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Abstract—IgG Fc receptors (Fc γ Rs) play a role in activating the immune system and in maintaining peripheral tolerance, but their role in atherosclerosis is unknown. We generated double-knockout (DKO) mice by crossing apolipoprotein E-deficient mice (apoE^{-/-}) with Fc γ R γ chain-deficient mice (γ ^{-/-}). The size of atherosclerotic lesions along the aorta was approximately 50% lower in DKO compared with apoE^{-/-} control mice, without differences in serum lipid levels. The macrophage and T-cell content of lesions in the DKO were reduced by 49 $\pm$ 6% and 56 $\pm$ 8%, respectively, compared with the content in apoE^{-/-} lesions. Furthermore, the expression of monocyte chemoattractant protein-1 (MCP-1), RANTES (Regulated on Activated Normal T-cell Expressed and Secreted), and intercellular adhesion molecule-1 (ICAM-1) and the activation of nuclear factor- κ B (NF- κ B) were significantly reduced in aortic lesions from DKO mice. In vitro, vascular smooth muscle cells (VSMCs) from both γ ^{-/-} and DKO mice failed to respond to immune complexes, as shown by impaired chemokine expression and NF- κ B activation. ApoE^{-/-} mice have higher levels of activating Fc γ RI and Fc γ RIIA, and inhibitory Fc γ RIIB, compared with wild-type mice. The DKO mice express only the inhibitory Fc γ RIIB receptor. We conclude that Fc γ R deficiency limits development and progression of atherosclerosis. In addition to leukocytes, Fc γ R activation in VSMCs contributes to the inflammatory process, in part, by regulating chemokine expression and leukocyte invasion of the vessel wall. These results underscore the critical role of Fc γ Rs in atherogenesis and support the use of immunotherapy in the treatment of this disease. (*Circ Res.* 2006;99:1188-1196.)

Key Words: Fc receptors ■ atherosclerosis ■ double-knockout mice ■ immune complexes

Atherosclerosis is a chronic inflammatory disease of the arterial wall characterized by progressive accumulation of lipids, cells, and extracellular matrix. In recent years, the immune processes associated to atherogenesis have received considerable attention. Studies in both humans and animals have demonstrated that atheromatous lesions at all stage of development contain a wide variety of cells and molecules characteristic of immune system, such as macrophages, lymphocytes, CD40, interferon- γ , major histocompatibility complex-II, complement, and antibodies (Abs).¹⁻⁴ In addition, congenital deficiency of macrophages, T and B lymphocytes, or even the inhibition of their mediators has resulted in the reduction of atherosclerotic lesion.^{1,3} One of the critical steps in atherogenesis is the accumulation within the arterial intima of cholesteryl ester-laden foam cells, many of them derived from macrophages, whose formation is ultimately dependent on the uptake of various forms of low-density lipoproteins (LDLs).^{1,2} Although emphasis has been placed on scavenger receptors, foam-cell development may also be influenced by lipoprotein interaction with other receptors,

such as LDL receptors, very-low-density lipoprotein (VLDL) receptors, and IgG Fc (Fc γ Rs) receptors.⁵ Different studies have demonstrated that Fc γ R is the most important macrophage receptor involved in the clearance of immune complexes (ICs) containing native or oxidized LDL.^{6,7} Indeed, modified LDL present in atherosclerotic lesions can trigger an autoimmune response in vivo, and auto-Abs (mainly IgG1 and IgG3) to oxidized LDL have been found in blood and plaques.^{4,8,9} These results suggest that the clearance of LDL-containing ICs by macrophage Fc γ Rs contributes to foam-cell development in vivo.

Previous studies found functional evidence of Fc γ Rs in monocytic cells from atherosclerotic patients, and differential expression of Fc γ Rs in the proliferative areas of human lesions.¹⁰⁻¹² Moreover, the beneficial effect of immunomodulation in atherosclerosis has recently been reported.¹³ Therapy with intact immunoglobulins (Igs) or Fc fragments has been tried in a wide range of immune and inflammatory disorders,^{14,15} and its potential use in chronic heart failure and cardiomyopathy has recently been suggested.^{16,17} Although

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different mechanisms may participate in the beneficial effects of Igs.^{14,18} FcγR blockade in macrophages and other effector cells is probably implicated in the protection from cardiovascular damage.^{13,18}

FcγRs are well-characterized cell surface glycoproteins that mediate phagocytosis or Ab-dependent cell cytotoxicity in immune and resident cells of many tissues.¹⁹ Two general classes of FcγRs are known, the activating and the inhibitory receptors, which transmit their signals via immunoreceptor tyrosine-based activation (ITAM) or inhibitory motifs (ITIM). In activating receptors, the ITAM motif can be either intrinsic to the receptor, as in FcγRIIA (a receptor not found in the mouse), or as part of an associated subunit, mainly the γ chain, as in FcγRI, FcγRIIIA, and FcγRIV (receptors conserved between mouse and human). The inhibitory receptor (FcγRIIB) contains the ITIM sequence in its cytoplasmic domain and binds IgG and ICs with low affinity.^{19,20} In the last years, studies in genetically modified mice have documented that FcγR activation is involved in several inflammatory and immune diseases.^{19,21–23} However, the *in vivo* role of FcγRs in atherosclerotic lesion formation has not been well examined. In this report, we analyze the contribution of FcγRs present in vascular and infiltrating cells to the inflammatory process during atherosclerosis. We have generated double-knockout (DKO) mice by crossing apolipoprotein E-deficient mice (apoE^{−/−}) with γ chain-deficient mice (γ^{−/−}), the FcγRI and FcγRIII of which are not functional. The study of lesion development in these DKO mice will be useful for determining the involvement of immune system in the modulation of atherosclerosis.

Materials and Methods

For more details, see the expanded Material and Methods section in the online data supplement, available at <http://circres.ahajournals.org>.

Mice and Atherosclerotic Model

Female apoE^{−/−} mice²⁴ were crossed with male γ^{−/−} mice (C57BL/6 background both),^{21,25} and the progeny was bred back to apoE^{−/−} mice for 7 generations to obtain the DKO lineage (apoE^{−/−} γ^{−/−}) and the littermate controls apoE^{−/−} (apoE^{−/−} γ^{+/+}) and γ-heterozygous (apoE^{−/−} γ^{+/−}). The apoE and γ genotypes were determined by genomic PCR, using specific primers.^{21,24,25} Mice (8 weeks) with apoE^{−/−}, γ-heterozygous, and DKO genotypes (males, n=11; females, n=8 per group) were fed a Western-type high cholesterol (HC) diet for 16 weeks. To compare the atherogenic diet effect, male apoE^{−/−}, DKO, and wild-type (WT) were placed on standard low-cholesterol (LC) diet for 16 weeks (n=8 per group). These animals were processed for histological examination. In other set of experiments, male apoE^{−/−}, DKO, and WT were fed a HC diet and used for mRNA analysis (n=10 per group). These studies were performed in accordance with the European Union normative and were approved by the ethical committee of our institution.

