

# IFN- $\gamma$ Deficiency Exerts Gender-Specific Effects on Atherogenesis in Apolipoprotein E<sup>-/-</sup> Mice

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## ABSTRACT

We have shown recently that administration of exogenous interferon- $\gamma$  (IFN- $\gamma$ ) to apolipoprotein E (apoE)<sup>-/-</sup> mice augmented atherogenesis. In the present study, we examined whether deficiency of endogenous IFN- $\gamma$  would reduce atherosclerosis in apoE<sup>-/-</sup> mice. Compound-deficient mice were generated by crossing strain-matched IFN- $\gamma$ <sup>-/-</sup> and apoE<sup>-/-</sup> mice and comparing them to apoE<sup>-/-</sup> mice. Groups of both genders were fed either a normal or a high-fat diet. IFN- $\gamma$  deficiency did not affect serum cholesterol concentrations or lipoprotein-cholesterol distributions in any groups. IFN- $\gamma$  deficiency had no effect on serum triglyceride concentrations, except for an increase noted in males fed a normal diet. The extent of atherosclerosis was determined in tissue sections of the ascending aorta and on the surface of the aortic arch. During feeding of normal diets, IFN- $\gamma$  deficiency had no effect on the extent of atherosclerosis in female mice in either vascular bed. In contrast, in male mice fed normal diet, IFN- $\gamma$  deficiency markedly decreased lesion size in both vascular beds. During feeding of high-fat diets, IFN- $\gamma$  deficiency also had no effect on lesion size in females but profoundly decreased lesion size in the aortic root of male mice. IFN- $\gamma$  deficiency had no effect on the abundance of T lymphocytes or MHC class II-positive cells in aortic root lesions of females. By comparison, both these parameters were reduced in lesions of male mice. Therefore, IFN- $\gamma$  deficiency decreased atherogenesis, potentially by decreasing T lymphocyte presence and cell activation, without influencing serum cholesterol concentrations. However, this effect is strikingly restricted to male mice.

## INTRODUCTION

T LYMPHOCYTES HAVE BEEN IDENTIFIED in both human and mouse atherosclerotic lesions at all stages of lesion development.<sup>(1)</sup> These cells express MHC class II and very late activation antigen-1 (VLA-1), which are consistent with activation that would lead to elaboration of cytokines.<sup>(2)</sup> A role of T lymphocytes in the development of atherosclerotic lesions is implied from animal studies in which pharmacologic and genetic approaches to immune suppression have modified the development of atherosclerotic lesions.<sup>(3-6)</sup>

Interferon- $\gamma$  (IFN- $\gamma$ ) is a major component of the acquired immune response and is a prominent cytokine secreted by activated T lymphocytes,<sup>(7)</sup> natural killer (NK) cells,<sup>(8)</sup> and macrophages.<sup>(9)</sup> IFN- $\gamma$  mRNA has been detected in atherosclerotic lesions from both humans<sup>(10-12)</sup> and mice.<sup>(13,14)</sup> IFN- $\gamma$  protein has also been detected by immunocytochemical analysis of human<sup>(10)</sup> and mouse lesions.<sup>(15,16)</sup> This cytokine has the potential to influence the development of atherosclerotic lesions

through a number of mechanisms that have been demonstrated in cultured cells. These include effects on 15-lipoxygenase,<sup>(17)</sup> lipoprotein metabolism,<sup>(18)</sup> lipoprotein modification,<sup>(19)</sup> lipoprotein cell recognition,<sup>(11,20,21)</sup> apoptosis,<sup>(22)</sup> extracellular matrix (ECM) synthesis,<sup>(23)</sup> and macrophage ABCA1 expression.<sup>(24)</sup> These diverse effects of IFN- $\gamma$  on cultured cells render it difficult to predict whether this cytokine would promote or retard atherogenesis.

The current limited evidence is consistent with IFN- $\gamma$  exerting a proatherogenic role. First, exogenous delivery of the cytokine by parenteral administration increased lesion size in mice.<sup>(25,26)</sup> Further evidence of a potential proatherogenic effect of IFN- $\gamma$  was provided by determining the effects of IFN- $\gamma$  receptor deficiency on the development of lesions in female apolipoprotein E<sup>-/-</sup> (apoE<sup>-/-</sup>) mice.<sup>(27)</sup> Deficiency of this cytokine receptor decreased lesion development in the aortic root and enriched the content of ECM. Deficiency of IFN- $\gamma$  receptors led to unexpected changes in plasma lipoprotein and apolipoprotein concentrations, and, therefore, it was difficult to

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determine if the effects on lesion formation were caused by peripheral or local effects. Furthermore, recent findings in strain-matched mice demonstrate that IFN- $\gamma$  receptor deficiencies do not necessarily mimic the effects of IFN- $\gamma$  deficiency.<sup>(28)</sup> This has led to speculation that there may be alternative ligands for the IFN- $\gamma$  receptor or more than one receptor class for this cytokine.<sup>(28)</sup>

To directly define the role of IFN- $\gamma$  in the development of atherosclerotic lesions, we determined the effects of IFN- $\gamma$  deficiency on lesion development in two arterial regions in age-matched, strain-matched, and gender-matched apoE<sup>-/-</sup> mice. Because diet has been shown previously to influence lesion development in lymphocyte-deficient apoE<sup>-/-</sup> mice, the effects of IFN- $\gamma$  deficiency were determined during feeding of both normal and high-fat diets.<sup>(5,6)</sup> Furthermore, as gender can affect immune responses,<sup>(25,29)</sup> we determined the effects of IFN- $\gamma$  deficiency in both female and male mice. We demonstrate that deficiency of IFN- $\gamma$  had no effect on atherosclerosis in either vascular bed during feeding of normal or high-fat diets in female apoE<sup>-/-</sup> mice. However, IFN- $\gamma$  deficiency had a striking effect in reducing the size of atherosclerotic lesions in male apoE<sup>-/-</sup> mice, without changing serum cholesterol concentrations. This effect was associated with a decreased abundance of T lymphocytes and MHC class II-positive cells.

