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Research Article

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IFN- γ Potentiates Atherosclerosis in ApoE Knock-out Mice

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Abstract

The early colocalization of T cells and the potent immunostimulatory cytokine IFN- γ to atherosclerotic lesions suggest that the immune system contributes to atherogenesis. Since mice with a targeted disruption of the apoE gene (apoE 0 mice) develop profound atherosclerosis, we examined the role of IFN- γ in this process. First, the presence of CD4⁺ and CD8⁺ cells, which secrete lesional IFN- γ , was documented in apoE 0 atheromata. Then, the apoE 0 mice were crossed with IFN- γ receptor (IFN γ R) 0 mice to generate apoE 0/IFN γ R 0 mice. Compared to the apoE 0 mice, the compound knock-out mice exhibited a substantial reduction in atherosclerotic lesion size, a 60% reduction in lesion lipid accumulation, a decrease in lesion cellularity, but a marked increase in lesion collagen content. Evaluation of the plasma lipoproteins showed that the compound knock-out mice had a marked increase in potentially atheroprotective phospholipid/apoA-IV rich particles as well. This correlated with an induction of hepatic apoA-IV transcripts. These observations suggest that IFN- γ promotes and modifies atherosclerosis through both local effects in the arterial wall as well as a systemic effect on plasma lipoproteins. Therefore, therapeutic inhibition of IFN- γ signaling may lead to the formation of more lipid-poor and stable atheromata. (*J. Clin. Invest.* 1997. 99:2752–2761.) Key words: IFN- γ • apolipoprotein-deficient mice • T cells • collagen

Introduction

The evolution of atherosclerotic lesions involves an interaction between four major cell types, endothelial cells (ECs), smooth muscle cells (SMCs), macrophages, and lymphocytes (1). The contribution of ECs, macrophages, and SMCs to lesion development has been well-documented. However, the role of lesional T cells in this process has been less well-characterized. Immunohistochemical studies have colocalized T cells and the potent immunostimulatory cytokine they secrete, IFN- γ , to human atherosclerotic lesions. In most of these studies, the CD4⁺ subset of T cells appears to predominate modestly (2, 3). Even though both the CD4⁺ (i.e., Th1 cells) and CD8⁺ cells secrete IFN- γ , the nature of the immune response they mediate

is quite distinct. CD4⁺ (Th1) cells restrict their response to antigens presented in the context of MHC II (i.e., foreign antigens). Moreover, macrophages play an important role in this response. Detailed studies on how T cells and macrophages interact to mediate an immune response in other systems indicate that IFN- γ plays a pivotal role in mediating this cellular immune response (4–6). However, studies on the potential role of IFN- γ and the immune system in the pathogenesis of atherosclerotic lesions have provided conflicting results. Although both T cells and MHC-II expressing macrophages and SMCs are early components of lesions (7–9), MHC II and T cell-deficient mice develop normal fatty streak lesions on atherogenic diets (10). Yet, mice deficient in MHC I exhibited a threefold increase in lesion area ($P = 0.01$), suggesting that CD8⁺ cells are atheroprotective (10). However, the latter observation is inconsistent with the parallel set of studies on the T cell-deficient mice suggesting T cells have no effect on lesion development. Additional studies, examining the atherosclerotic potential of IFN- γ have yielded mixed results as well. On one hand, IFN- γ stimulates the expression of VCAM-1 on ECs (8), MHC-II on macrophages and SMCs (8, 9), and lipoprotein receptors in SMC (11), all potentially proatherogenic properties. On the other hand, IFN- γ decreases lipoprotein receptor expression on macrophages (12, 13), decreases collagen synthesis in SMCs (14), and blocks SMC proliferation (15, 16), all potentially antiatherogenic effects. Hence, the overall impact of the immune system and IFN- γ in diet-induced atherosclerosis remains controversial.

The critical role IFN- γ plays in modulating the cellular immune responses has been recently confirmed by the detailed characterization of the intracellular signaling pathways stimulated by IFN- γ (17–20), and by the generation of mice with a targeted disruption of IFN- γ (21) or the IFN- γ receptor (6). As predicted, these mice display a profound impairment of their immune response to several intracellular pathogens (6, 21). They also exhibit a hyperproliferative response in their Th2 subset of T cells after stimulation with other antigens, highlighting the important role IFN- γ plays in maintaining the balance between the Th1 subset (involved in cellular immunity) and the Th2 subset (involved in humoral immunity) of T cells (6, 20, 21). The availability of IFN- γ receptor-deficient (i.e., IFN γ R 0) mice has provided a unique opportunity to directly examine the role of IFN- γ in atherosclerosis. Moreover, since standard strains of mice with immune system defects do not develop extensive or complex atherosclerotic lesions, we have crossed the IFN γ R 0 mice into the hypercholesterolemia and atherosclerosis-prone apoE knockout (apoE 0) background (22–24). These compound knock-out mice demonstrate a significant decrease in their proclivity to develop atheromata, providing strong evidence that IFN- γ promotes atherosclerosis.

Methods

Mice. The IFN γ R 0 mice, in a 129 background, were a generous gift from M. Aguet (Swiss Institute of Experimental Cancer Research,

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1. Abbreviations used in this paper: ECs, endothelial cells; IFN γ R, IFN γ receptor; SMCs, smooth muscle cells.