Histological and Immunohistochemical Analysis

Aortas were dissected out from euthanized mice and frozen. Serial 8-μm sections of the aortic root were stained with Oil red O/hematoxylin,²⁶ and intimal lesion areas were quantified by morphometry. Infiltrating cells and RANTES (Regulated on Activated Normal T-cell Expressed and Secreted) were analyzed by indirect immunoperoxidase with MOMA-2 (macrophages), CD4 and CD8 (T lymphocytes), CD22 (B lymphocytes), and RANTES Abs. Activated nuclear factor κB (NF-κB) was detected by Southwestern histochemistry with digoxigenin-labeled probes, using competition and mutant probe as specificity controls.²⁷ Immunostaining was expressed as

percentage of positive staining per millimeter squared (macrophages and RANTES) and positive nuclear staining per millimeter squared (NF-κB).^{28,29} Cells positive for CD4, CD8, and CD22 were recorded per millimeter squared.

Cell Culture

Murine VSMCs were cultured in DMEM containing 10% FBS and characterized by immunostaining (α-actin positive, factor VIII negative).³⁰ Cells (second to seventh passage) were stimulated with ICs (heat-aggregated mouse IgG)³¹ and lipopolysaccharide (LPS).

mRNA and Protein Expression Analysis

Total RNA from aorta and VSMCs was isolated with TRIzol.^{30,31} Gene expression of murine monocyte chemoattractant protein-1 (MCP-1), adhesion molecule-1 (ICAM-1), RANTES, FcγRI, FcγRIIB, and FcγRIIIA was analyzed by real-time PCR. Chemokine expression was also analyzed by RNase protection assay. Target gene expression was normalized to housekeeping gene (GAPDH).

Cytosolic proteins (25 μg) were resolved on SDS-PAGE gels and immunoblotted with RANTES and macrophage inflammatory protein-1β (MIP-1β) Abs.³¹ MCP-1 concentration in VSMC supernatants was measured with a commercial ELISA.

NF-κB Activity

For electrophoretic mobility shift assay (EMSA), nuclear proteins (10 μg) were incubated in buffer containing 0.035 pmol of [³²P]NF-κB oligonucleotide, then electrophoresed and autoradiographed. Specificity was confirmed using a 100-fold excess of unlabeled probe.³² Nuclear detection of NF-κB subunits was detected in fixed, permeabilized cells with p50 and p65 Abs, followed by fluorescein isothiocyanate (FITC)-labeled Ab, and counterstained with diamidino-2-phenylindole (DAPI).

Statistical Analysis

Results are given as mean±SD. Differences between the groups were considered significant at *P*<0.05 using the ANOVA and Tukey–Kramer tests.

Results

FcγR Deficiency Reduces Atherosclerotic Lesions

To evaluate the contribution of FcγRs to atherogenesis, we generated DKO mice with targeted deletion of apoE and γ-chain genes. Aortic roots were investigated in DKO mice and their littermate controls (apoE^{−/−} and γ-heterozygous) after feeding with either atherogenic or standard diet. At 16 weeks of HC diet, female apoE^{−/−} mice exhibited significantly greater lesions than diet-matched males and, as expected, than LC diet-fed males (Figure 1A and 1C). Compared with their respective apoE^{−/−} controls, γ-heterozygous mice showed smaller lesions, whereas a drastic decrease was observed in DKO (Figure 1A). Plaque quantification revealed significant reduction in the extension (Figure 1B) and size (Figure 1C) of the lesions in both male and female DKO (percentage decrease versus apoE^{−/−}: 53±10 and 57±4). The atheroprotective effect of FcγR deficiency was also seen in the LC diet model (54±6% decrease). Animals from the 3 genotypes showed no differences in cholesterol and triglycerides, suggesting that FcγR deficiency did not affect the lipid metabolism (Table 1). Body weights were also unaltered. Moreover, DKO survived longer than apoE^{−/−} mice (survival rate at 10 months: 90% versus 50%).

The inflammatory infiltrate was analyzed by immunohistochemistry with MOMA-2 (macrophages), CD4/CD8 (T cells), and CD22 (B cells) Abs. Numerous macrophages

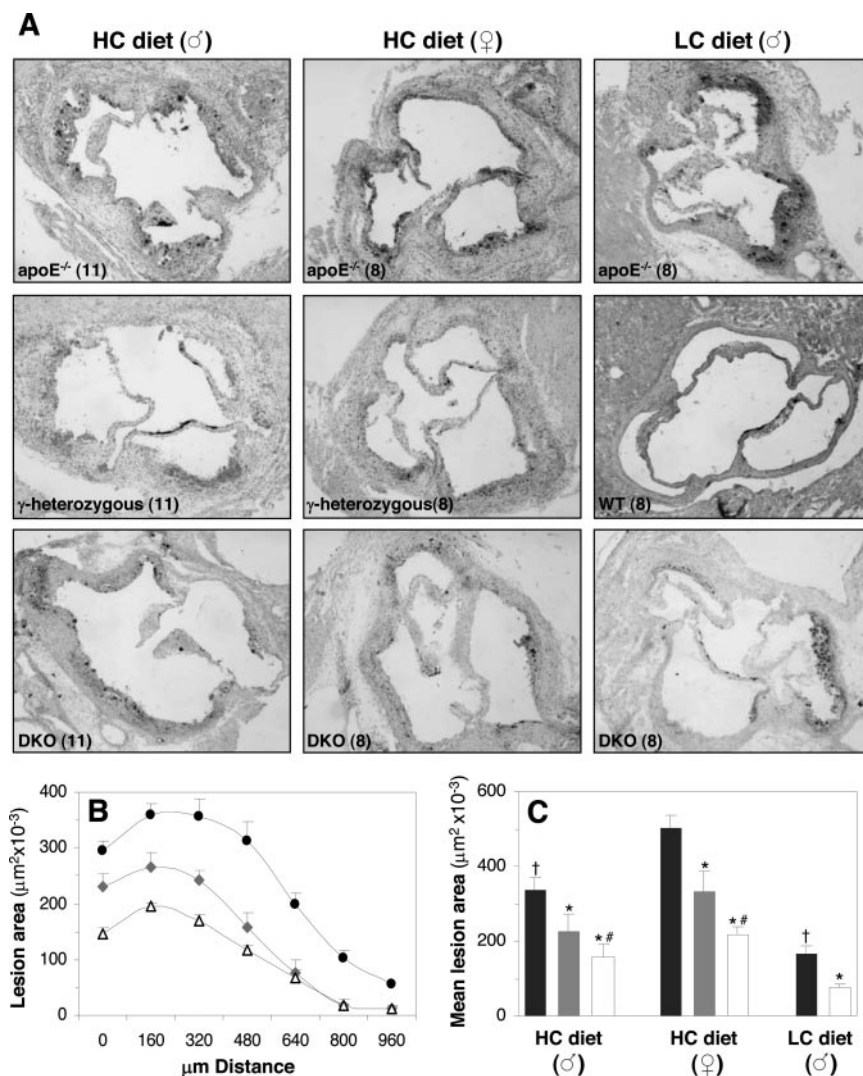


Figure 1. FcγR gene deficiency diminished the extension and size of atherosclerotic lesions. A, Oil red O/hematoxylin staining in aortic sections from mice fed a HC or LC diet (sex and number of animals are indicated). Magnification, ×40. B, Lesion area throughout the studied region in male apoE^{-/-} (black), γ-heterozygous (gray), and DKO (white) mice fed a HC diet. C, Average of maximal lesion areas from each group of apoE^{-/-} (black), γ-heterozygous (gray), and DKO (white) mice. **P*<0.01 vs apoE^{-/-}, #*P*<0.05 vs γ heterozygous, †*P*<0.01 vs HC diet (female).