## MATERIALS AND METHODS

### Animals and diets

apoE<sup>-/-</sup> and IFN- $\gamma$ <sup>-/-</sup> mice, backcrossed 10 times to the C57BL/6 strain, were obtained from the Jackson Laboratory (Bar Harbor, ME). IFN- $\gamma$ <sup>-/-</sup> mice were bred to apoE<sup>-/-</sup> mice to generate compound-deficient mice. PCR, as described previously,<sup>(30,31)</sup> was used to identify mice homozygous for both apoE and IFN- $\gamma$  deficiency.

Mice were kept on a standard laboratory diet for 9 months or, at 8 weeks of age, were placed on a diet supplemented with 0.15% (w/w) cholesterol and 20% (w/w) butterfat (high-fat diet) (TD 88137, Harlan Teklad, Madison, WI) for 3 months. Mice

were housed in a pathogen-free facility, and all procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

### Blood collection

Blood samples at termination of the study were collected from anesthetized mice by puncture of the right ventricle. Blood was allowed to clot, and serum was obtained by centrifugation.

### Serum lipid and lipoprotein quantification

Serum cholesterol and triglyceride concentrations were determined using commercially available assays (Wako Bioproducts, Richmond, VA) as described previously.<sup>(25,32)</sup> Serum cholesterol measurements were standardized to samples provided by the Core Laboratory for Clinical Studies at Washington University. Lipoprotein-cholesterol and lipoprotein-triglyceride distributions from individual serum samples (4–6 mice per group) were determined following resolution by size exclusion chromatography (Biologic Workstation, Bio-Rad, Hercules, CA) using a Superose 6 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) as described previously.<sup>(25,32)</sup>

### Tissue collection and lesion analysis

Mice were perfused with phosphate-buffered saline (PBS), and the hearts were separated from the aorta at the base, embedded in OCT, and frozen at -20°C. Aortic tissue was removed from the ascending aorta to the iliac bifurcation and placed in fresh 4% paraformaldehyde overnight at ambient temperature.

The extent of atherosclerosis in the ascending aorta was determined as described previously<sup>(25,33,34)</sup> from Oil Red O-stained serial sections, 8  $\mu$ m thick and collected 80  $\mu$ m apart, starting at the region where the aortic sinus becomes the ascending aorta. The cross-sectional area of all lesions in a section was measured and summed to determine the total lesion area per section. Lesion size was determined by calculating the mean area in four sections. *En face* measurements of the percentage of intimal surface area covered by atherosclerotic lesions were determined for

TABLE 1. SERUM LIPID CONCENTRATIONS

Diet	Gender	IFN- $\gamma$ genotype	n	Serum concentration (mg/dl)	
				Total cholesterol	Triglyceride
Normal diet	M	+/+	11	218 $\pm$ 13 <sup>a</sup>	105 $\pm$ 19
		-/-	10	284 $\pm$ 21	211 $\pm$ 20*
	F	+/+	9	185 $\pm$ 20	70 $\pm$ 22
		-/-	10	217 $\pm$ 34	74 $\pm$ 20**
Modified diet <sup>b</sup>	M	+/+	10	543 $\pm$ 33	295 $\pm$ 46
		-/-	10	592 $\pm$ 44	348 $\pm$ 41
	F	+/+	10	516 $\pm$ 13	258 $\pm$ 12
		-/-	10	515 $\pm$ 15	271 $\pm$ 29

<sup>a</sup>Means  $\pm$  SEM.

<sup>b</sup>Standard laboratory mouse feed supplemented with 0.15% (wt/wt) cholesterol and 20% (wt/wt) butterfat (TD 88137, Harlan Teklad).

\* $p$  = 0.01 vs. male ApoE<sup>-/-</sup>  $\times$  IFN- $\gamma$ <sup>+/+</sup> mice.

\*\* $p$  = 0.01 vs. male ApoE<sup>-/-</sup>  $\times$  IFN- $\gamma$ <sup>-/-</sup> mice.

the aortic arch, as described previously.<sup>(5,32)</sup> Measurements of lesion size were verified by two observers.

#### Cellular and ECM characterization of atherosclerotic lesions

Immunocytochemistry and ECM staining were performed on serial sections of the ascending aorta adjacent to those stained with Oil Red O, as described previously.<sup>(25,32)</sup> For immunocytochemistry, the following sera/monoclonal antibodies (mAb) were used: rabbit antisera to mouse macrophages (AI-AD31240, 1:3000 dilution) (Accurate Chemical and Scientific Corp., Westbury, NY), an antimouse Thy 1.2 antibody (01011D, 7  $\mu$ g/ml), (PharMingen, San Diego, CA), and an antimouse MHC class II antibody (ALU0041, 1:5 dilution) (Biosource International, Camarillo, CA). Endogenous tissue peroxidase was ablated prior to immunostaining by exposing tissue to H<sub>2</sub>O<sub>2</sub>, and the extent of nonspecific staining was examined by staining serial sections in the absence of primary antibody. Extracellular collagen and elastin were visualized with Gomori trichrome and Verhoeff's stains, respectively.