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CD4 Staining

CD8 Staining

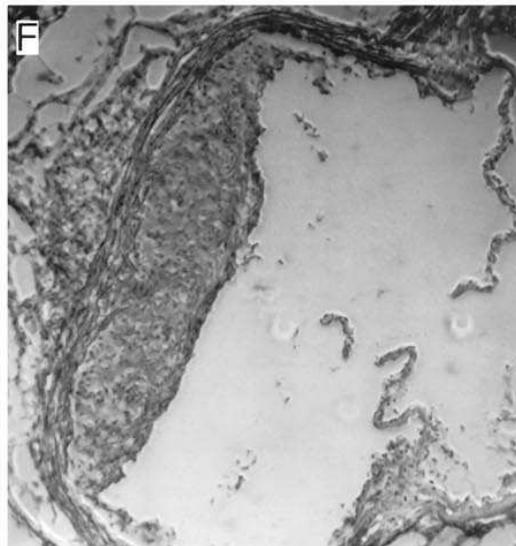
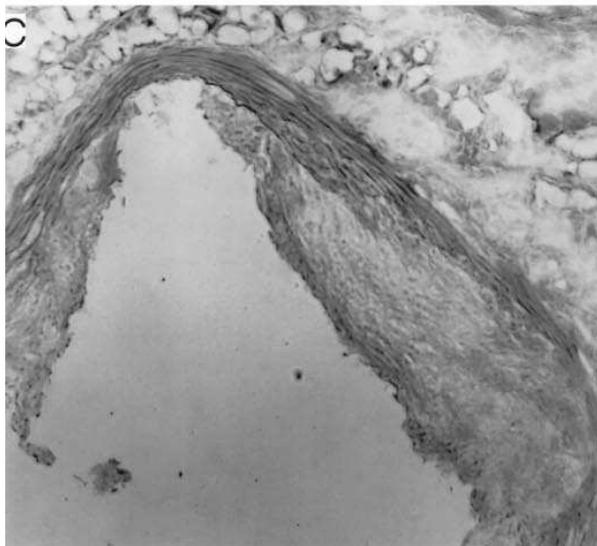
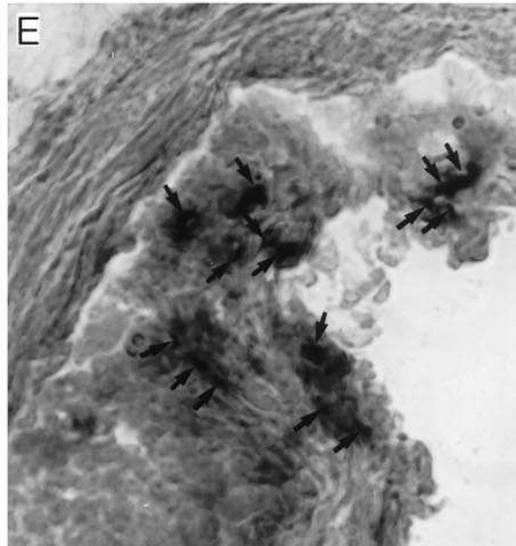
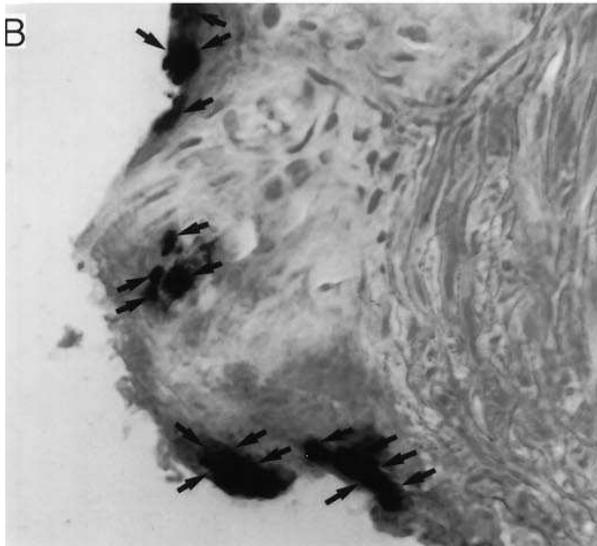
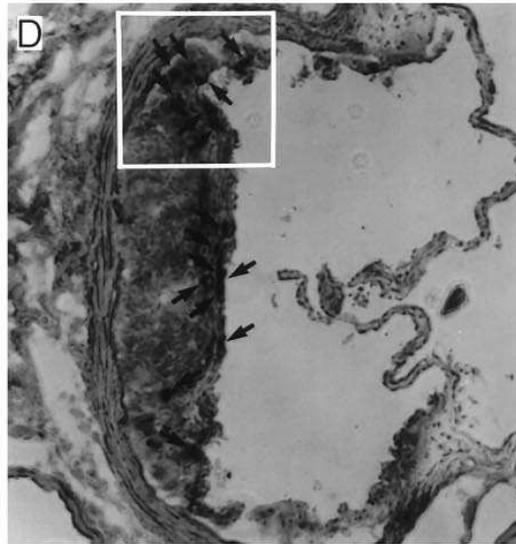


Figure 1. Localization of CD4⁺ and CD8⁺ T cells to apoE 0 atherosclerotic lesions. Sections of proximal aorta from 17-wk-old apoE 0 mice, fed the Western type diet, were immunostained with CD4 (A and B) or CD8 (D and E) monoclonal antibodies. The sections were then counter-