infiltrate the lesion in apoE^{-/-} controls (Figure 2A). Lipid accumulation (Oil red O) coincided with MOMA staining, indicating the presence of foam cell-rich areas. CD4⁺ T cells were also detected within lesions, with the CD8⁺ T cells and CD22⁺ B cells being somewhat less abundant (not shown). Quantification revealed significant reduction of macrophages and T lymphocytes in γ-heterozygous and DKO mice fed either a LC or HC diet. However, FcγR deficiency was not able to decrease the B-cell content of lesions (Table 2).

Reduced Expression of Proinflammatory Genes in FcγR-Deficient Mice

We next evaluated the expression of genes involved in the leukocyte recruitment to the intima at the earliest stages of atherosclerosis.^{33–35} In male apoE^{-/-} fed the HC diet, RANTES protein was detected in the intima and media, and its expression diminished in γ-heterozygous and DKO mice (Figure 2C, 2D, and 2I). Likewise, mRNA expressions of MCP-1 and ICAM-1 were significantly higher in apoE^{-/-} than in DKO (Figure 2J).

TABLE 1. Body Weights and Serum Concentrations of Cholesterol and Triglycerides in Mice With the Different Genotypes

	ApoE ^{-/-}			γ-Heterozygous		DKO		
	HC (Male, n=11)	HC (Female, n=8)	LC (Male, n=8)	HC (Male, n=11)	HC (Female, n=8)	HC (Male, n=11)	HC (Female, n=8)	LC (Male, n=8)
BW, g	25.3±0.3	20.2±0.4	23.2±0.3	26.3±0.2	20.6±0.9	25.4±0.3	19.8±0.4	23.5±0.3
CHO, mg/dL	495±57*	579±52*	260±21	467±31	512±46	423±35*	582±33*	252±29
TG, mg/dL	129±17*	82±16	85±10	95±4	92±7	107±8	95±11	89±8

BW indicates body weight; CHO, serum concentration of cholesterol; TG, serum concentration of triglycerides. Results are mean±SD. Sex and no. of animals are indicated. **P*<0.05 vs LC (male).

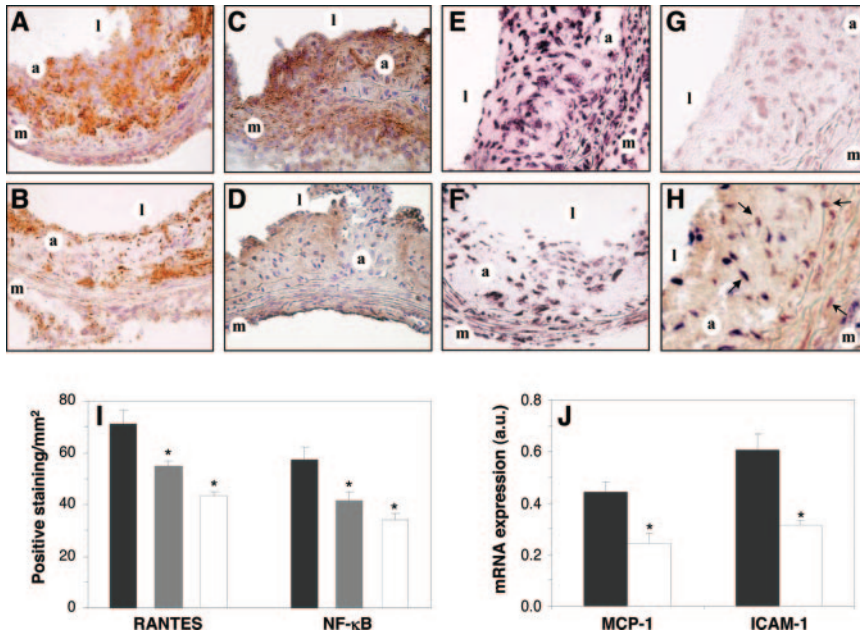


Figure 2. Fc γ R absence reduces inflammatory responses in aorta from apoE^{-/-} mice. Histological examination of macrophages (A and B), RANTES (C and D), and NF- κ B (E through H) in aorta from male mice fed a HC diet. Aortic roots from apoE^{-/-} contain a substantial number of macrophages in the intima (A) and abundant RANTES (C) and NF- κ B (E) in both intima and media. These parameters were decreased in DKO mice (B, D, and F, respectively). G, NF- κ B-specific binding in apoE^{-/-} sample was verified with 200-fold excess of unlabeled probe. H, Colocalization of NF- κ B (nuclear staining in blue) and RANTES (cytoplasmic staining in brown) in apoE^{-/-}. Arrows denote representative cells with double staining. Original magnification, $\times 200$. Atheroma (a), media (m), and lumen (l) are indicated. I, Quantification of area with positive staining. J, MCP-1 and ICAM-1 expression in aorta from apoE^{-/-} and DKO mice (n=10 per group) were determined by real-time PCR and expressed in arbitrary units. * $P < 0.05$ vs apoE^{-/-}. Black bars indicate apoE^{-/-}; gray bars, γ -heterozygous; white bars, DKO.

Among the transcription factors regulating proinflammatory genes during atherogenesis,^{28–30,36} we analyzed NF- κ B activation. Southwestern histochemistry in apoE^{-/-} sections revealed an intense nuclear staining in intimal and medial cells (Figure 2E), which colocalized with RANTES (Figure 2H), suggesting that NF- κ B regulates RANTES expression in the lesions. Number of NF- κ B-positive cells were decreased in aortas from γ -heterozygous and DKO mice (Figure 2F and 2I).

ICs Induce Chemokine Expression and NF- κ B Activation in VSMCs

Because RANTES and NF- κ B were detected not only in the intima but also in the media of vessels, mainly constituted by VSMCs, we next examined whether cultured VSMCs are sensitive to ICs. In VSMCs from WT mice, ICs enhanced the expression of chemokines known to be implicated in athero-

genesis, mainly RANTES, MIP-1 β , and MCP-1 (Figure 3A), as evaluated by RNase protection assay. Time-dependent mRNA expression of MCP-1 and RANTES, determined by real-time PCR (Figure 3B), was maximal at 8 and 24 hours, respectively. Protein expression profile followed similar kinetics, as determined by Western blot (MIP-1 β and RANTES; Figure 3C) and ELISA (MCP-1; Figure 3D). The involvement of Fc γ R in chemokine expression was demonstrated by using VSMCs from γ ^{-/-} and DKO mice. Both cell types identically responded to IC stimulation, exhibiting a reduced mRNA (Figure 3A and 3B) and protein (Figure 3C and 3D) expression of chemokines, when compared with WT cells.