T lymphocytes and MHC class II-positive cells were quantified by manually counting the number of immunoreactive cells, as described previously.<sup>(25,33,35)</sup>

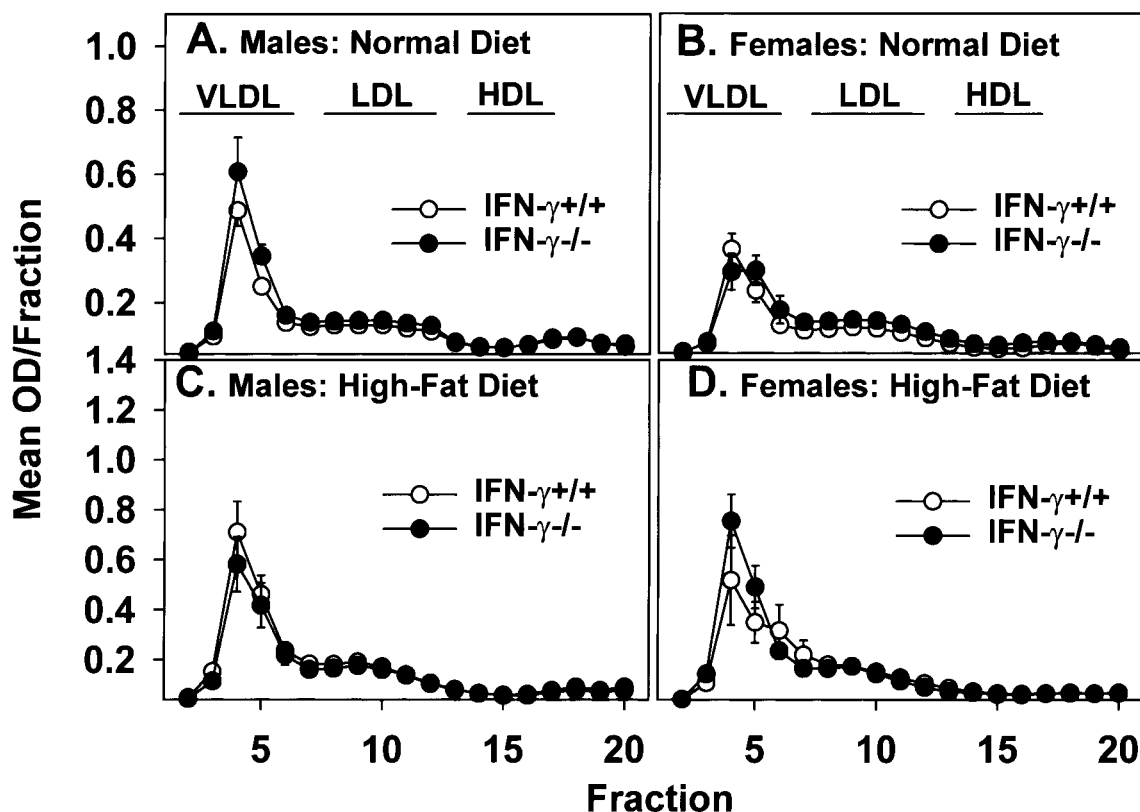
#### Statistics

Data analysis was performed using SigmaStat 2.03 software (SPSS Inc., Chicago, IL). Differences between experimental groups were evaluated using a one-way ANOVA with all pairwise comparisons of the mean responses to the different treatment groups conducted using the Tukey test after assuring that the data complied with the constraints of parametric analyses. When the normality test failed while performing the one-way ANOVA, a one-way ANOVA on Ranks (Kruskal-Wallis test) was performed, and subsequent pairwise comparisons of the median values for the treatment groups to a designated control group were done using the Dunnett's method. Values with  $p < 0.05$  were considered to be statistically significant. Values are represented as mean  $\pm$  standard error of mean (SEM).

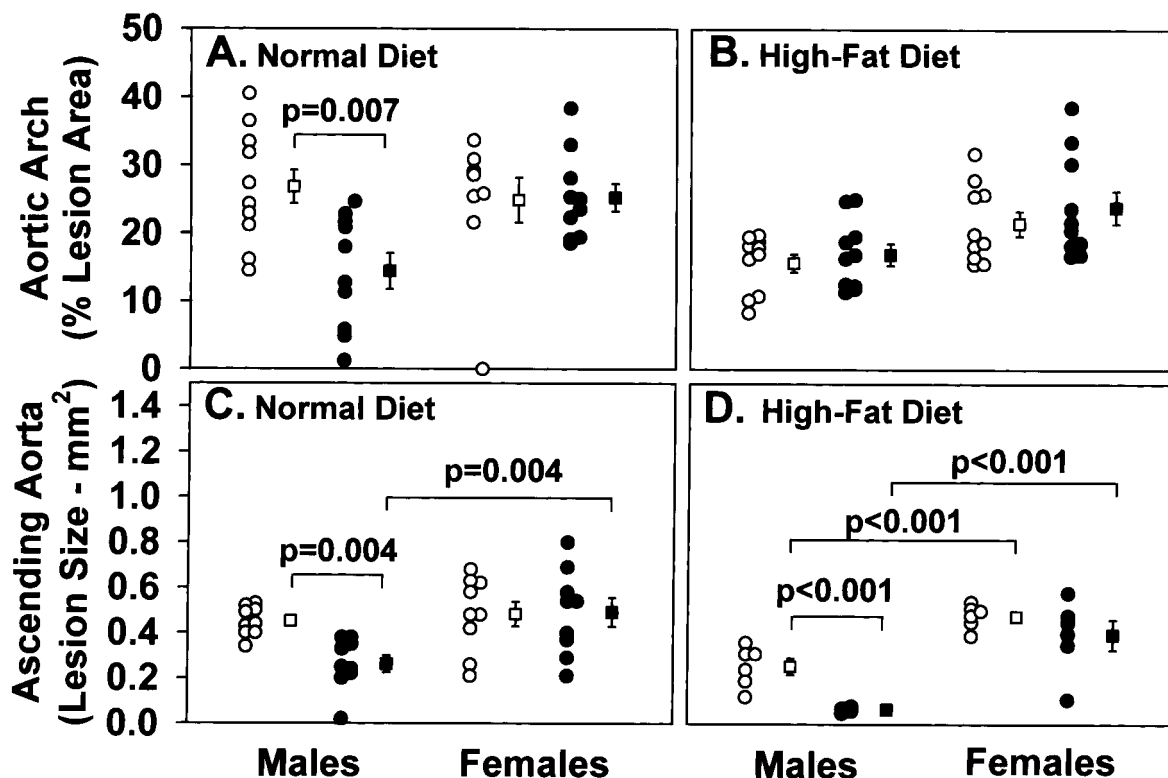
## RESULTS

#### Serum lipid and lipoproteins

IFN- $\gamma$  deficiency did not affect serum cholesterol concentrations in any of the groups studied (Table 1). Lipoprotein-cholesterol distribution was also not influenced by IFN- $\gamma$  deficiency in apoE<sup>-/-</sup> mice of either gender fed normal or high-fat



