Cell Metabolism
Article

Myeloid Type I Interferon Signaling Promotes Atherosclerosis by Stimulating Macrophage Recruitment to Lesions

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DOI 10.1016/j.cmet.2010.06.008

SUMMARY

Inflammatory cytokines are well-recognized mediators of atherosclerosis. Depending on the pathological context, type I interferons (IFNs; IFNα and IFNβ) exert either pro- or anti-inflammatory immune functions, but their exact role in atherogenesis has not been clarified. Here, we demonstrate that IFNβ enhances macrophage-endothelial cell adhesion and promotes leukocyte attraction to atherosclerosis-prone sites in mice in a chemokine-dependent manner. Moreover, IFNβ treatment accelerates lesion formation in two different mouse models of atherosclerosis and increases macrophage accumulation in the plaques. Concomitantly, absence of endogenous type I IFN signaling in myeloid cells inhibits lesion development, protects against lesional accumulation of macrophages, and prevents necrotic core formation. Finally, we show that type I IFN signaling is upregulated in ruptured human atherosclerotic plaques. Hereby, we identify type I IFNs as proatherosclerotic cytokines that may serve as additional targets for prevention or treatment.

INTRODUCTION

The important contribution of inflammatory cytokines to atherosclerosis development is well recognized (Hansson and Libby, 2006; Tedgui and Mallat, 2006; Weber et al., 2008). The macrophage is both an important source and a major target of these inflammatory mediators. Specific cytokines influence macrophage effector functions and thereby affect plaque initiation, progression, and cellular composition. Ultimately, macrophages hereby alter important plaque stability factors such as necrotic core formation and inflammatory cell content. Hence, the macrophage is a very attractive target for designing therapeutic interventions (Li and Glass, 2002), making it crucial to understand how cytokines regulate macrophage function in atherogenesis.

Cytokines of the type I interferon (IFN) family (IFNα and IFNβ) are produced by immune cells in response to pathogenic challenges such as viruses, bacteria, and tumor cells. They induce antiviral responses (Borden et al., 2007; Katze et al., 2002), have immunomodulating activities, and are clinically used to treat viral diseases, multiple sclerosis, and certain tumors (Borden et al., 2007; Paty and Li, 1993; Takaoka and Yanai, 2006). Recent data indicate that IFNβ is also necessary for sustaining TNF-driven inflammation (Yarilina et al., 2008), promotes TNF-induced lethal shock (Huys et al., 2009), and mediates MHC-I induction by TNF (Leeuwenberg et al., 1987). In addition, constitutive low levels of type I IFN amplify IFNγ- and interleukin-6 (IL-6)-driven inflammatory responses (Mitani et al., 2001; Takaoka et al., 2000). Thus, type I IFNs also modulate immune responses that are not directly induced by pathogens. In sharp contrast to the clinical application of IFNβ as treatment for the pathologies mentioned above, type I IFNs have also been shown to promote disease in systemic lupus erythematosus (SLE), where inhibition strategies are being considered for therapy (Rönnblom et al., 2006). Of interest, it was recently postulated that the increased risk of cardiovascular mortality in SLE patients (McMahon and Hahn, 2007) is linked to their elevated levels of type I IFNs (Lee et al., 2007). Thus, depending on the context, type I IFN may have either disease-promoting or disease-inhibiting properties.

The important immunomodulatory activities of type I IFNs prompted us to hypothesize that they also have a role in atherogenesis. We show that IFNβ treatment induces chemokine-dependent adhesion and migration of leukocytes and promotes

atherosclerosis development in vivo. Consequently, we show that inhibition of type I IFN signaling in myeloid cells hampers cellular recruitment to lesions and thus atherosclerosis development. Finally, we found that type I IFN signaling is an integral feature of human atherosclerosis as well and is upregulated in plaque instability.