NF- κ B activity was analyzed by EMSA (Figure 4A) and immunofluorescence with p65 and p50 Abs (Figure 4B and not shown). Consistent with chemokine expression, IC-induced NF- κ B activity was attenuated in γ ^{-/-} when com-

TABLE 2. Inflammatory Cell Infiltrate in the Atherosclerotic Lesions

Group	MOMA-2	CD4	CD8	CD22
LC diet (male)				
ApoE ^{-/-} (n=8)	65.0 $\pm$ 3.0	126.6 $\pm$ 18.7	49.1 $\pm$ 9.0	27.1 $\pm$ 10.3
DKO (n=8)	38.1 $\pm$ 4.0*	51.2 $\pm$ 3.9*	23.1 $\pm$ 3.2*	23.4 $\pm$ 7.2
HC diet (male)				
ApoE ^{-/-} (n=11)	57.8 $\pm$ 5.3	129.8 $\pm$ 14	53.4 $\pm$ 8.7	40.1 $\pm$ 5.4
γ -Heterozygous (n=11)	45.0 $\pm$ 5.1*	79.1 $\pm$ 6.9*	42.1 $\pm$ 4.1*	33.1 $\pm$ 5.6
DKO (n=11)	29.6 $\pm$ 3.7*†	49.4 $\pm$ 6.0*†	23.1 $\pm$ 4.2*†	29.8 $\pm$ 2.8
HC diet (female)				
ApoE ^{-/-} (n=8)	68.5 $\pm$ 2.3	73.8 $\pm$ 1.6	40.2 $\pm$ 1.7	20.1 $\pm$ 1.5
γ -Heterozygous (n=8)	50.9 $\pm$ 5.1*	45.6 $\pm$ 1.3*	24.0 $\pm$ 2.6*	19.9 $\pm$ 6.7
DKO (n=8)	34.7 $\pm$ 4.1*†	27.1 $\pm$ 1.7*†	14.6 $\pm$ 3.5*	17.9 $\pm$ 2.7

Immunohistochemical analysis of macrophages and T and B lymphocytes in aortic sections from the different animals (sex and no. are indicated). Positive staining is expressed as percentage of positivity per millimeter squared (MOMA-2) or no. of cells per millimeter squared (CD4, CD8, and CD22). * $P < 0.05$ vs apoE^{-/-}, † $P < 0.05$ vs γ heterozygous.

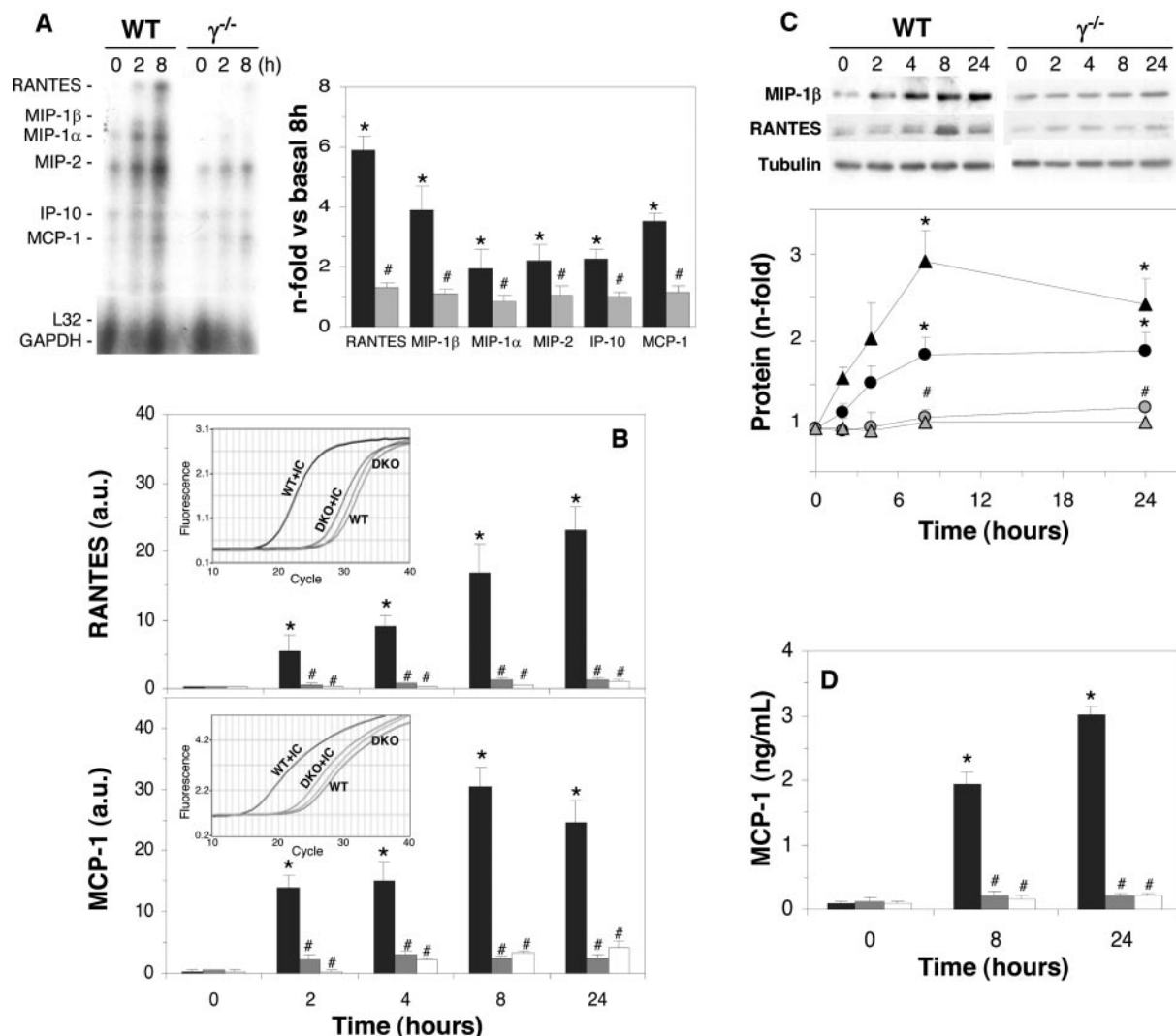


Figure 3. Chemokine expression in VSMCs stimulated with ICs. VSMCs from WT (black bars), $\gamma^{-/-}$ (gray bars), and DKO (white bars) mice were incubated with 150 μ g/mL ICs for different periods of time. A, The mRNA expression of the indicated chemokines was measured by RNase protection assay. B, Real-time PCR for RANTES and MCP-1. Insets, Fluorescence intensity vs cycle number plots in WT and DKO cells at 0 and 8 hours of stimulation. C, Western blot for MIP-1 β (triangles) and RANTES (circles) protein in VSMCs from WT (black) and $\gamma^{-/-}$ (gray) mice. D, Secreted MCP-1 protein was measured by ELISA. Expression data were normalized to GAPDH (A and B), tubulin (C), and cell protein content (D) and are mean $\pm$ SD of 4 to 5 experiments. * P <0.05 vs basal, # P <0.05 vs WT.

pared with WT cells. Nevertheless, the response to the positive control (LPS) was similar in both cell types.