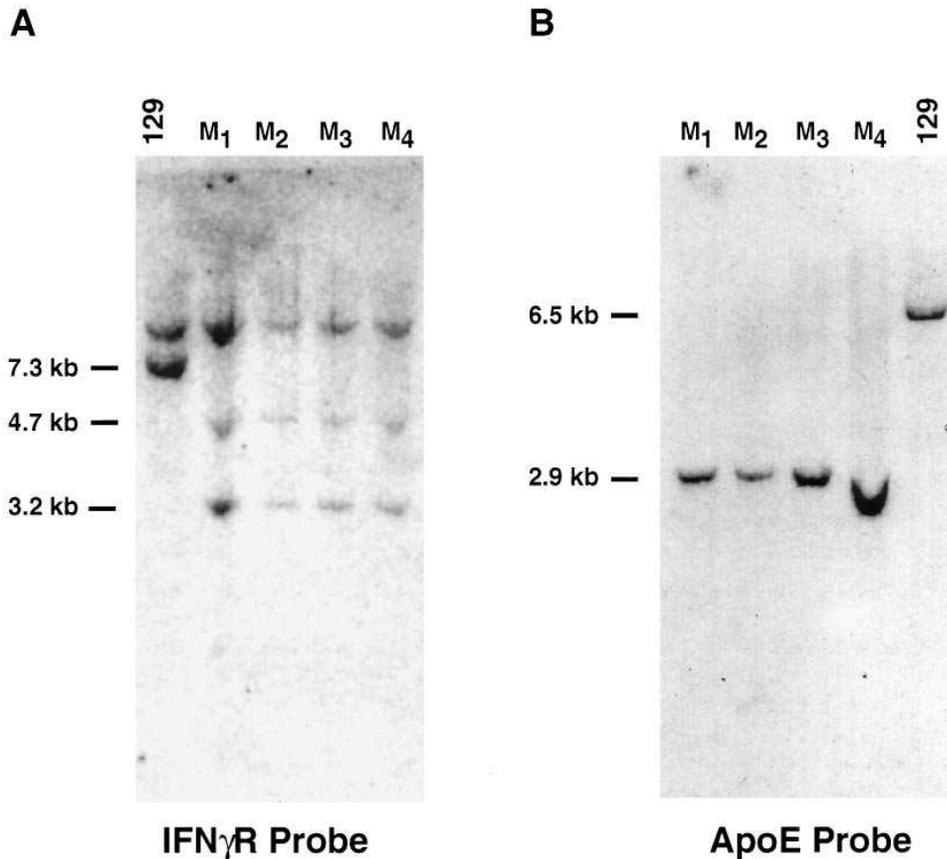


Figure 2. Genotyping of the apoE 0/IFN γ R 0 mice by Southern blotting. Murine tail DNA preparations were digested with BamHI (A) or HindIII (B) and hybridized with IFN γ R (A) or apoE (B) probes as described previously (6, 22). Mobilities of the wild-type 7.3-kb and the knock-out 4.7- and 3.2-kb IFN γ R bands are indicated to the left of A. A larger band of \sim 9 kb arises from hybridization to a BamHI fragment outside of the targeted locus. The mobilities of the wild-type 6.5-kb and the knock-out 2.9-kb apoE bands are indicated to the right of B. 129 represents DNA prepared from wild-type 129 mice, whereas M₁–M₄ represent DNA prepared from the original four compound knock-out mice. All subsequent apoE 0/IFN γ R 0 mice were progeny of M₁–M₄.

Lausanne, Switzerland) (6). The apoE 0 mice, in a mixed 129/C57Bl/6J background, were a generous gift from J.L. Breslow (Rockefeller University, New York) (22). The apoE 0 and apoE 0/IFN γ R 0 mice used in these studies were all derived from the offspring of a single cross between the original set of IFN γ R 0 and apoE 0 mice. For many of the studies, the mice studied arose from one additional intercross between the apoE 0 and apoE 0/IFN γ R 0 mice, in an effort to homogenize genetic backgrounds. All genotyping was initially carried out by a PCR-based assay and then confirmed with Southern blotting as described previously (6, 22). Northern blotting was done as described previously (25).

Once sufficient numbers of single and double knock-out mice were available, 5–10 age-matched (5 wk old) females were placed on a Western type diet (21% fat, 0.15% cholesterol; Harlan/Teklad, Madison, WI) for 3 mo. After this period the mice were killed and both plasma samples and hearts/proximal aorta preparations were collected.

Histochemistry. Hearts were perfused with phosphate-buffered saline, embedded in O.C.T. compound (Tissue-Tek; Miles Laboratories, Elkhart, IN) and snap-frozen. 10- μ m-thick transverse sections were collected from the proximal aorta, fixed in cold acetone, and stained with CD4 or CD8 monoclonal antibodies, as recommended by the manufacturer (GIBCO BRL, Gaithersburg, MD). Bound antibodies were detected with a biotin coupled alkaline phosphatase kit (Vectastain ABC-AP; Vector Laboratories Inc., Burlingame, CA) after treating the specimens with levamisole to reduce background.

Specimens were then visualized with a Nikon Optiphot microscope with \times 4, \times 10, and \times 40 objectives.

For quantitation, 10- μ m-thick transverse sections of atherosclerotic lesions (covering a length of 350–400 μ m) from the proximal aorta were stained with oil red-O, hematoxylin and eosin, or trichrome after fixation in 4% formaldehyde. For the samples stained with oil red-O, every eighth section, for a total of eight sections, were quantitated for accumulation of intimal lipid by video microscopy as described previously (22, 26, 27), and a cumulative value was determined for each mouse.

Lipid studies. Plasma samples, collected at the time of killing, were pooled as indicated and evaluated for phospholipid, cholesteryl ester, and free cholesterol by a 4-aminoantipyrine-based enzymatic assay (Wako Bioproducts, Richmond, VA). Samples were also fractionated by FPLC (Superose 6; Pharmacia LKB Biotechnology Inc., Piscataway, NJ) or by sequential buoyant ultracentrifugation and SDS/PAGE gel as described previously (22, 28).

Results

CD4⁺ and CD8⁺ cells colocalize to atherosclerotic lesions in apoE 0 mice. Since studies on the role of lesional IFN- γ on atherosclerosis are predicated on the presence of T cells within lesions, the apoE 0 lesions were evaluated for the presence of CD4⁺ and CD8⁺ cells. Frozen sections were prepared from the

Figure 1 legend (Continued)

stained with nuclear fast red, and examined with a Nikon Optiphot microscope at \times 100 (A, C, D, and F) and at \times 400 (B and E). The areas magnified at \times 400 are indicated by white boxes in A and D. The sections shown in C and F were only stained with secondary antibodies and serve as negative controls. Arrows, CD4⁺ and CD8⁺ staining.

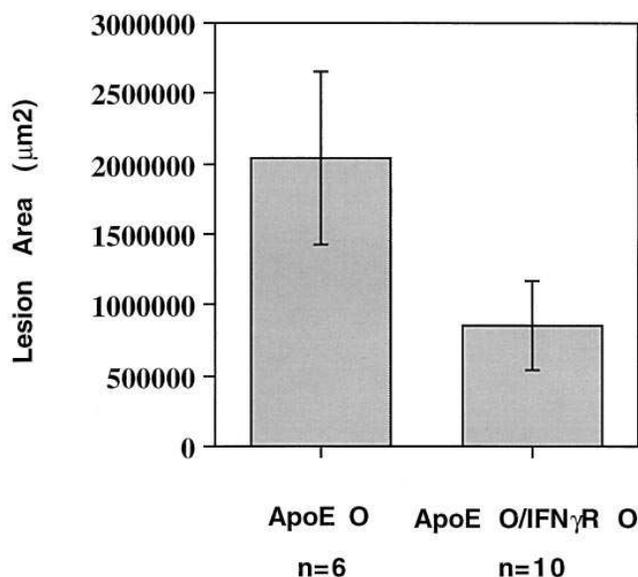


Figure 3. Quantitative comparison of atherosclerotic lesions in apoE 0 and apoE 0/IFN γ R 0 mice. Sections of proximal aorta from 17-wk-old female apoE 0 ($n = 6$) and apoE 0/IFN γ R 0 ($n = 10$) mice, fed a Western type diet for 12 wk, were stained with oil red-O, and subsequently quantitated for accumulation of subintimal lipid by video microscopy. Then the total (i.e., cumulative) values of subintimal lipid accumulations for each mouse were appropriately averaged to obtain the values represented by each bar.