**FIG. 1.** Serum lipoprotein distribution of mice fed a normal and high-fat diet. Serum from either (A,C) male or (B,D) female apoE<sup>-/-</sup>  $\times$  IFN- $\gamma$ <sup>+/+</sup> (open circle) or apoE<sup>-/-</sup>  $\times$  IFN- $\gamma$ <sup>-/-</sup> (closed circle) mice fed a normal diet for 9 months (A,B) or a diet enriched in cholesterol and saturated fat for 3 months (C,D) was resolved by size exclusion chromatography using a Superose 6 column, and cholesterol concentrations were determined in each fraction. Volumes of serum were loaded onto the column in approximate proportion to the serum cholesterol concentrations (200  $\mu$ l for normal diet and 50  $\mu$ l for high-fat diet). Values are represented as mean  $\pm$  SEM of individual profiles from 6–8 animals per group (A,B) and 4–6 animals per group (C,D).



**FIG. 2.** Aortic lesion size. The extent of intimal surface covered by grossly discernible lesions was determined from (A,B) *en face* preparations of the aortic arch and from (C,D) serial sections of the ascending aorta from male and female  $\text{apoE}^{-/-} \times \text{IFN-}\gamma^{+/+}$  (open symbols) and  $\text{apoE}^{-/-} \times \text{IFN-}\gamma^{-/-}$  (closed symbols) mice fed either (A,C) a normal diet for 9 months or (B,D) a high-fat diet for 3 months. Circles, values of individual mice; squares, means; bars, SEM.

diets (Fig. 1).  $\text{IFN-}\gamma$  deficiency had no effect on serum triglyceride concentrations, except for the increase noted in  $\text{apoE}^{-/-}$  male mice fed a normal diet (Table 1).

#### Quantification of atherosclerosis

In female  $\text{apoE}^{-/-}$  mice,  $\text{IFN-}\gamma$  deficiency did not reduce the size of atherosclerotic lesions in either the aortic arch (Fig. 2A,B) or the ascending aorta (Fig. 2C,D) during feeding of either normal or high-fat diets. Interestingly, lesions from the ascending aorta in female mice were larger than in males (female vs. male  $\text{IFN-}\gamma^{-/-}$  mice fed a normal diet,  $p = 0.004$  [Fig. 2C] and female vs. male  $\text{IFN-}\gamma^{+/+}$  and  $\text{IFN-}\gamma^{-/-}$  mice fed a high-fat diet,  $p < 0.001$  [Fig. 2D]).

In contrast to females,  $\text{IFN-}\gamma$  deficiency reduced atherosclerosis in male  $\text{apoE}^{-/-}$  mice fed a normal diet for 9 months in both the aortic arch ( $26.8\% \pm 2.5\%$  vs.  $14.4\% \pm 2.7\%$ ,  $p = 0.007$ ) (Fig. 2A) and ascending aorta ( $0.452 \pm 0.022 \text{ mm}^2$  vs.  $0.262 \pm 0.038 \text{ mm}^2$ ,  $p \leq 0.004$ ) (Fig. 2C). In male  $\text{apoE}^{-/-}$  mice fed a high-fat diet, although there was no significant effect of  $\text{IFN-}\gamma$  deficiency on the development of atherosclerosis in the aortic arch (Fig. 2B), there was a 74% decrease in lesion size in the ascending aorta ( $0.255 \pm 0.036 \text{ mm}^2$  vs.  $0.067 \pm 0.008 \text{ mm}^2$ ,  $p \leq 0.001$ ) (Fig. 2D).

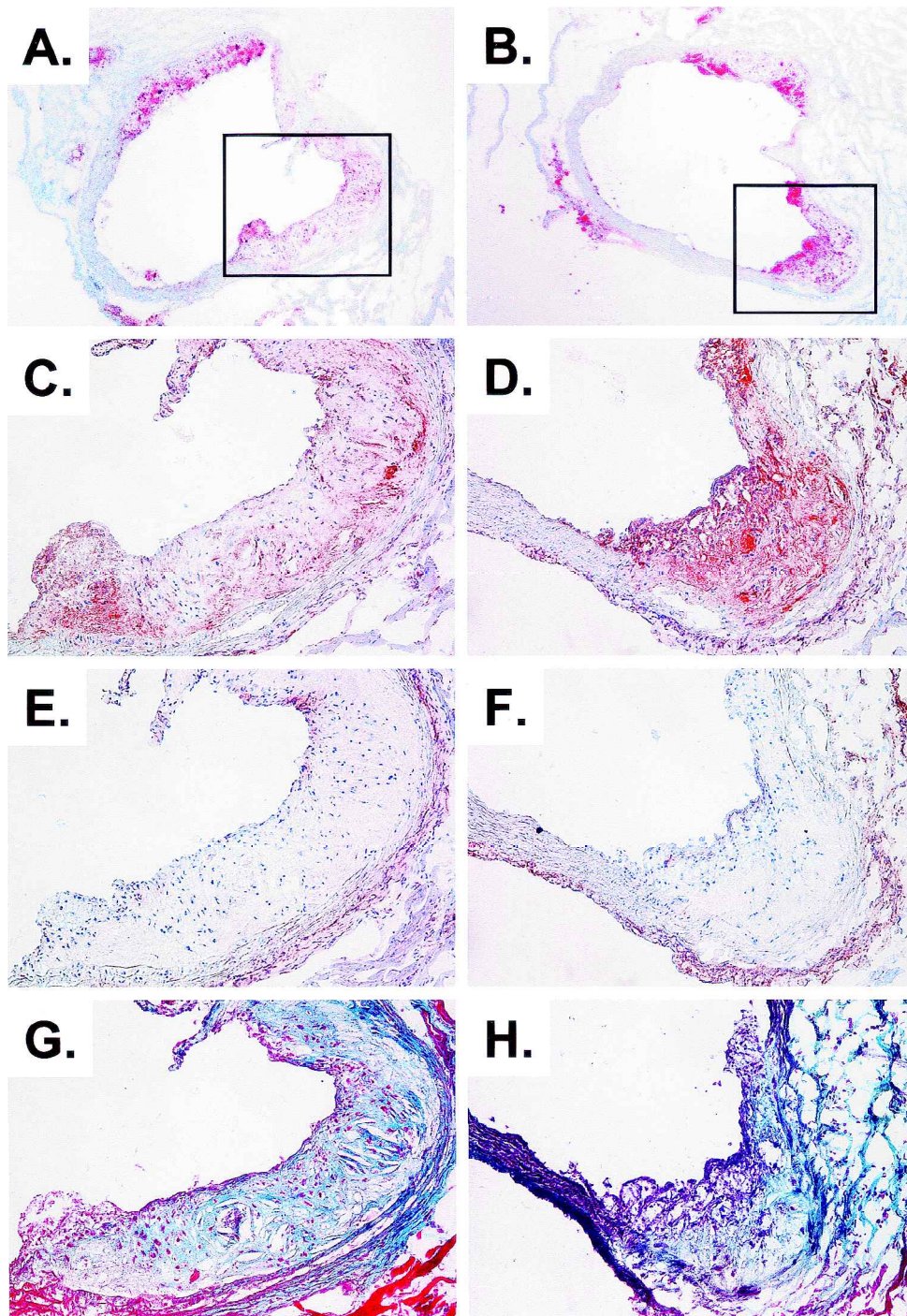
#### Characteristics of atherosclerotic lesions