Regulation of Fc γ R Expression in Mice and Cells

Next, we analyzed the Fc γ RI, Fc γ RIIB, and Fc γ RIIIA expression in aorta from HC diet-fed mice. In WT mice, Fc γ RIIB and Fc γ RIIIA were found modestly increased, whereas apoE $^{-/-}$ displayed markedly upregulation of the 3 receptor types, mainly Fc γ RIIIA. As expected, lack of detectable expression of γ chain–dependent receptors (Fc γ RI and Fc γ RIIIA) was observed in DKO, but, interestingly, Fc γ RIIB was found to be clearly upregulated (Figure 5A). Based on the mRNA expression profile, the activating/inhibitory ratio of WT, apoE $^{-/-}$, and DKO (1.4, 2.5, and 0.4, respectively) revealed a change from activation to inhibitory state in Fc γ R-deficient mice.

Next, we analyzed whether ICs influence Fc γ R expression in vitro. In basal conditions, VSMCs from WT mice ex-

pressed low levels of Fc γ R, which were time-dependently increased by ICs. Fc γ RI and Fc γ RIIIA signals did not temporally vary in DKO cells treated for up to 24 hours with ICs. In contrast, the Fc γ RIIB mRNA expression, although detectable in control conditions, was clearly induced after IC incubation (Figure 5B and 5C).

Discussion

There is accumulating evidence that immunologic mechanisms contribute to atherogenesis.^{1,2} Several studies support the idea that, in addition to scavenger receptors, Fc γ R could be a second mechanism for phagocytosis and clearance of LDL-containing ICs.^{6,7} Fc γ R expression has been described in blood cells and plaques of cardiovascular patients¹¹ and in cultured vascular and endothelial cells.¹¹ However, whether Fc γ R activation influences atheroma formation in vivo has hardly been studied. In this work, we demonstrate that Fc γ R gene deficiency protects against atherosclerosis development.

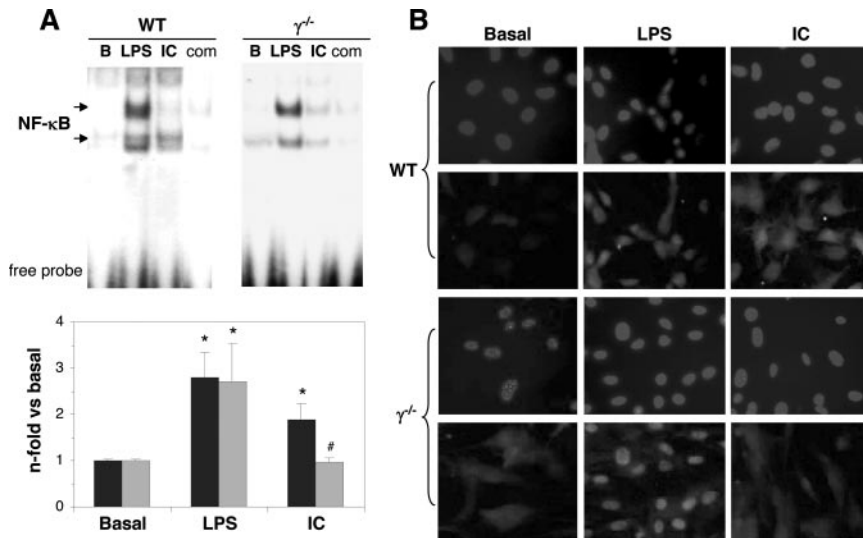


Figure 4. IC-induced NF-κB activation is reduced in FcγR-deficient cells. VSMCs from WT (black) and γ^{-/-} (gray) mice were stimulated with ICs (150 μg/mL) or LPS (1 μg/mL) for 60 minutes. A, NF-κB activity in nuclear extracts was analyzed by EMSA. com indicates competition assay in sample from IC-stimulated cells. Arrows indicate specific bands. Densitometric data are mean±SD of 4 experiments (**P*<0.05 vs basal, #*P*<0.05 vs WT). B, Nuclear translocation of activated complex was visualized by immunofluorescence with p65 Ab (green) in combination with DAPI (blue). Representative of 3 independent experiments.

We studied for the first time DKO mice lacking genes for apoE and γ chain, the common activation subunit required for surface assembly and signaling of FcγRI, FcγRIII, and FcεRI.¹⁹ Independently of sex and diet, DKO mice exhibited a significant reduction in the lesion area compared with apoE^{-/-} controls, thus underscoring the critical role of FcγRs in atherogenesis. These data confirm previous studies describing that FcγR blockade is among the underlying mechanisms of the beneficial effects of Ig therapy in cardiovascular diseases.^{13,16–18}

In the present study, we observed that FcγR deficiency led to a reduction in macrophage accumulation in the lesions, a key step in atherosclerosis.² In monocytes, FcγR activation triggers the production of cytokines, proteolytic enzymes, and reactive oxygen intermediates.^{37–39} In addition to the direct clearance of LDL-ICs,⁴⁰ FcγRs are involved in the upregulation of LDL and scavenger receptors,^{39,41} affecting the ability to take up modified LDL through a non-FcγR-mediated pathway. Moreover, LDL-ICs promote survival of monocytes through FcγRI, then constituting a novel mecha-