proximal aorta and control spleens (data not shown) of apoE 0 mice fed a Western type diet for 3 mo and stained with murine specific CD4 and CD8 antibodies. Similar to studies published as this manuscript was being prepared demonstrating the presence of Thy 1.2, CD4, and CD8 positive cells (i.e., T cells) in apoE 0 atheromata (29, 30), we found numerous CD4⁺ and CD8⁺ cells in apoE 0 lesions (Fig. 1). Although a majority of these cells were observed within the lesion, many were near the luminal surface. CD8⁺ cells were also detected, but they were somewhat less abundant. Likewise, they were more evident near the luminal surface. However, they tended to be associated with areas of the lesions where the endothelial surface appeared to be compromised (Fig. 1). These studies are consistent with reports on human atheromata, and indicate that the apoE 0 model is appropriate for the study of the role of lesional IFN- γ in atherogenesis. Moreover, as in human studies (2, 3), we found CD4⁺ cells to be modestly more abundant than the CD8⁺ cells.

Generation of IFN γ R/ApoE compound knock-out mice. Initial studies on the ability of IFN γ R 0 mice to develop atherosclerotic lesion on an atherosclerosis-prone diet were uninformative because mice in a 129 background do not develop significant atherosclerotic lesions (data not shown). To increase the sensitivity of the experimental system and to be able to evaluate either increases or decreases in lesion size, IFN γ R 0 mice were crossed with the hypercholesterolemia and atherosclerosis-prone apoE 0 mice (22–24). F1 mice were interbred and genotyped (Fig. 2) (6, 22) in order to obtain apoE 0 and apoE 0/IFN γ R 0 mice. The final population of apoE 0/IFN γ R 0 mice was homozygous for both the 3.2- and 4.7-kb

bands of the IFN γ R knock-out allele and the 2.9-kb band of the apoE knock-out allele (Fig. 2, M_1 – M_4). The final population of apoE 0 mice was only homozygous for the 2.9-kb band of the apoE knock-out allele (data not shown).

Atherosclerosis in apoE 0/IFN γ R compound knock-out mice. To evaluate atherosclerotic lesion development, a matched set of apoE 0 and apoE 0/IFN γ R 0 mice was placed on a Western type diet (22). After 3 mo, the mice were killed and the atherosclerotic lesions in the proximal aorta was evaluated. Standard histology indicated that the lesions in the apoE 0/IFN γ R 0 mice were substantially smaller than those in the apoE 0 mice (see Fig. 4). As other murine studies had validated assessment of subintimal lipid accumulation as an effective method for the quantitation of murine atherosclerotic lesions (22, 26, 27), such an analysis was undertaken with these mice. Evaluation of 6 apoE 0 and 10 apoE 0/IFN γ R 0 mice, matched for age and sex, determined a significant 59% ($P < 0.0001$) reduction in lesional lipid content in the compound knock-out mice (Fig. 3). Consistent with these observations, evaluation of a temporally distinct set of age- and sex-matched apoE 0 ($n = 5$) and apoE 0/IFN γ R 0 ($n = 6$) mice demonstrated a 58% reduction ($P < 0.002$) in subintimal lipid accumulation (data not shown). Not only did these observations support our initial impression that lesions in apoE 0/IFN γ R 0 mice are smaller than those in apoE 0 mice, but the fact that our P values were low in the setting of a relatively large sample size indicates that individual genetic variation had little effect on these results.

To determine whether there were any qualitative differences in lesions in addition to those of lipid content, a careful histological analysis was carried out. Hematoxylin and eosin sections, prepared from three mice from the control (apoE 0) and study groups (apoE 0/IFN γ R 0), were evaluated blindly. In each case, lesions from the apoE 0 mice were larger and more cellular (Fig. 4). Consistent with what has been reported previously, the structural features observed in these sections, as well as those determined with specialized stains (e.g., oil red-O staining, Fig. 3; CD4 and CD8 immunohistochemistry, Fig. 1; and a trichrome stain, Fig. 5), suggested that foam cells, SMCs, and lymphocytes were present in the apoE 0 lesions (22, 24, 29, 30). In contrast, the apoE 0/IFN γ R 0 lesions were strikingly less cellular (Fig. 4), but still contained many of the same cell types, including CD4 and CD8 positive cells (data not shown). To evaluate the possibility that the decreased cellularity of the compound knock-out lesions might reflect an increase in extracellular matrix, additional sections were evaluated with a trichrome stain. These sections demonstrated a marked increase in blue staining collagen, spanning the entire thickness of the lesion. This pattern was distinct from the apoE

Table I. Plasma Lipid Concentration (Milligrams per Deciliter)

Mice	Phospholipid	Cholesteryl ester	Free cholesterol
129	227 \pm 31	66 \pm 5	11 \pm 0.4
IFN γ R 0	216 \pm 22	57 \pm 11	10 \pm 1.7
apoE 0	320 \pm 20	448 \pm 66	281 \pm 42
apoE 0/IFN γ R 0	487 \pm 62*	501 \pm 52	342 \pm 39 [‡]

Pooled plasma from apoE 0 and apoE 0/IFN γ R 0 mice (Fig. 2) was evaluated for total phospholipid, cholesteryl ester, and free cholesterol ester content. * $P < 0.001$ when compared to apoE 0; [‡] $P < 0.01$ when compared to apoE 0.

