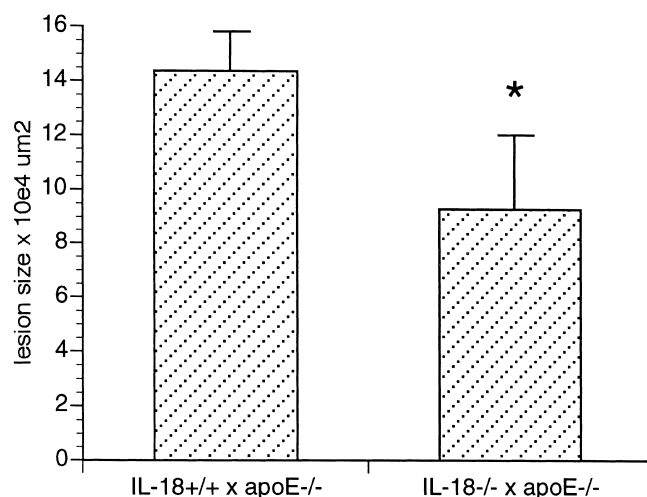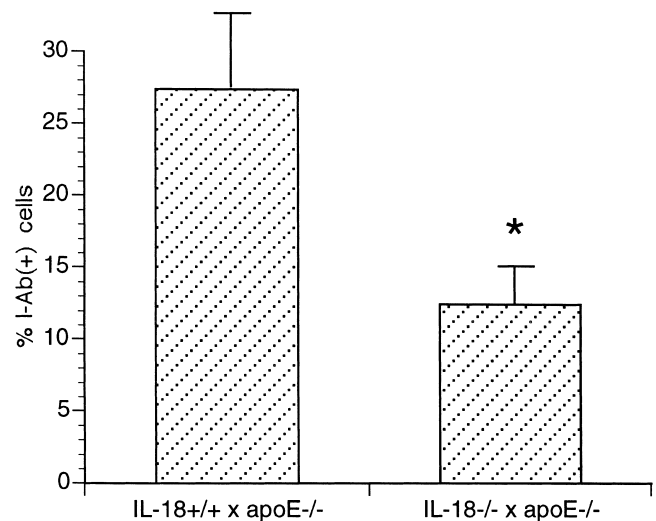

Fig. 4. Proportion of cells expressing I-A^b (% of all lesion cells) in apoE$^{-/-}$ IL-18$^{+/+}$, compared to apoE$^{-/-}$ IL-18$^{-/-}$ mice. Cryostat sections of the aortic root were stained for I-A^b with avidin–biotin–peroxidase immunostaining, and counterstained with hematoxylin. Antibody positive cells and the total number of cells were counted at 400× magnification. Data show mean±S.E.M. (n=10 per group), *P= 0.000265.

to a switch from Th1 to Th2 in the immune response to this autoantigen.

4. Discussion

The present data support the notion that Th1 signalling aggravates atherosclerosis and implicate IL-18 as an important mediator of this effect. When compared with IL-18 competent littermates, the compound IL-18$^{-/-}$× apoE$^{-/-}$ mice exhibited a substantial, 35% reduction in the size of atherosclerotic lesions. Furthermore, lesions contained an increased proportion of α-SM-actin+ smooth muscle cells and reduced interferon-γ dependent gene expression, implying increased lesion stability with reduced immune activation. This is in line with previous findings of reduced IFN-γ production in IL-18 deficient mice and serum IFN-γ level [17–20]. Remarkably, the reduction in atherosclerosis occurred in spite of a 50% increase in serum cholesterol in the IL-18 deficient animals. These data clearly demonstrate that IL-18 is a proatherogenic mediator.

Several previous studies have implicated IL-18 in the pathogenesis of atherosclerosis. Mallat et al. showed that this cytokine is expressed in human atherosclerotic plaques and up-regulated in unstable plaques [21]. Functional IL-18 receptors are expressed by vascular cells and transduce IL-18 signals, which may also be produced by cells present in atherosclerotic lesions [22]. Treatment of atherosclerotic mice with recombinant IL-18 aggravates disease [13], while transfection with an inhibitory IL-18 binding protein plasmid reduces advanced disease in apoE$^{-/-}$ mice [12].