Representative sections of atherosclerotic lesions from the ascending aorta of male  $\text{IFN-}\gamma^{+/+}$  and  $\text{IFN-}\gamma^{-/-}$  mice fed ei-

ther normal or high-fat diets are shown in Figures 3 and 4, respectively. Serial sections have been stained for neutral lipid (Figs. 3A,B and 4A,B), macrophages (Figs. 3C,D and 4C,D), T lymphocytes (Figs. 3E,F and 4E,F), and collagen (Figs. 3G,H and 4G,H). Despite the reduction in the size of lesions in male  $\text{IFN-}\gamma^{-/-} \times \text{apoE}^{-/-}$  mice, there were no obvious changes in gross morphology of lesions between the wild-type and cytokine-deficient mice. In all groups of mice, lesions were predominantly composed of macrophages, many of which stained for neutral lipid (Figs. 3A,B,C,D and 4A,B,C,D). No gross differences in ECM elements of elastin or collagen were noted in lesions from  $\text{apoE}^{-/-}$  male  $\text{IFN-}\gamma^{+/+}$  vs.  $\text{IFN-}\gamma^{-/-}$  mice, following visualization with Verhoeff's and Gomori stains (Figs. 3G,H and 4G,H) (data not shown).

#### Quantification of cellularity of lesions

After visually detecting a difference in morphologic staining patterns for T lymphocytes, we conducted extensive quantitative analysis of both T lymphocyte-positive and MHC class II-positive cell populations within these lesions. No difference in lesion T lymphocyte numbers was found between  $\text{apoE}^{-/-}$  female  $\text{IFN-}\gamma^{+/+}$  and  $\text{IFN-}\gamma^{-/-}$  mice fed either diet (Fig. 5A,B). Comparison between genders revealed that lesions in female  $\text{IFN-}\gamma^{-/-}$  mice had nearly twice as many T lymphocytes as lesions from male  $\text{IFN-}\gamma^{-/-}$  mice fed a normal diet ( $p = 0.037$ ) (Fig. 5A). T lymphocyte abundance did not change in lesions from  $\text{apoE}^{-/-}$  male  $\text{IFN-}\gamma^{+/+}$  vs.  $\text{IFN-}\gamma^{-/-}$  mice fed a normal



**FIG. 3.** Aortic lesion histology from mice fed a normal diet. Representative histologic sections from a region where the aortic sinus becomes the ascending aorta of a male (A,C,E,G) apoE<sup>-/-</sup> × IFN- $\gamma$ <sup>+/+</sup> mouse and a male (B,D,F,H) apoE<sup>-/-</sup> × IFN- $\gamma$ <sup>-/-</sup> mouse fed a normal diet for 9 months. A segment of heart tissue spanning the aortic sinus and ascending aorta was embedded in OCT, sectioned, and stained with (A,B) Oil Red O for neutral lipids, (C,D) rabbit antisera to mouse macrophages (1:3000 dilution), (E,F) a monoclonal antimouse Thy1.2 antibody (7  $\mu$ g/ml), (G,H) Gomori trichrome to detect collagen. A,B, × 40; C-H, × 200. (A, inset) and (B, inset) Areas contained in C,E,G and D,F,H, respectively.

diet ( $60 \pm 10$  vs.  $38 \pm 10$  mean lesion cell number per section) (Fig. 5A) but was significantly decreased in mice fed a high-fat diet ( $50 \pm 8$  vs.  $15 \pm 5$  mean lesion cell number per section,  $p = 0.01$ ) (Fig. 5B).

Substantial changes in the number of cells expressing MHC class II antigens at the sites of inflammation are indicative of the intensity of an immune response. Therefore, we determined whether IFN- $\gamma$  deficiency affected MHC class II expression. IFN- $\gamma$  deficiency had no effect on the number of cells expressing MHC class II in female apoE $^{-/-}$  mice (Fig. 5C,D). MHC class II-expressing cells were significantly reduced in IFN- $\gamma^{-/-}$   $\times$  apoE $^{-/-}$  male mice fed a normal diet ( $52 \pm 13$  vs.  $13 \pm 3$  cell number per section,  $p = 0.004$ ) (Fig. 5C) but not in lesions from mice fed a high-fat diet ( $24 \pm 6$  vs.  $9 \pm 4$  mean cell number per section) (Fig. 5D).