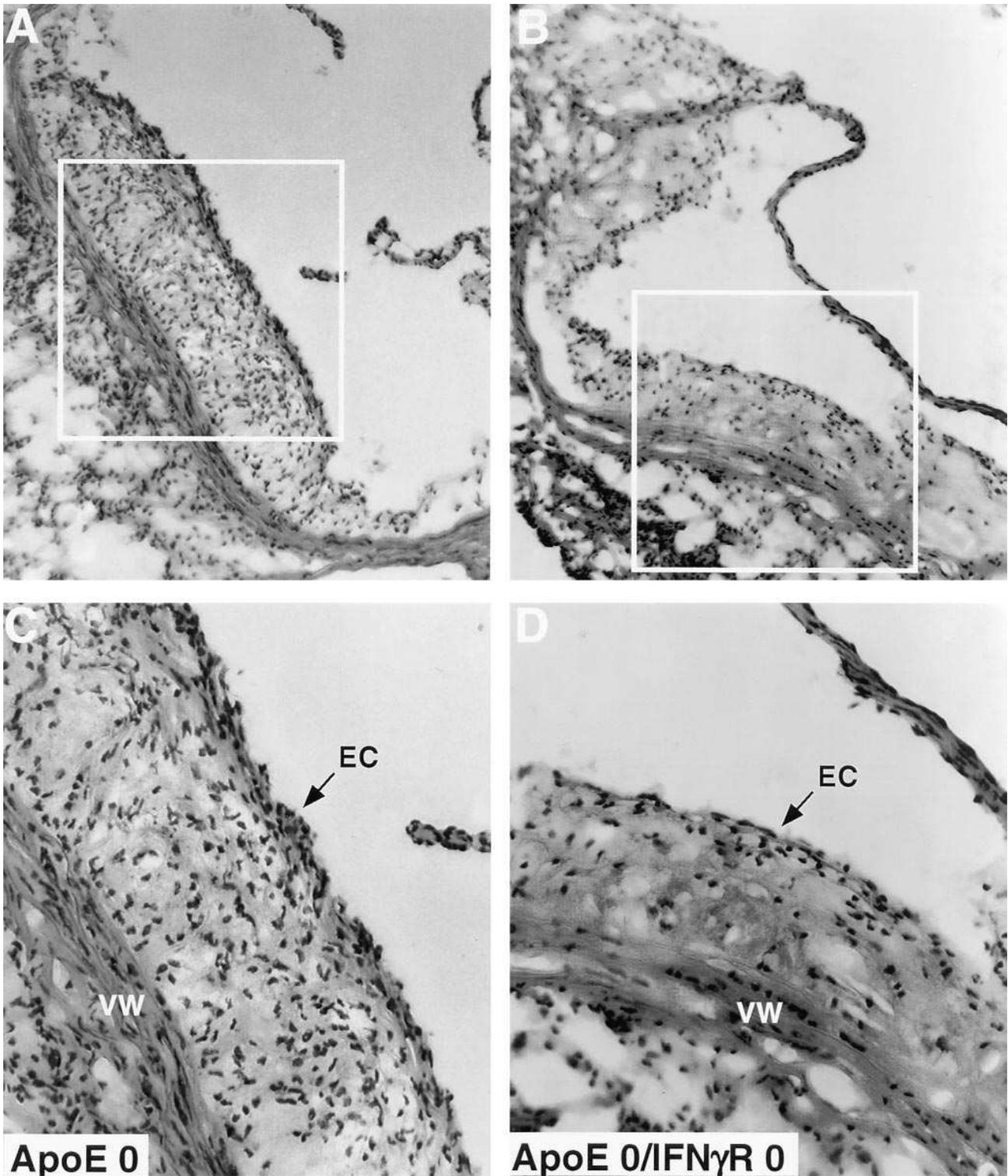


Figure 4. Hematoxylin and eosin stain of atherosclerotic lesions from apoE 0 and apoE 0/IFN γ R 0 mice. Sections prepared from apoE 0 (A and EC) and apoE 0/IFN γ R 0 (B and D) mice, as described in Fig. 3, were stained with hematoxylin and eosin and examined with a Nikon Optiphot microscope at $\times 100$ (A and B) and $\times 400$ (C and D). The areas magnified at $\times 400$ are indicated by white boxes in A and B. EC and vessel wall (VW) are indicated.

0 mice, where the majority of the collagen staining was evident at the adluminal surface of the lesion. These studies indicate that a loss in the ability to signal through IFN- γ leads to substantial changes in lesion size and structure.

Serum lipoprotein profiles in IFN γ R/ApoE compound knock-out mice. Next, the plasma lipoprotein levels in apoE 0 and apoE 0/IFN γ R 0 mice were examined. First, pooled plasma samples were fractionated by FPLC. The apoE 0 mice