Fig. 3. Atherosclerotic lesions in apoE$^{-/-}$ IL-18$^{+/+}$ and apoE$^{-/-}$ IL-18$^{-/-}$ mice (n=10 in each group, mean±S.E.M.). Data show the average lesion size in cross-sections from the aortic root. The two groups were significantly different, *P=0.005.
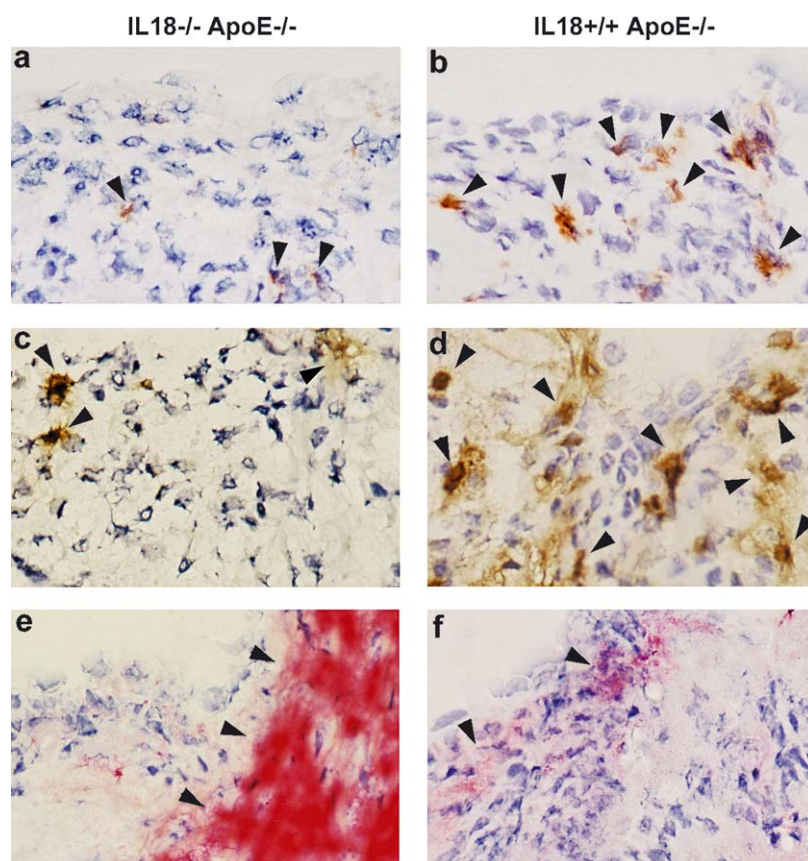
Fig. 5. Immunohistochemical analysis of lesions in IL-18$^{-/-}$×apoE$^{-/-}$ and IL-18$^{+/+}$×apoE$^{-/-}$ mice. Cryostat sections were stained for I-A^b (a, b) and CD4 (c, d) using immunoperoxidase (brown staining), and for α-actin in smooth muscle cells (α-SM-actin) by alkaline phosphatase-labelled antibodies (e, f; red staining). Sections (a), (c) and (e) are from IL-18$^{-/-}$×apoE$^{-/-}$ mice, while (b), (d) and (f) are from IL-18$^{+/+}$×apoE$^{-/-}$ mice. Magnification 400× (scale: 1 cm=190 μm).
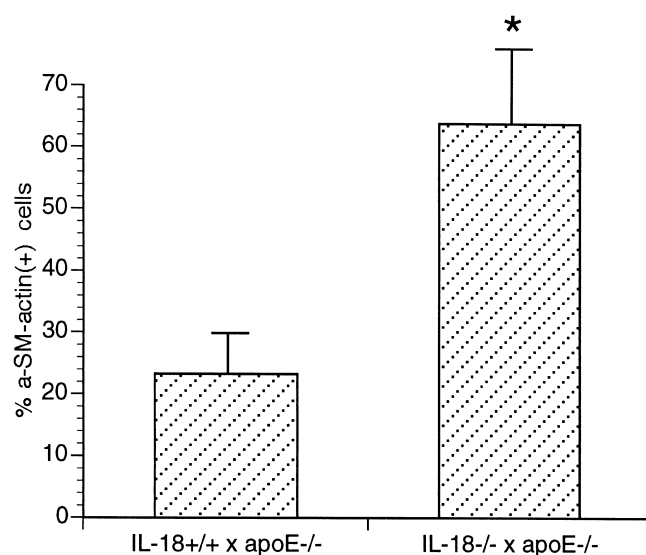

Fig. 6. Proportion of smooth muscle cells expressing α-actin (α-SM-actin) in atherosclerotic lesions. Cryostat sections of the aortic root were stained for α-SM-actin by alkaline phosphatase-labelled antibodies and counterstained with hematoxylin. Antibody-positive cells and the total number of cells per lesion were counted at 400× magnification. Data represent antibody-positive cells as percent of all cells (n=10 per group, mean±S.E.M.), *P=0.0085.
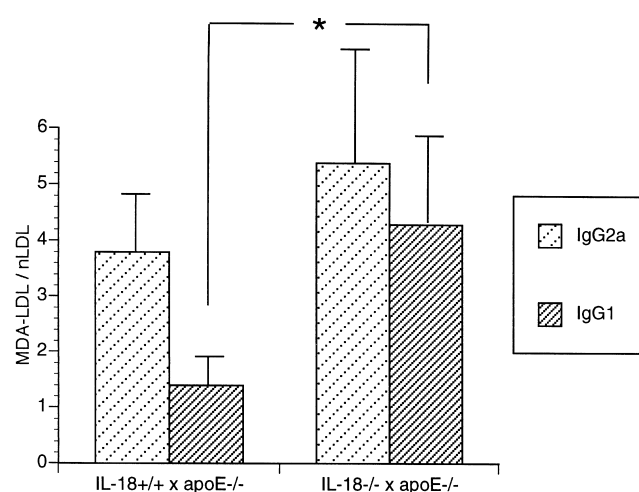