## DISCUSSION

Previously, we have demonstrated that administration of exogenous IFN- $\gamma$  significantly increased atherosclerosis in apoE $^{-/-}$  male mice.<sup>(25)</sup> The present study provides further evidence that IFN- $\gamma$  is proatherogenic by demonstrating that endogenous deficiency of the cytokine in apoE $^{-/-}$  mice decreases atherosclerosis. However, the effects of IFN- $\gamma$  deficiency on the development of atherosclerosis are restricted to male mice.

Although both our study and that using IFN- $\gamma$  receptor-deficient mice<sup>(27)</sup> led to the same conclusion regarding the proatherogenic effects of this cytokine, there are several major differences between the studies. Perhaps the most striking difference is that IFN- $\gamma$  receptor deficiency reduced atherosclerosis in females,<sup>(27)</sup> whereas in our study, deficiency of the cytokine had no effect on this gender. The effects of IFN- $\gamma$  receptor deficiency on the development of atherosclerosis in male mice has not been reported. Also, in contrast to the receptor-deficient animals,<sup>(23)</sup> we were not able to detect an effect of IFN- $\gamma$  deficiency on serum concentrations of total cholesterol or lipoprotein-cholesterol distribution. Furthermore, IFN- $\gamma$  receptor-deficient animals had a grossly increased ECM content of lesions.<sup>(23)</sup> By comparison, we were unable to detect any overt changes in the content of collagen and elastin in cytokine-deficient apoE $^{-/-}$  mice.

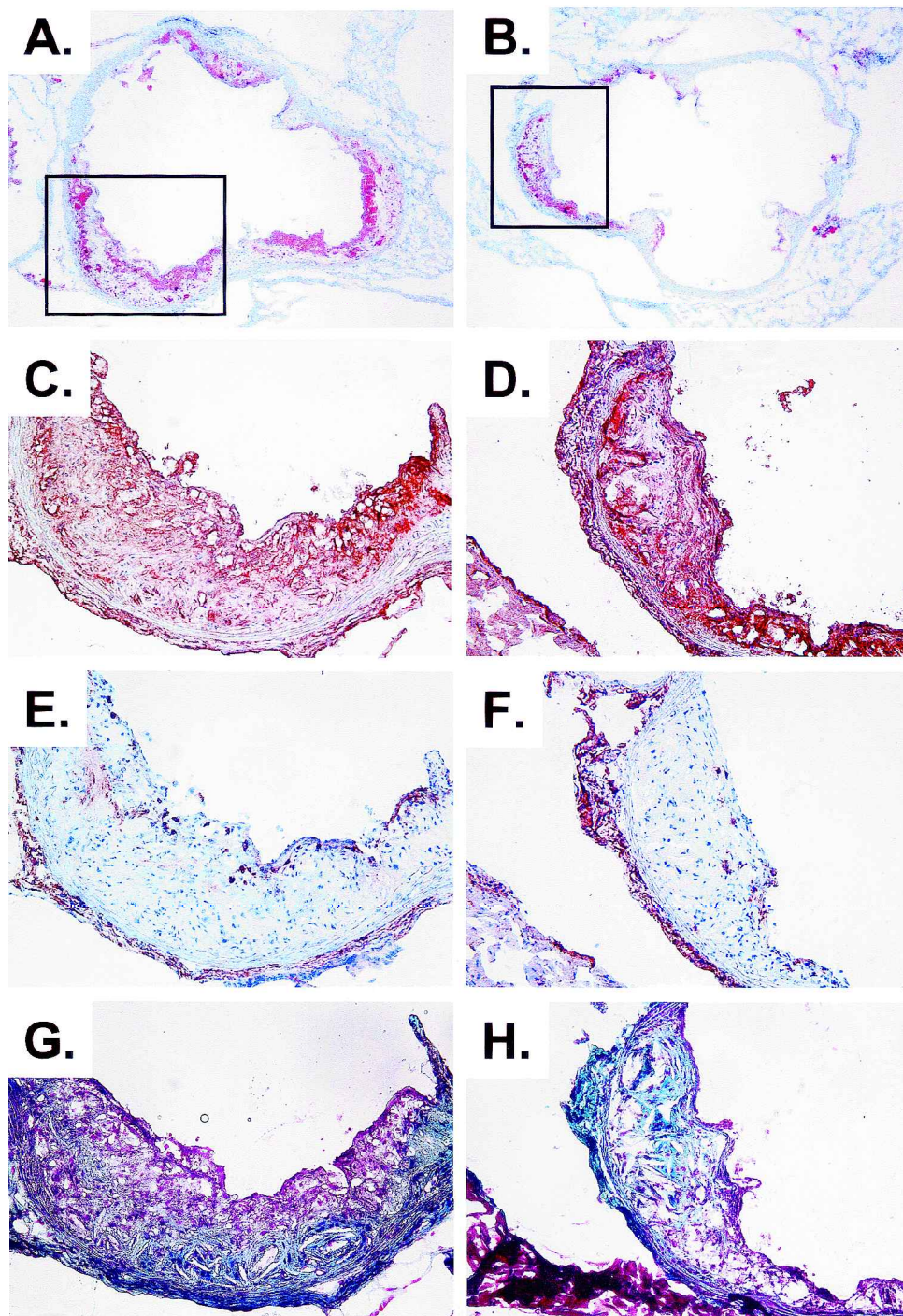
As noted earlier, the differences in the atherogenic process in apoE $^{-/-}$  mice that lack either the cytokine or receptor may be accounted for by a disparate phenotype of IFN- $\gamma$  and IFN- $\gamma$  receptor-deficient mice. The possibility of ligands other than IFN- $\gamma$  interacting with the IFN- $\gamma$  receptor has been inferred previously in response to viral infection,<sup>(28)</sup> in a manner analogous to the chemokine system.<sup>(36)</sup> However, there is currently no evidence that the difference is attributable to the existence of more than one IFN- $\gamma$  receptor.<sup>(37)</sup> In addition to the potential for differences between receptor and cytokine deficiency, the discrepancies between the two studies may also be attributable to the different strains of mice. Gupta et al.<sup>(27)</sup> used a hybrid of 129 and C57BL/6 in both the single and compound-deficient mice. Both the apoE $^{-/-}$  and IFN- $\gamma^{-/-}$  mice used in the present study were backcrossed 10 times into a C57BL/6 background. In addition to ensuring strain equivalency in the single and compound-deficient mice, this strain would be one of the most responsive to IFN- $\gamma$  deficiency, as C57BL/6 mice are considered among the most Th1-responsive strains.<sup>(30)</sup>