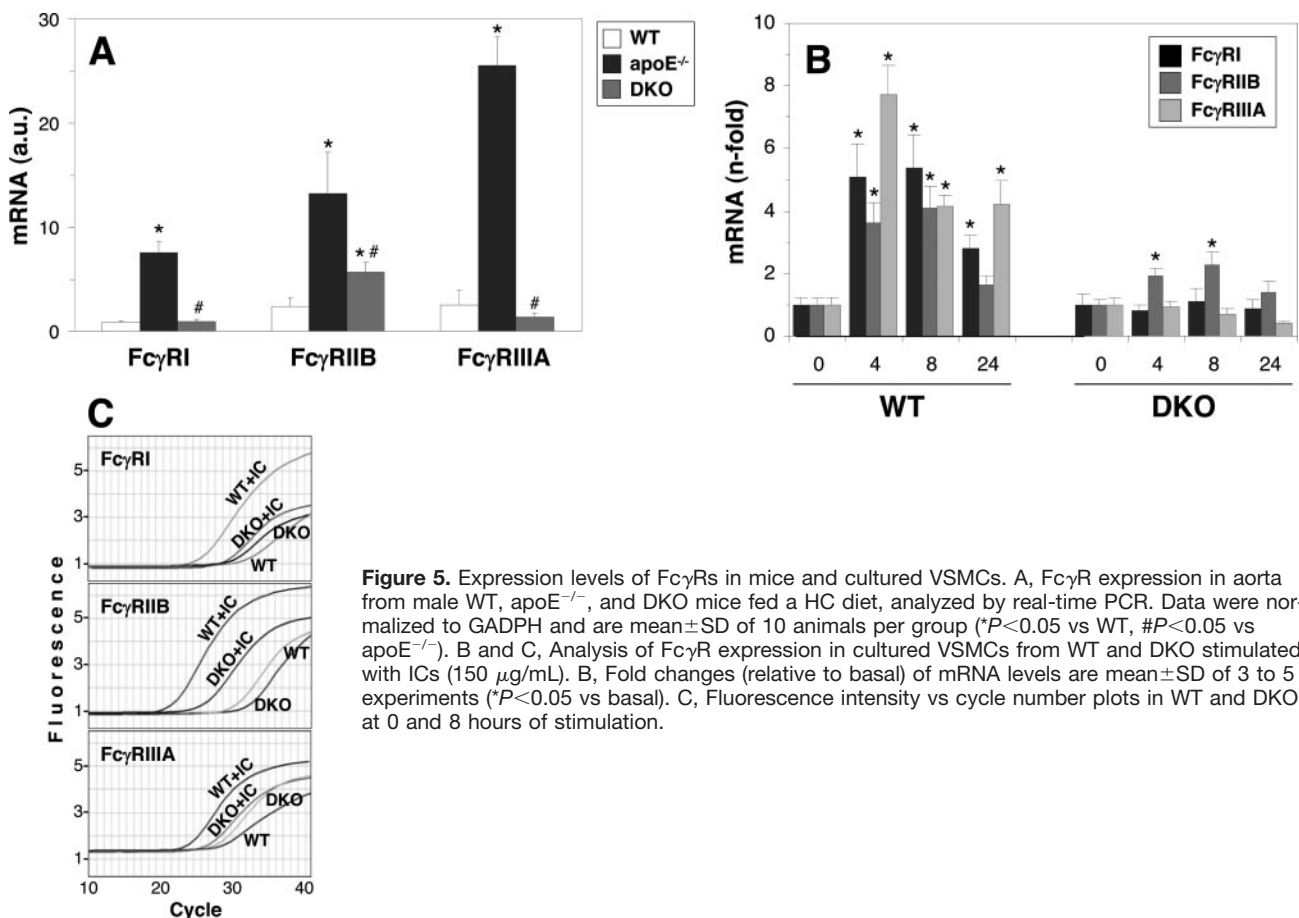


Figure 5. Expression levels of FcγRs in mice and cultured VSMCs. A, FcγR expression in aorta from male WT, apoE^{-/-}, and DKO mice fed a HC diet, analyzed by real-time PCR. Data were normalized to GAPDH and are mean±SD of 10 animals per group (**P*<0.05 vs WT, #*P*<0.05 vs apoE^{-/-}). B and C, Analysis of FcγR expression in cultured VSMCs from WT and DKO stimulated with ICs (150 μg/mL). B, Fold changes (relative to basal) of mRNA levels are mean±SD of 3 to 5 experiments (**P*<0.05 vs basal). C, Fluorescence intensity vs cycle number plots in WT and DKO at 0 and 8 hours of stimulation.

nism by which immune reaction regulates lesion formation.⁴² Based on these findings, we postulate that Fc γ R deficiency may suppress the accumulation, activation, and maintenance of intimal macrophages in atherosclerotic lesions. We have also observed a lower infiltration rate of CD4⁺ and CD8⁺ T lymphocytes in lesions of DKO mice, whereas the number of B lymphocytes was not affected. It has been reported that most plaque T cells are proinflammatory CD4⁺ Th1 effector cells.^{3,43} Moreover, downregulation of Th1 responses reduces atherogenesis, whereas transfer of CD4⁺ T cells aggravates atherosclerosis.³ By contrast, B-cell infusion reduces lesion size and T-cell infiltration,^{1,44} suggesting that protective immunity may also occur in atherosclerosis. Then, a conceivable consequence of Fc γ R deficiency would be the reduction of proatherogenic immune activation in the artery, which may explain the decrease in the severity of atherosclerosis. The recent finding that γ chain deficiency decreases leukocyte attachment to the vascular wall and neointimal hyperplasia after balloon vascular injury is in agreement with such interpretation.⁴⁵

In parallel with the reduction in leukocyte infiltration, DKO exhibited a blunted expression of chemokines and adhesion molecules. These genes were found increased in cardiovascular diseases,^{2,29,30,33,46} and inhibition of their function markedly decreases plaque formation.^{33,34} In vitro, ICs trigger the expression of chemokines in immune and tissue cells.^{32,47} Moreover, it has recently been demonstrated that Ig therapy has direct effects on leukocyte recruitment through inhibition of adhesion molecules.⁴⁸ Our data are compatible with those suggesting that the atheroprotective effect of Fc γ R deficiency in apoE^{-/-} mice is mediated, in part, by the reduced generation of proinflammatory molecules.

NF- κ B is an important transcription factor required for the cooperative induction of proinflammatory genes^{47,49} that has been found activated in atherosclerotic plaques.^{28,30,36} However, the benefit of inhibiting NF- κ B pathway in atherosclerosis is a current controversy, because it has been proposed that NF- κ B could be either proinflammatory regulator in early inflammation²⁹ or antiinflammatory regulator in the resolution phases.^{36,50} In this work, we observed a decreased NF- κ B activation in DKO mice. Moreover, NF- κ B colocalized with RANTES in cells of the intima and media, suggesting that, in addition to infiltrating cells, the resident vascular cells are involved in the inflammatory responses mediated by Fc γ R in vivo. This is in line with recent reports describing the active role that VSMCs play in vascular injury, mediating both early inflammatory and thrombotic responses, such as extracellular matrix synthesis, LDL uptake, and cytokine secretion.^{51,52} Here, we reveal that VSMCs are sensitive to ICs. Then, Fc γ R engagement in VSMCs may result in NF- κ B activation and expression of chemokines, responsible for leukocyte recruitment, thereby accelerating lesion formation. However, we cannot rule out the idea that Fc γ R gene deficiency in inflammation may be nonspecific for atherosclerosis and may be a general antiinflammatory pathway. Therefore, the possibility that Fc γ R deficiency may prevent the activation of other intracellular pathways regulating proinflammatory genes needs to be evaluated in the future. In this sense, it has recently been reported that Fc γ RIIB, via

protein phosphatase 2A, mediates the inhibition of endothelial NO synthase by C reactive protein.⁵³