RESULTS

IFNβ Treatment of Macrophages Induces Chemotactic Factors

To study the effect of type I IFN on macrophages, we first performed in vitro studies with cultured bone marrow-derived macrophages (BMM) and analyzed cytokine secretion, uptake pathways, and factors mediating adhesion and migration. Though TNF and IL-12 were not induced by IFNβ, treatment did induce the expression of the anti-inflammatory cytokine IL-10 (Figure 1A). Next, we analyzed uptake pathways by BMM that had been pretreated with IFNβ. Neither endocytosis of oxidatively modified LDL (oxLDL) nor phagocytosis of fluorescently labeled latex beads was affected by treatment of cells with IFNβ (Figures 1B and 1C). We further focused on molecules mediating attraction of cells to atherosclerotic lesions. FACS analysis of IFNβ-treated macrophages showed no differences for any of the major integrins necessary for rolling and arrest at inflammatory sites (Figure 1D). Of interest, gene expression of the chemokine and chemokine receptors (Figures 1E and 1F) CCL5 (RANTES) and its receptor CCR5, as well as CCR2, was upregulated in macrophages activated with IFNβ. However, further FACS analysis showed no significant upregulation of surface expression of both CCR2 and CCR5 (Figure 1G). In contrast, CCL5 secretion was strongly increased by activation of macrophages with IFNβ (Figure 1H). Next, we studied the role of the main receptor for type I IFN, IFNAR1, and for this, macrophages from mice with a myeloid-specific deletion of *IFNAR1* (Prinz et al., 2008) were used. Quantitative PCR showed a deletion efficiency of *IFNAR1* of 73.2% ± 1.4%, and CCL5 induction by IFNβ was reduced to a similar extent in *IFNAR1*-deleted cells ($IFNAR1^{del}$) compared to control cells ($IFNAR1^{WT}$) (Figure 1I). Ablation of signal transducer and activator of transcription 1 (STAT1) signaling, as a key downstream transcription factor of type I IFN signaling, almost completely abolished induction of CCL5 by IFNβ (Figure 1J). CCL5 ELISA showed similar reduction in secreted CCL5 for $IFNAR1^{del}$ and $STAT1^{-/-}$ cells (Figure S1 available online). Treatment of macrophages with another type I IFN, IFNα, showed comparable effects on CCR5 and CCL5 (Figure S2). Thus, in addition to inducing IL-10, type I IFN treatment of primary macrophages induces chemotactic factors, which may influence leukocyte attraction and atherosclerosis development.

IFNβ Treatment Enhances In Vitro Macrophage Adhesion and Promotes In Vivo Leukocyte Arrest

In line with our data described above, we found that static adhesion of IFNβ-treated macrophages to endothelial cells was enhanced (Figure 2A). Endothelial adhesion of macrophages lacking *IFNAR1* or *STAT1* was not inducible by IFNβ (Figures 2B and 2C). In line with a major role for CCL5-CCR5 signaling, we found that IFNβ-induced adhesion was not affected in *CCR1*-deficient macrophages but was completely blunted in macrophages lacking *CCR5* (Figure 2D). Absence of *CCR2* showed an intermediate phenotype, with a moderate effect of IFNβ. In vivo, 4 days treatment of high-fat fed apolipoprotein E-deficient ($apoe^{-/-}$) mice with IFNβ led to a strong increase of leukocyte arrest in the carotid arteries of these animals (Figure 2E). Cotreatment of mice with the CCL5 antagonist Met-Rantes just prior to analyzing leukocyte arrest reduced leukocyte arrest in untreated mice and fully restored leukocyte adhesion to control levels in IFNβ-treated animals (Figure 2E). Thus, IFNβ treatment in vitro and in vivo promotes leukocyte adhesion and attraction to the endothelium through CCL5-CCR5-dependent mechanisms.

IFNβ Treatment Accelerates Atherogenesis in $apoe^{-/-}$ and $ldlr^{-/-}$ Mice

Next, we analyzed the effect of IFNβ on atherogenesis in two well-established mouse models of atherosclerosis. Daily injections of IFNβ in a collar-induced atherosclerosis model in $apoe^{-/-}$ mice (von der Thüsen et al., 2001) did not alter plasma cholesterol levels (27.89 ± 2.10 and 26.98 ± 2.04 mM for the ctrl and IFNβ treated, respectively) but increased plasma levels of IL-10 (3.90 ± 2.1 and 19.21 ± 5.8 pg/ml, $p < 0.05$, for the ctrl and IFNβ treated, respectively). Atherosclerotic lesion analysis (Figures 3A and 3B) showed a strong increase in lesion formation in mice that had been treated with IFNβ. The same effect was seen in low-density lipoprotein receptor-deficient ($ldlr^{-/-}$) mice in which IFNβ treatment increased aortic root atherosclerotic lesion size by almost 2-fold (Figures 3C and 3D) without any effects on plasma cholesterol (26.30 ± 1.78 and 26.86 ± 1.66 for the ctrl and IFNβ treated, respectively) or blood leukocyte levels ($B220^+$ B cells, $CD3^+$ T cells, $Gr1^{hi}CD11b^+$ neutrophils, and $Gr1^{int/-}CD11b^+$ monocytes; data not shown). Staining for macrophage content showed an ~2-fold increase in absolute macrophage area in IFNβ-treated mice (Figures 3E and 3F). Gene expression analysis of aortic arches from these mice also showed increased expression of the macrophage marker CD68 (Figure 3G), further indicating increased macrophage accumulation in atherosclerotic lesions. More extensive immunohistochemical analysis of the aortic root lesions did not show any significant difference with respect to neutrophil or T cell accumulation, TUNEL-positive cells, or necrotic core formation (data not shown). Of interest, we did find an induction of circulating CCL5 levels in IFNβ-treated mice (Figure 3H). Thus, despite the induction of IL-10, IFNβ treatment enhances atherosclerosis development, coinciding with increased levels of CCL5.