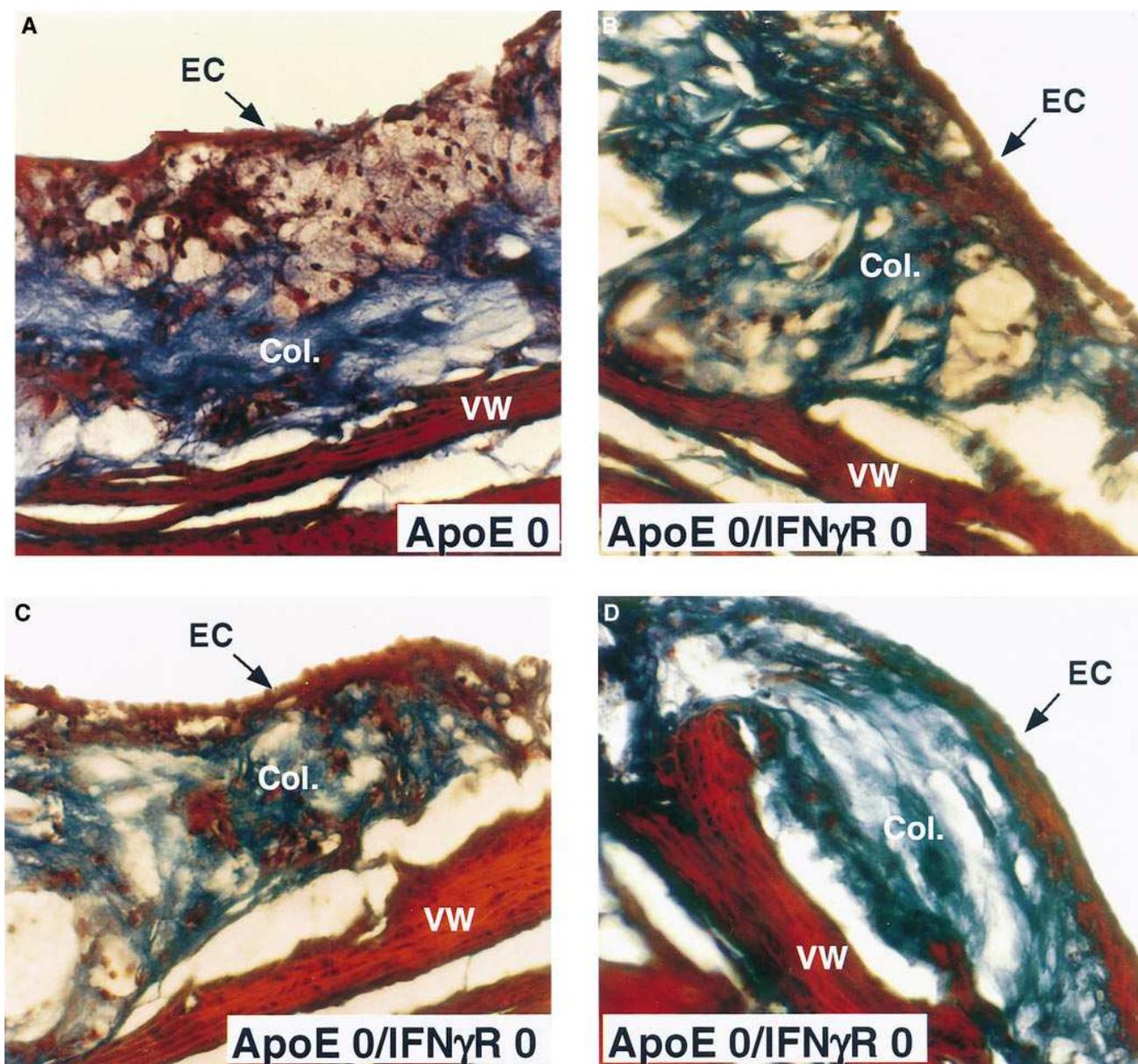


Figure 5. Trichrome stain of atherosclerotic lesions from apoE 0 and apoE 0/IFN γ R 0 mice. Sections prepared from apoE 0 (A) and apoE 0/IFN γ R 0 (B, C, and D) mice, as described in Fig. 3 were stained with trichrome and examined with a Nikon Optiphot microscope at $\times 400$. EC, vessel wall (VW), and collagen (Col.) are indicated.

demonstrated the anticipated large VLDL peak eluting in the void volume (Fig. 6 A) (22, 24). Intriguingly, the FPLC profiles of the apoE 0/IFN γ R 0 mice exhibited an increase in cholesterol rich lipoproteins between fractions 15 and 22 (i.e., a shoulder to the right of the VLDL peak; Fig. 6 A, arrow). Furthermore, there was a marked increase in phospholipids eluting in the VLDL region and extending into the LDL region (Fig. 6 B). These observations were confirmed by an evaluation of the second set of matched apoE 0 and apoE 0/IFN γ R 0 mice (data not shown). Control studies on plasma prepared from IFN γ R 0 and 129 mice revealed only small LDL peaks, typical of wild-type mice (data not shown). Phospholipid and cholesterol levels of unfractionated plasma were also consis-

tent with these studies, demonstrating an increase in the relative levels of phospholipid and free cholesterol in the apoE 0/IFN γ R 0 mice (Table I). Plasma triglycerides were similar in both groups. Thus, the loss in IFN- γ signaling led to an increase in free cholesterol and phospholipid rich lipoprotein particles in the apoE 0 background.

To evaluate changes in plasma lipoproteins in more detail, pooled plasma samples from both sets of apoE 0 and apoE 0/IFN γ R 0 mice were fractionated sequentially by buoyant density ultracentrifugation and analyzed by SDS/PAGE in several independent experiments (28). Results from three studies with chow-fed mice determined a 9.5-fold increase ($P < 0.00001$) in apoA-IV levels in the VLDL and/or LDL plasma fractions

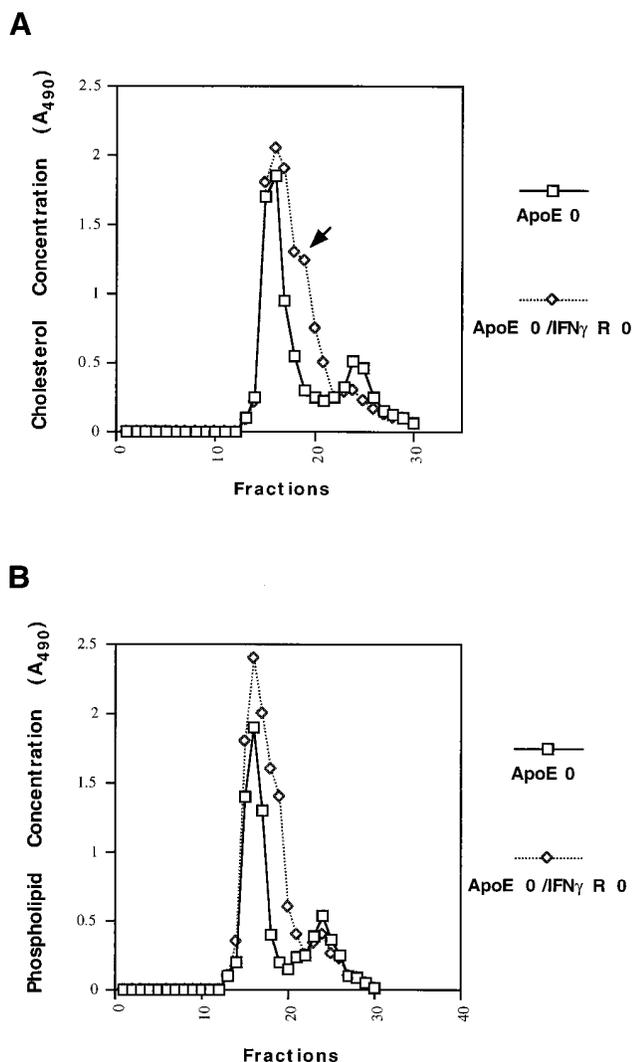


Figure 6. Plasma cholesterol and phospholipid FPLC profiles. Pooled samples of plasma ($n = 5$) from Western type diet fed apoE 0 and apoE 0/IFN γ R 0 mice were fractionated by FPLC and evaluated for cholesterol (A) or phospholipid content (B). Profiles of apoE 0 mice are shown with a solid line and profiles of the apoE 0/IFN γ R 0 mice with a dotted line. The x axes represent FPLC column fractions and the y axes represent the relative cholesterol or phospholipid content as determined spectrophotometrically (absorbance at a wavelength of 490 nm) by a colorimetric assay. The LDL shoulder is indicated by an arrow in A.

from compound knock-out mice in comparison to apoE 0 mice. Similarly, three studies on mice fed a Western type diet identified a comparative 7.6-fold increase ($P < 0.001$) in compound knock-out plasma apoA-IV levels. The results of one of these studies, where chow and Western type diets are compared directly are shown in Fig. 7. In this experiment there was also a small decrease in apoA-I in the HDL fractions and a corresponding increase in the LDL fractions, which is consistent with a redistribution of HDL particles to lower density secondary to the increased phospholipid content (31). In contrast to the results from the apoE 0 and apoE 0/IFN γ R 0 mice, evaluation of the control 129 mice did not reveal any increase in the apoA-IV in the VLDL and LDL plasma fractions.