In this study, we describe that Fc γ RI, Fc γ RIIB, and Fc γ RIIIA were upregulated in atherosclerotic mice and were also time-dependently increased by ICs in cultured VSMCs, thus providing evidence that Fc γ R expression on infiltrating and/or intrinsic vascular cells is associated with vascular inflammation. It has been reported that the γ chain is required for Fc γ RI, Fc γ RIII, and Fc ϵ R expression, and for effector functions such as IC phagocytosis.^{19,20,54} Consistent with this, our DKO mice were shown not to express Fc γ RI and Fc γ RIIIA, but, interestingly, the Fc γ RIIB was potentiated. Thus, it is very likely that activating Fc γ RI and Fc γ RIIIA are required for initiating IC-mediated inflammation in aorta, but limited in their response by inhibitory Fc γ RIIB expression. This compelling concept is supported by the recent observation that Fc γ RIIB plays an immunoregulatory role in the development of immune diseases.²³ Moreover, the antiinflammatory activity of intravenous Igs in experimental myocarditis and atherosclerosis is linked to the Fc γ RIIB expression on effector cells.^{18,20,53} However, we cannot rule out that Fc γ RIIIA may be functioning even in the absence of γ chain if it associates with 2 ζ chains, as previously reported.^{19,20} It has been described that the ratio of activating to inhibitory Fc γ Rs on immune cells may be regulated by different factors. Thus, inflammatory mediators upregulate activating receptors, whereas antiinflammatory cytokines increase Fc γ RIIB expression, thereby setting high thresholds for cell activation.⁵⁵ Although other factors, such as affinity and isotype regulation, can influence the interaction of ICs with Fc γ Rs in cells of the lesions, modulation of expression levels and activating/inhibitory ratios may represent another link between immune system and atherosclerosis progression.

In summary, these data provide evidence that Fc γ RI and Fc γ RIII activation in leukocytes and VSMCs are involved in the IC-mediated responses during atherogenesis. Our findings contribute to the understanding of the immune mechanisms associated to atherosclerosis and support the use of immunotherapy in the treatment of this disease.

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Disclosures

None.

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MATERIAL AND METHODS: EXPANDED VERSION**Mice and atherosclerotic model**

Female apoE^{-/-} mice (C57BL/6 background; Jackson Laboratory; Bar Harbor, ME) (24) were crossed with male γ^{-/-} (C57BL/6) (21;25), and the progeny was bred back to apoE^{-/-} mice for 7 generations to obtain the DKO lineage (apoE^{-/-} γ^{-/-}) and the littermate controls apoE^{-/-} (apoE^{-/-} γ^{+/+}) and γ-heterozygous (apoE^{-/-} γ^{+/-}). The apoE and γ-chain genotypes were determined by PCR assays from genomic DNA, using specific primers as previously described (21;24;25). Mice (8 weeks) with apoE^{-/-}, γ-heterozygous, and DKO genotypes (males, n=11; females, n=8 per group) were fed on Western-type (HC) diet for 16 weeks. This HC diet contained 21% fat (0.15 % cholesterol) and 17% protein. To compare the effects of the atherogenic diet, male apoE^{-/-}, DKO and wild type (WT) mice were placed on standard low cholesterol (LC) diet for 16 weeks (n=8 per group). These animals were processed for histological examination. In other set of experiments, male apoE^{-/-}, DKO and WT mice fed on HC diet were used for mRNA analysis (n=10 per group). All these studies were performed in accordance to the EU normative and were approved by the ethical committee of our institution.

Tissue preparation and histology

Aortas were dissected out from euthanized mice and frozen. Serial 8μm sections were obtained covering 1000 μm of the aortic root beginning when valve leaflets appeared (26). Seven sections at 160 μm intervals were stained with Oil red O and hematoxylin. The intimal area was quantified using CAS-200 Imaging System. The mean lesion area was determined for each mouse by averaging the values for 4 sections. The individual mean lesion areas were further averaged to determine the mean lesion area for each group.

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Macrophage, T- and B-cell content, and chemokine production were analyzed by indirect immunoperoxidase in acetone-fixed cryosections using Abs against MOMA-2 (0.6 μg/mL; Serotec, Oxford, UK), CD4, CD8, CD22 (20 μg/mL BD Biosciences, Erembodegem, Belgium), and RANTES (10 μg/mL; Chemicon, Hofheim, Germany), respectively. After a washing step,

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Southwestern histochemistry was developed for *in situ* detection of activated NF-κB (27). Frozen aortic sections were fixed in 0.5% paraformaldehyde and incubated with 0.1 mg/mL DNaseI. The DNA binding reaction was performed by incubation with 6 pmol of digoxigenin-labeled DNA probe in buffer containing 0.25% BSA and 2 µg/mL poly (dI-dC) followed by alkaline phosphatase conjugated anti-digoxigenin IgG, and colorimetric detection. Preparations without probe were used as negative controls and a 200-fold excess of unlabeled probe was used to test the specificity of the technique. In some cases double immunohistochemistry for RANTES was carried out on slides directly from the final wash of the Southwestern histochemistry protocol (counterstain with hematoxylin was omitted).

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Blood samples were collected from the orbital venous plexus after an overnight fasting period at the time of sacrifice. Total serum cholesterol and triglycerides concentrations were measured using standard enzymatic methods with commercially available test kits (Sigma Chemicals, St. Louis, MO).

Cell culture

Murine VSMC were isolated from aortas of WT or $\gamma^{-/-}$ mice by enzymatic digestion as previously described (30). Briefly, all external fat, connective tissue and adventitial area were detached by blunt dissection from the thoracic aorta. Vessels were cut and incubated for 45 min

with 4 mg/mL collagenase type II (Sigma) at 37°C. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine (Life Technologies, Rockville, MD, USA) and used between the 2nd and 7th passage. VSMC were characterized by phase-contrast microscopy and immunostaining (positive for smooth muscle α -actin and negative for factor VIII, excluding endothelial cells). Cells were made quiescent by 24h incubation in medium without FBS, and then stimulated with IC (heat-aggregated mouse IgG) (31) and LPS (Sigma).

mRNA expression analysis

Total RNA from aortic tissue and VSMC was isolated with Trizol (30:31). Gene expression of murine MCP-1, ICAM-1, RANTES, FcγRI, FcγRIIB, and FcγRIIIA was analyzed by real-time PCR. In brief, 1 µg of RNA underwent random primed reverse transcription using a modified Maloney murine leukemia virus reverse transcriptase (Superscript II; Life Technologies, Gaithersburg, MD) for 2 h at 37°C. Real-time PCR was performed on a TaqMan ABI 7700 Sequence Detection System using heat-activated *TaqDNA* polymerase (Amplitaq Gold). After an initial hold of 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s. GAPDH served as housekeeping gene and was amplified in parallel with the genes of interest. The expression of each target gene was normalized to both different housekeeping transcripts. Target gene, forward and reverse primers, and probes were designed using Primer Express 1.5 software (Applied Biosystems, Foster City, CA) and searched against the public databases to confirm unique amplification products. All primers, probes, and reagents were obtained from Applied Biosystems. All measurements were performed in duplicate.