Myeloid *IFNAR1* Deletion Reduces Atherosclerosis Development

Subsequently, we investigated whether endogenous production of type I IFN also contributes to atherosclerosis development. We first analyzed whether typical type I IFN signature genes were expressed in lesions of atherosclerotic mice. Indeed, expression of OAS1, OAS2, MX2, and IRF9 could be readily detected in aortic arches from $ldlr^{-/-}$ mice that had been fed a high-fat diet for 6 or 9 weeks (Figure S3), indicating that endogenous type I IFN signaling is present. We then performed a transplantation using bone marrow from $IFNAR1^{WT}$ and $IFNAR1^{del}$ mice,

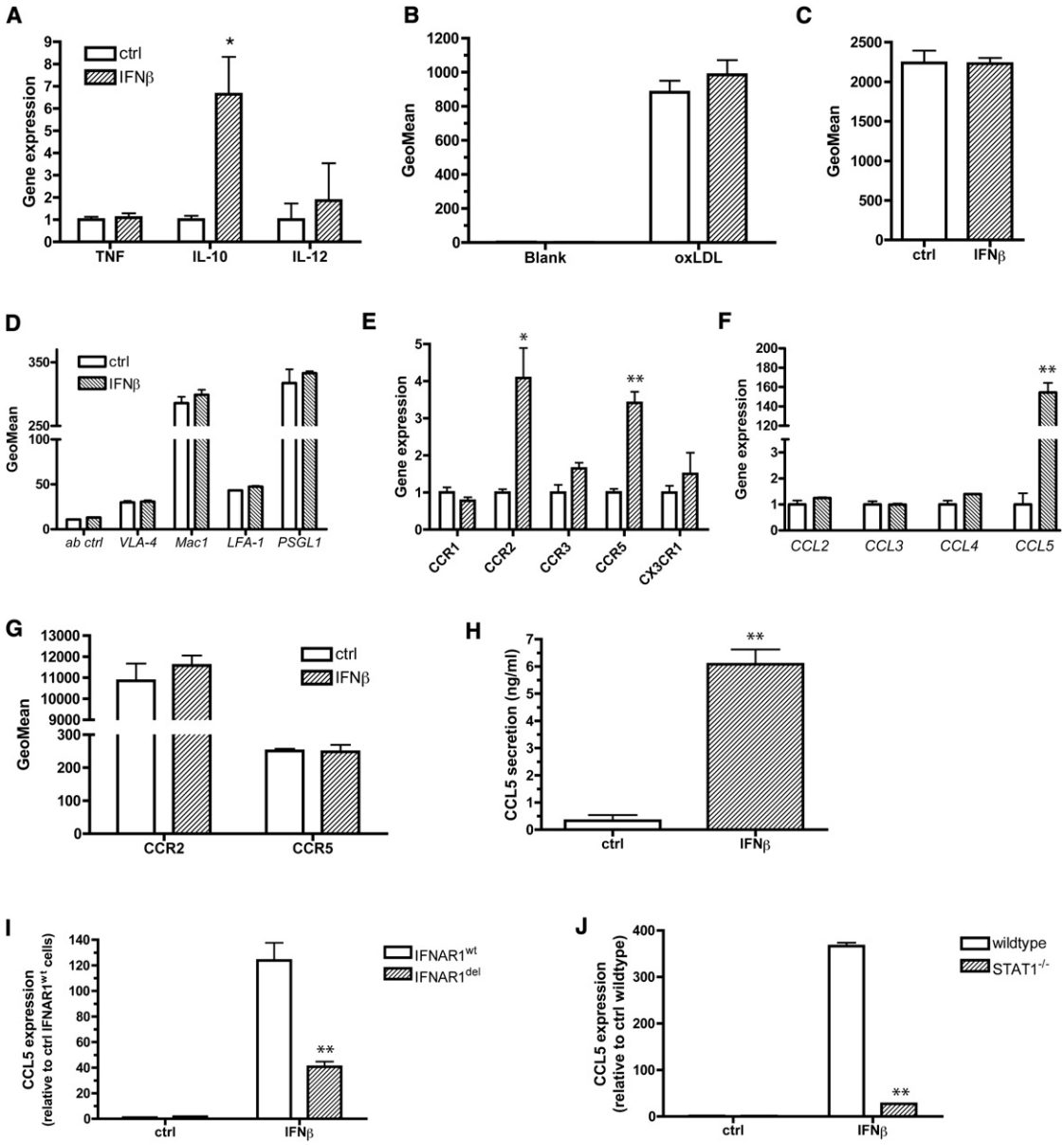

Figure 1. The Effect of IFNβ on Cultured Bone Marrow-Derived Macrophages

(A) Treatment of macrophages with IFNβ induces IL-10 expression without affecting TNF or IL-12.
(B) Uptake of DiI-labeled oxLDL by control (ctrl)- or IFNβ-treated macrophages.
(C) Uptake of fluorescent latex beads by ctrl- or IFNβ-treated macrophages.
(D) Surface expression of VLA-4, Mac1, LFA-1, and PSGL1 in ctrl- or IFNβ-treated macrophages.
(E) Relative gene expression of chemokine receptors in ctrl- or IFNβ-treated macrophages.
(F) Chemokine expression after IFNβ treatment of macrophages.
(G) FACS analysis of CCR2 and CCR5 after treatment with IFNβ.