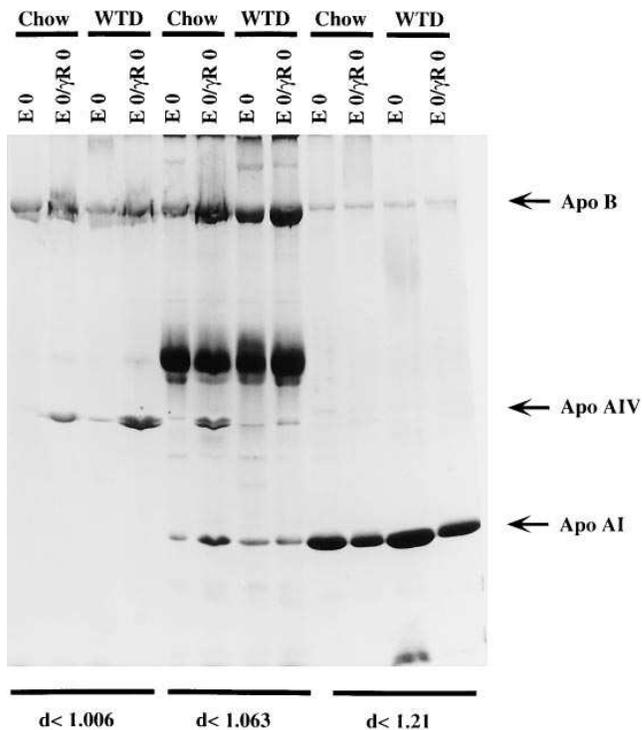


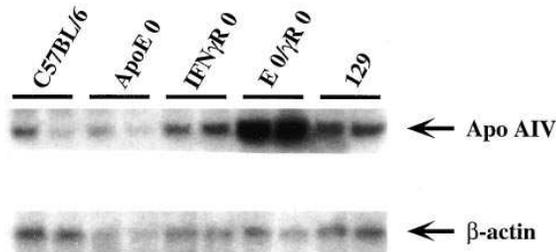
Figure 7. SDS-PAGE analysis of plasma lipoproteins. Pooled samples of serum of apoE 0 ($n = 5$) and apoE 0/IFN γ R 0 ($n = 5$) mice fed either chow or Western type diet were fractionated by sequential buoyant density ultracentrifugation and SDS-PAGE, and visualized by Coomassie blue staining. Lipoprotein densities were as indicated: VLDL, $d < 1.006$ g/dl; LDL, $d = 1.006$ – 1.063 g/dl; and HDL, $d = 1.0063$ – 1.210 g/dl. The mobilities of apoB, apoA-IV, and apoA-I are also indicated.

The large rise in apoA-IV levels observed in the compound knock-out mice suggested that IFN γ regulates apoA-IV levels. To investigate the potential mechanism behind this regulation, apoA-IV gene expression patterns were examined in several candidate tissues. As hepatic expression was most robust, the comparison between expression in apoE 0, apoE 0/IFN γ R 0 mice, and the parental strains (i.e., 129 and C57Bl/6J) was undertaken in this tissue. Although we did observe a good level of expression in the 129 mice (Fig. 8), the difference with respect to C57Bl/6J was more modest than had been reported previously (32). In contrast, the apoE 0/IFN γ R 0 mice exhibited a more marked (i.e., greater than or equal to threefold, $P < 0.001$) increase in apoA-IV expression (Fig. 8). This increase was even more striking when one considers that apoA-IV expression in 129-C57Bl/6J interbred mice has been reported to be less than that of 129 mice (32). This provides compelling evidence that in the setting of hypercholesterolemia IFN γ exerts an important level of regulation on apoA-IV expression. These studies suggest that the ability of IFN γ to downregulate the expression of an atheroprotective (33, 34) apolipoprotein apoA-IV contributes to atherogenesis in apoE 0 mice.

Discussion

The early colocalization of T cells and macrophages to atherosclerotic lesions supports a role for the immune system in the

A



B

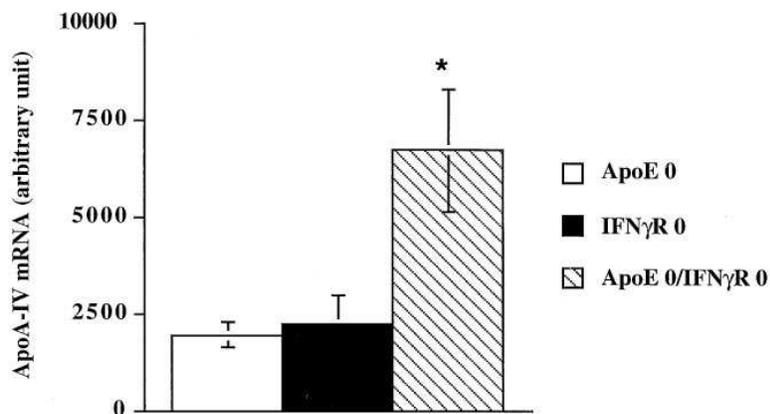


Figure 8. Pattern of hepatic apoA-IV expression varies with the strain of different strains of mice. (A) 20 μ g of total hepatic RNA, from 3–6-mo old chow fed mice, was evaluated after fractionation in a formaldehyde gel by hybridization with a 427-bp Afl III/Pst I fragment of the murine apoA-IV cDNA (53), or a 245-bp KpnI/XbaI murine β -actin probe (Ambion Inc., Austin, TX) as described previously (25). Each strain including 129, C57Bl/6J, apoE 0 (mixed 129/C57Bl/6J), apoE 0/IFN γ R 0 (mixed 129/C57Bl/6J) was evaluated in duplicate as shown. (B) Hybridization intensity of apoA-IV bands from four samples (two from A and two additional samples) of knock-out mice (i.e., apoE 0, IFN γ R 0, and apoE 0/IFN γ R 0) was determined (by Scanner; Molecular Devices, Sunnyvale, CA), compiled, and represented graphically.