Fig. 7. Immunoglobulin G isotypes of antibodies to malondialdehyde-modified low density lipoproteins (MDA-LDL). ELISA analysis of antibody titers (mean±S.E.M.); values represent absorbance to plates coated with MDA-LDL divided by the absorbance to plates with native LDL, *P=0.024.

Interestingly, no disease aggravating effect of IL-18 could be discerned in interferon-γ deficient apoE$^{-/-}$ mice, implying that IL-18 enhances atherosclerosis through release of interferon-γ [13]. However, these short-term treatment studies did not clarify whether IL-18 acts on the vessel wall or through lipid metabolism when it worsens atherosclerosis. The first generation of a double knockout mouse permitted us to address this question and to determine the long-term effects of IL-18 deficiency on atherosclerosis.

Our present data show that IL-18 deficiency reduces atherosclerosis in spite of increased serum cholesterol. The latter effect was not due to a shift from VLDL/LDL to HDL since all lipoprotein fractions were affected to approximately the same extent. These findings suggest that IL-18 does not act on a single, specific step in lipoprotein conversion such as a lipase. Instead, it might modulate cholesterol uptake or synthesis. However, the effect of IL-18 on cholesterol metabolism requires further study.

IL-18 deficient mice exhibited reduced I-A^b expression, implying reduced interferon-γ signalling, in their atherosclerotic lesions. This is in line with the recent finding that the proatherogenic effect of recombinant IL-18 is abrogated in interferon-γ deficient mice [13] and suggests that IL-18 aggravates atherosclerosis by inducing interferon-γ, which acts on lesion cells to accelerate the disease process. Importantly, interferon-γ is a macrophage-activating cytokine, upregulates adhesion molecule expression on endothelial cells, and inhibits smooth muscle proliferation and α-actin expression [23]. The increased proportion of α-SM-actin+ smooth muscle cells in IL-18$^{-/-}$×apoE$^{-/-}$ mice may therefore reflect reduced interferon-γ signalling. Since differentiated smooth muscle cells contribute tensile strength to arterial tissue, the increased proportion of such cells in the IL-18$^{-/-}$×apoE$^{-/-}$ mice suggests that their lesions were more stable than those in IL-18 competent apoE$^{-/-}$ mice. This, in turn, may imply that IL-18 secretion contributes to lesion destabilization, plaque rupture, and arterial thrombosis. Again, further studies will be required to clarify whether this is the case.

The primary target for IL-18 is likely to be the CD4+ T cell. Under the influence of IL-18 and IL-12, antigen-exposed naïve CD4+ T cells differentiate into Th1 effector cells, which secrete interferon-γ. Since atherosclerotic lesions contain abundant CD4+ T cells as well as interferon-γ [24], both CD4+ T cells and interferon-γ have been shown to aggravate disease [25,26], and the proatherogenic effect of recombinant IL-18 is mediated through interferon-γ [13], it is likely that an absence or paucity of Th1 effector cells in lesions accounts for the reduced atherosclerosis in IL-18$^{-/-}$×apoE$^{-/-}$ mice. The isotype switch observed for T-cell dependent antibodies to MDA-LDL indicates that these mice have a profound, systemic Th1→Th2 switch. In addition to lack of IL-18, this switch may be prompted by severe hypercholesterolemia, which occurred in IL-18 deficient mice [16].

However, it is likely that the lack of IL-18 played a decisive role for the Th1→Th2 switch observed in the present study.

It is possible that the proatherogenic effect of IL-18 is exerted in the secondary lymphoid organs, where it promotes Th1 differentiation. If correct, this would imply that the reduced interferon-γ signalling in lesions is due to a systemic reduction in Th1 cells rather than to a local action of IL-18 in the lesions. This hypothesis is in line with the finding of changes in systemic adaptive immunity in parallel with the effects on the disease process in the artery wall.

To summarize, our analysis of IL-18$^{-/-}$×apoE$^{-/-}$ mice has shown reduced atherosclerosis despite increased serum cholesterol and provided data suggesting reduced local interferon-γ signalling and increased plaque stability. These findings corroborate the notion that IL-18 is a proatherogenic cytokine which acts by promoting the development of interferon-γ secreting Th1 type CD4+ T cells. They suggest that inhibition of the IL-18/Th1/interferon-γ pathway could be an attractive approach for treatment of atherosclerosis.

Acknowledgements

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