During the course of this study, we attempted to detect IFN- $\gamma$  protein in atherosclerotic lesions by immunocytochemical techniques. Previous studies have observed IFN- $\gamma$  in lesions by this technique in human<sup>(10)</sup> and mouse tissue.<sup>(39)</sup> Also, IFN- $\gamma$  protein has been detected in plasma<sup>(40)</sup> and spleens<sup>(13)</sup> of atherosclerotic animals, although its relationship to the local events within vascular lesions is unclear. In agreement with Mallat et al.,<sup>(16)</sup> however, we were unable to detect IFN- $\gamma$  protein in atherosclerotic lesions from apoE $^{-/-}$  mice. Part of the reason for the inability to discern IFN- $\gamma$  may relate to the protein's being secreted at intervals other than those at which the tissue was acquired. In addition, very small amounts of IFN- $\gamma$ , which may be below the limits of detection, exert pronounced biologic effects through polarized delivery. Therefore, the inability to detect this cytokine does not diminish its role in the disease. Most importantly, in spite of our inability to detect IFN- $\gamma$  protein in the lesions of mice that were wild-type for this cytokine, the decrease in atherosclerosis in the IFN- $\gamma^{-/-}$  mice demonstrates that the cytokine is involved in the disease process.

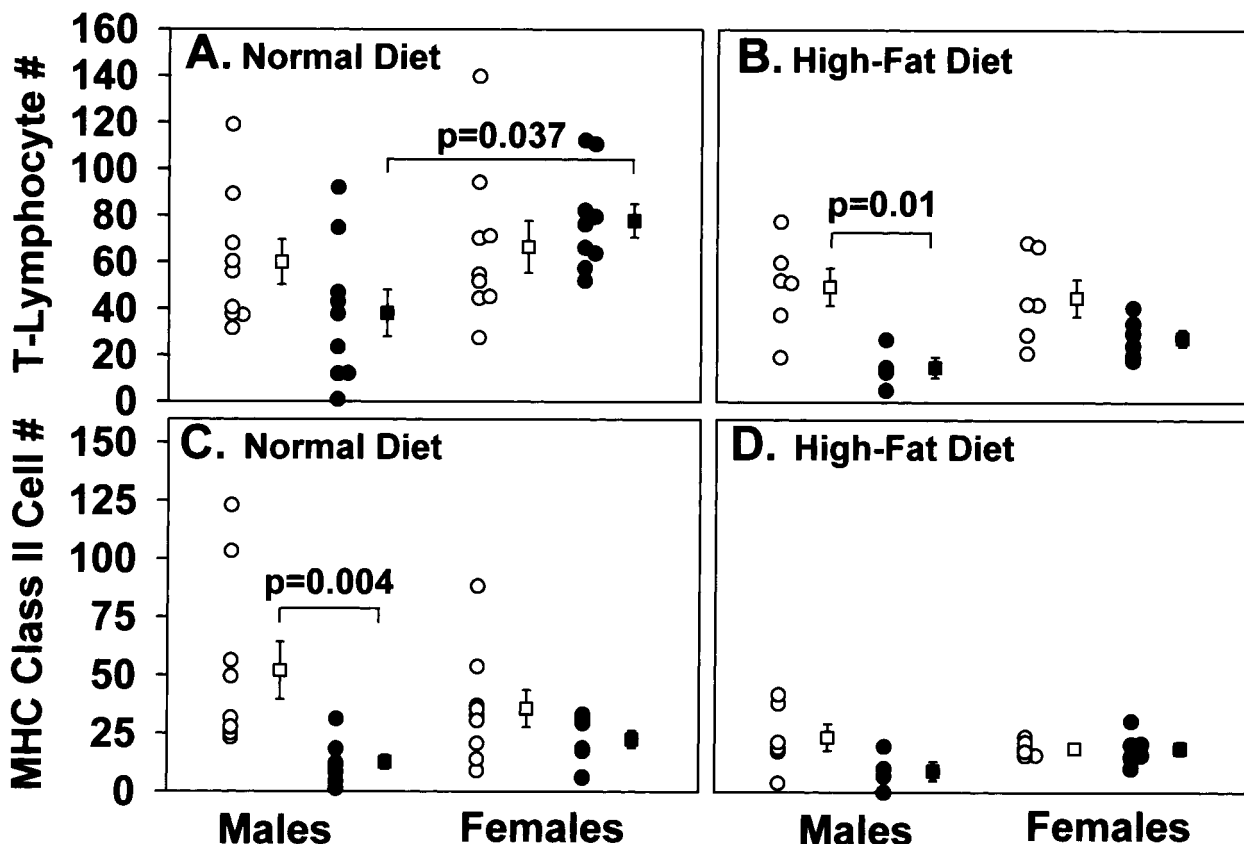
Previous studies have demonstrated that there is a correlation between the extent of atherosclerosis in the aortic root and throughout the entire aorta in apoE $^{-/-}$  mice,<sup>(38)</sup> although differences have been noted.<sup>(41,42)</sup> Therefore, we chose to analyze lesion development in both these regions. For mice fed a normal diet, we noted equivalent changes in both the aortic root and the intimal area of the aortic arch. However, discrepancies in effect of these two regions were noted in mice fed a high-fat diet. Under these conditions, IFN- $\gamma$  deficiency promoted pronounced decreases in lesion size in the aortic root but had no effect on the *en face* analysis of the aortic intima. The reasons for disparities in the atherogenesis in different vascular beds that have been described in this and other studies<sup>(37,39)</sup> is not known.<sup>(42,43)</sup>