RNA expression for different chemokines was analyzed using RiboQuantTM RNase protection Assay System with the mCK-5c multiprobe template set (BD Biosciences, Erembodegem, Belgium) following the manufacturer's instructions. RNA probes were synthesized with α -[³²P]UTP (Amersham) and used within 2 days. Five micrograms of total RNA was used in each hybridization and RNase-protected probe fragments were resolved on 5% polyacrylamide gels. The gels were dried and exposed to X-OMAT AS films (Eastman Kodak, Madrid, Spain).

Autoradiograms were quantified by scanning densitometry (BioRad Lab., California, USA) and L32 and GAPDH were used for normalization.

Protein expression analysis

Stimulated cells were washed in cold PBS and lysed in 10 mmol/L Tris-HCl pH 7.4 containing 150 mmol/L NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM EDTA, 1mM EGTA, 0.2 mM Na₃VO₄, 10 mM NaF, 0.2 mM PMSF, and protease inhibitors cocktail. Protein concentration was determined by the BCA method. Cytosolic proteins (25 µg) were resolved on SDS-PAGE gels, transferred onto polyvinylidene difluoride membranes and immunoblotted with rabbit anti-mouse RANTES and goat anti-mouse MIP-1β (0.5 µg/mL; Santa Cruz Biotechnology, Santa Cruz, CA). After visualizing with ECL system, membranes were reblotted with anti-tubulin as loading control (31). MCP-1 concentration in supernatants from VSMC was measured by a commercial ELISA (R&D systems, Minneapolis, MI).

NF-κB activity

For electrophoretic mobility shift assay (EMSA), nuclear proteins (10 µg) from VSMC were incubated in buffer containing 50 µg/mL poly(dI-dC) and 0.035 pmol of [³²P]-NF-κB oligonucleotide. Reactions were analyzed on 4% nondenaturing PAGE gels and autoradiographed. Specificity was confirmed using a 100-fold excess of unlabeled probe (32).

For nuclear detection of NF-κB complex subunits, VSMC were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100, and then incubated with 3 µg/mL of rabbit anti-p50 and rabbit anti-p65 Abs (sc-7178 and sc-372; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by FITC-labeled anti-IgG and counterstained with diamidino-2-phenylindole (DAPI, Vector Laboratories Peterborough, UK). Negative controls without primary Abs were run in parallel.

Statistical analysis

Results are given as mean±SD. Differences between the groups were considered significant at $p < 0.05$ using the Analysis of Variance or Tukey-Kramer tests.

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with 4 mg/mL collagenase type II (Sigma) at 37°C. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM glutamine (Life Technologies, Rockville, MD, USA) and used between the 2nd and 7th passage. VSMC were characterized by phase-contrast microscopy and immunostaining (positive for smooth muscle α -actin and negative for factor VIII, excluding endothelial cells). Cells were made quiescent by 24h incubation in medium without FBS, and then stimulated with IC (heat-aggregated mouse IgG) (31) and LPS (Sigma).

mRNA expression analysis

Total RNA from aortic tissue and VSMC was isolated with Trizol (30:31). Gene expression of murine MCP-1, ICAM-1, RANTES, FcγRI, FcγRIIB, and FcγRIIIA was analyzed by real-time PCR. In brief, 1 µg of RNA underwent random primed reverse transcription using a modified Maloney murine leukemia virus reverse transcriptase (Superscript II; Life Technologies, Gaithersburg, MD) for 2 h at 37°C. Real-time PCR was performed on a TaqMan ABI 7700 Sequence Detection System using heat-activated *Taq*DNA polymerase (Amplitaq Gold). After an initial hold of 2 min at 50°C and 10 min at 95°C, the samples were cycled 40 times at 95°C for 15 s and 60°C for 60 s. GAPDH served as housekeeping gene and was amplified in parallel with the genes of interest. The expression of each target gene was normalized to both different housekeeping transcripts. Target gene, forward and reverse primers, and probes were designed using Primer Express 1.5 software (Applied Biosystems, Foster City, CA) and searched against the public databases to confirm unique amplification products. All primers, probes, and reagents were obtained from Applied Biosystems. All measurements were performed in duplicate.

RNA expression for different chemokines was analyzed using RiboQuantTM RNase protection Assay System with the mCK-5c multiprobe template set (BD Biosciences, Erembodegem, Belgium) following the manufacturer's instructions. RNA probes were synthesized with α -[³²P]UTP (Amersham) and used within 2 days. Five micrograms of total RNA was used in each hybridization and RNase-protected probe fragments were resolved on 5% polyacrylamide gels. The gels were dried and exposed to X-OMAT AS films (Eastman Kodak, Madrid, Spain).

Autoradiograms were quantified by scanning densitometry (BioRad Lab., California, USA) and L32 and GAPDH were used for normalization.

Protein expression analysis

Stimulated cells were washed in cold PBS and lysed in 10 mmol/L Tris-HCl pH 7.4 containing 150 mmol/L NaCl, 1% Triton X-100, 0.5% NP-40, 1 mM EDTA, 1mM EGTA, 0.2 mM Na₃VO₄, 10 mM NaF, 0.2 mM PMSF, and protease inhibitors cocktail. Protein concentration was determined by the BCA method. Cytosolic proteins (25 µg) were resolved on SDS-PAGE gels, transferred onto polyvinylidene difluoride membranes and immunoblotted with rabbit anti-mouse RANTES and goat anti-mouse MIP-1β (0.5 µg/mL; Santa Cruz Biotechnology, Santa Cruz, CA). After visualizing with ECL system, membranes were reblotted with anti-tubulin as loading control (31). MCP-1 concentration in supernatants from VSMC was measured by a commercial ELISA (R&D systems, Minneapolis, MI).

NF-κB activity

For electrophoretic mobility shift assay (EMSA), nuclear proteins (10 µg) from VSMC were incubated in buffer containing 50 µg/mL poly(dI-dC) and 0.035 pmol of [³²P]-NF-κB oligonucleotide. Reactions were analyzed on 4% nondenaturing PAGE gels and autoradiographed. Specificity was confirmed using a 100-fold excess of unlabeled probe (32).

For nuclear detection of NF-κB complex subunits, VSMC were fixed with 3% paraformaldehyde, permeabilized with 0.5% Triton X-100, and then incubated with 3 µg/mL of rabbit anti-p50 and rabbit anti-p65 Abs (sc-7178 and sc-372; Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by FITC-labeled anti-IgG and counterstained with diamidino-2-phenylindole (DAPI, Vector Laboratories Peterborough, UK). Negative controls without primary Abs were run in parallel.

Statistical analysis

Results are given as mean±SD. Differences between the groups were considered significant at $p < 0.05$ using the Analysis of Variance or Tukey-Kramer tests.