pathogenesis of atherosclerotic lesions (2, 3, 7, 35, 36). Although the nature of the involved immune response is not known, immunohistochemical studies have identified several potentially important components. Both major classes of T cells (i.e., CD4⁺ and CD8⁺) and the potent immunomodulatory cytokine they secrete, IFN- γ , have been colocalized to lesions (2, 3). Moreover, IFN- γ has been shown to have important effects on the cellular components of atherosclerotic lesions. In addition to being a potent coactivator of macrophages (17), IFN- γ has been shown to downregulate the expression of the scavenger receptor A, the low density lipoprotein related receptor, and lipoprotein lipase (LPL) in some cell types (12, 13, 37, 38), begetting the question of whether this potent cytokine is a protagonist or antagonist in the pathogenesis of atherosclerotic lesions. The observation that IFN- γ blocks the ability of SMC to proliferate and synthesize collagen (14, 15), both hallmarks of atherogenesis (1, 14), has further fueled this controversy. These latter observations have led to a proposal that IFN- γ does not play an important role in plaque growth, but rather contributes to plaque rupture by promoting a thinning and destabilization of the fibrous cap at the plaque's shoulders (14, 36). Consistent with this model, T cells appear to localize to the shoulder region, and IFN- γ has recently been shown to stimulate the secretion of cathepsin-S, a neutral cysteine protease, from macrophages (14).

In an effort to clarify the role of IFN- γ in atherosclerosis,

we have examined the development of lesions in IFN γ R 0 mice crossed with the hypercholesterolemia and atherosclerosis-prone apoE 0 mice. When compared to apoE 0 mice, lesions in the apoE 0/IFN γ R 0 mice were significantly smaller, with a 60% decrease in lipid content. These observations support a model where IFN- γ potentiates an immune response, which in turn promotes atherogenesis. Likewise, detailed studies on how T cells and macrophages interact to mediate an immune responses in other systems have delimited a pivotal role of IFN- γ (4–6). These studies indicate that an immune response is initiated by a pathogen-mediated macrophage stress (5, 17), which may be functionally analogous to an initiating stress in atherosclerosis (e.g., oxidant stress in the setting of elevated lipids, or LDL aggregation). This initiating stress may also be the stimulus that draws both T cells and macrophages into early lesions. Subsequently, T cells (i.e., the Th1 subset) are stimulated to secrete IFN- γ (5, 17), and potentially other growth factors (39, 40), in an antigen-dependent process. Although the nature of the stimulating antigen has not been determined for atherosclerosis, recent studies have implicated oxidized LDL, providing another potential link to an established risk factor of atherogenesis (41). The secreted IFN- γ is likely to have potent activity on SMCs (14), as well as macrophages, where it acts to prime these cells to destroy offending agents, in part through the generation of reactive oxygen species (5, 17, 42). A secondary stimulus, required by mac-

rophages to attain their full inflammatory potential (5, 17), is likely to be provided by other factors that have been localized to atherosclerotic lesions as well (e.g., IL-1, TNF, or oxidative stress) (1, 14, 43–45). Consistent with this model, recent studies with macrophage-deficient apoE 0 mice demonstrate that macrophages play an important role in atherogenesis (46). Moreover, our studies on murine diet induced atherosclerosis, and other studies on murine transplant atherosclerosis, have directly implicated IFN- γ in the pathogenesis of atherosclerotic lesions (47, 48). Furthermore, we find that lesions in the apoE 0/IFN γ R 0 mice are not only smaller, but significantly less cellular, with a concomitant increase in extracellular collagen. Although our studies do not discriminate between a potential effect of IFN- γ on SMC collagen synthesis (i.e., down-regulation) (14) or collagenase activity (14), they strongly implicate IFN- γ in the regulation of the level of lesional collagen. Intriguingly, extracellular fibrillar collagen has been shown recently to block SMC proliferation, potentially accounting for some of this decrease in cellularity (49), and highlighting the potential importance of IFN- γ 's regulation of lesional collagen content. As decreases in lesional collagen content correlate with plaque instability (14, 36), IFN- γ antagonists may serve to stabilize plaques.

In addition to the intralesional effects, IFN- γ was found to affect lipoprotein metabolism as well. Plasma lipoproteins from apoE 0/IFN γ R 0 mice exhibited a significant increase in a distinct population of lipoprotein particles that are rich in apoA-IV, phospholipid, and free cholesterol. As both an increase in expression of transgenic apoA-IV (33, 34) and phospholipid infusions (50, 51) have been shown to be antiatherogenic, it is very likely that the apoA-IV/phospholipids particle observed in the compound knock-out mice contributed to the observed reduction in lesion size, perhaps by decreasing subintimal lipid accumulation. Notably, expression of human apoA-IV transgenes in mice does not lead to an increase in plasma phospholipid levels, suggesting that the increase in phospholipids observed in the compound knock-out mice may be an independent property of that background (Jiang, X., and A. Tall, unpublished observation). However, the threefold increase in hepatic apoA-IV expression in compound knock-out mice directly implicates the loss in IFN- γ signaling to the increase in plasma apoA-IV levels. Consistent with the emerging concept that the liver may be a physiologically important target tissue of cytokines released into the portal circulation (52), these studies provide evidence that the liver is an important target of IFN- γ . Moreover, IFN- γ , or its receptor, may be one of the previously alluded to *trans*-acting factors that contribute to species-specific patterns of apoA-IV expression (32). In summary, our studies demonstrate that IFN- γ has important proatherogenic effects that are mediated by both intralesional and extralesional targets. The reduced size and lipid content of apoE 0/IFN γ R 0 atheromata, together with a striking increase in lesional collagen, suggests that inhibition of IFN- γ activity could be a therapeutic strategy to stabilize human atherosclerotic plaques.

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