Atherosclerotic lesions of apoE $^{-/-}$  mice contain T lymphocytes.<sup>(23,27,35,44)</sup> Therefore, lesions in these mice have the appropriate cellular constituents to study the role of this cell type in the disease process. Total lymphocyte deficiency decreases atherosclerosis in apoE $^{-/-}$  mice, although this effect is not manifested during feeding of diets that promote a severe hypercholesterolemic response (serum cholesterol > 1000 mg/dl).<sup>(5,6)</sup> In contrast to the effects of high-fat diets ablating the role of lymphocytes in atherogenesis in wild-type mice, IFN- $\gamma$  deficiency reduced atherosclerosis when male mice were fed either normal or high-fat diets. Future studies will address whether this difference is due to release of IFN- $\gamma$  from nonlymphocytic sources.<sup>(45)</sup>

Despite not observing any gross differences in overt cellular or ECM content between lesions from apoE $^{-/-}$  male IFN- $\gamma^{-/-}$  vs. IFN- $\gamma^{+/+}$  mice, we did observe a significant reduction in the number of lesion-associated T lymphocytes and MHC class II-positive cells, suggesting that deficiency of the cytokine reduced an inflammatory component of lesion formation. IFN- $\gamma$  deficiency does not affect systemic lymphocyte populations,<sup>(31)</sup> suggesting that one of the effects of IFN- $\gamma$  on the pathology is to enhance T lymphocyte numbers, via recruitment or clonal expansion, and the activation of immunologic cells within the developing atherosclerotic lesion. Another potential source of IFN- $\gamma$  may be NK cells that express this cytokine abundantly<sup>(8)</sup> and have been found in human and mouse atherosclerotic lesions.<sup>(46)</sup>



**FIG. 4.** Aortic lesion histology from mice fed a high-fat diet. Representative histologic sections from a region where the aortic sinus becomes the ascending aorta of a male (A,C,E,G) apoE<sup>-/-</sup> × IFN- $\gamma$ <sup>+/+</sup> mouse and a male (B,D,F,H) apoE<sup>-/-</sup> × IFN- $\gamma$ <sup>-/-</sup> mouse fed a cholesterol and fat-enriched diet for 3 months. A segment of heart tissue spanning the aortic sinus and ascending aorta was embedded in OCT, sectioned, and stained with (A,B) Oil Red O for neutral lipids, (C,D) rabbit antisera to mouse macrophages (1:3000 dilution), (E,F) a monoclonal antimouse Thy1.2 antibody (7  $\mu$ g/ml), (G,H) Gomori trichrome to detect collagen. A,B,  $\times$ 40; C-H,  $\times$ 200. (A, inset) and (B, inset) Areas contained in C,E,G and D,F,H, respectively.



**FIG. 5.** Aortic lesion T lymphocyte and MHC class II-positive cell numbers. Numbers of lesion-associated (A,B) T lymphocyte-positive and (C,D) MHC class II-positive cells were determined from cross-sections of the ascending aorta of male and female apoE<sup>-/-</sup> × IFN-γ<sup>-/-</sup> (open symbols) and apoE<sup>-/-</sup> × IFN-γ<sup>+/+</sup> (closed symbols) mice fed either (A,C) a normal diet for 9 months or (B,D) a high-fat diet for 3 months. Circles, values of individual mice; squares, means; bars, SEM. Segments of heart tissue spanning the aortic sinus and ascending aorta were embedded in OCT, sectioned, and stained with (A,B) a monoclonal antimouse Thy1.2 antibody (7 μm/ml) or (C,D) a monoclonal antimouse MHC II antibody (1:5 dilution). Values are derived from 9 mice per group in A and C and from 6 mice per group in B and D.

At present, the contribution of this cell type to IFN-γ secretion within lesions and progression of the disease is unknown.<sup>(46)</sup>

Female C56BL/6 mice develop more atherosclerosis than males,<sup>(47)</sup> but the data on gender differences have been more mixed in apoE<sup>-/-</sup> mice. Some have indicated an increased extent of lesion formation in female mice.<sup>(40,48)</sup> However, this has not been a uniformly observed difference and may depend on factors, such as age.<sup>(6,49)</sup> In the present study, the difference was apparent only during the feeding of high-fat diets and only in the ascending aorta. In agreement with the effects we noted for IFN-γ deficiency, gender-specific effects have also been observed on the extent of atherosclerosis development in infections<sup>(50)</sup> and total lymphocyte deficiency.<sup>(6,42)</sup> In both cases, males exhibited an effect on atherosclerosis in response to the intervention, whereas females were unaffected. Gender-specific differences in the relative lymphocyte subtype responses have been described in both humans and mice, with females exhibiting more pronounced Th2 responses compared with Th1-oriented response in males.<sup>(29,51)</sup> Although the

basis for this difference in responsiveness has not been defined, a hormonal basis is indicated by the switch in Th1:Th2 responses that occurs during pregnancy.<sup>(52,53)</sup> Furthermore, these changes revert to normal in the postpartum phase.<sup>(54,55)</sup> Therefore, it remains to be determined whether a specific hormonal balance is responsible for lack of effect of IFN-γ deficiency on the size and cellular characteristics of lesions in female apoE<sup>-/-</sup> mice.

In summary, we found a striking difference between male and female mice in the effect of IFN-γ deficiency on atherosclerosis. Deficiency of endogenous IFN-γ decreased the development of atherosclerotic lesions in male mice. This decrease was associated with a decreased abundance of T lymphocytes and MHC class II-positive cells. Because the ability to mount an effective immunologic response involves an intricate array of cytokines from various immunologic cells, more work must be done to determine the mediators underlying this difference. In future studies, we will determine the role of gender-specific hormones, particularly estrogen, on the effects of IFN-γ on the atherogenic process.<sup>(56)</sup>

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