(H) CCL5 secretion by macrophages after treatment with IFNβ.
(I) CCL5 expression in ctrl- and IFNβ-stimulated IFNAR1^{WT} and IFNAR1^{del} macrophages.
(J) CCL5 expression in ctrl- or IFNβ-treated wild-type and *STAT1*$^{-/-}$ macrophages.
Graphs are representative for at least two independent experiments. Bars represent mean of triplicate wells ± SEM. *p < 0.05, **p < 0.01.

specifically lacking *IFNAR1* in their myeloid lineage, to *ldlr*$^{-/-}$ mice to yield atherosclerosis-susceptible mice that were either wild-type (IFNAR1^{WT}) or deleted (IFNAR1^{del}) for *IFNAR1* in their myeloid cells. Compared with IFNAR1^{WT} mice, mice lacking myeloid *IFNAR1* showed a strong reduction in atherosclerotic lesion size (−34%) (Figures 4A and 4B) after 11 weeks of high-fat diet. Lesions were mainly composed of macrophages (Figure 4C), and absolute macrophage area in the lesions was reduced in the IFNAR1^{del} mice (Figure 4D). Furthermore, the lesions were of intermediate phenotype, consisting mainly of

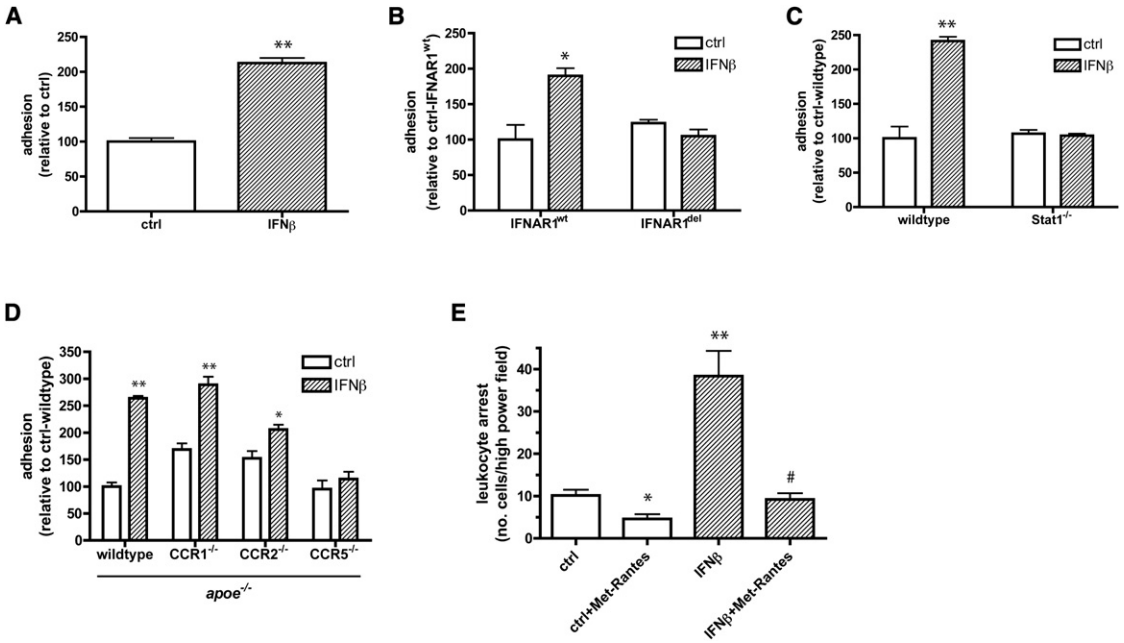

Figure 2. IFN Affects Static In Vitro Adhesion and In Vivo Leukocyte Arrest

(A) Adhesion of untreated macrophages (ctrl) to endothelial cells compared to IFNβ-treated macrophages (**$p < 0.01$).

(B) Adhesion of ctrl- or IFNβ-treated IFNAR1^{WT} and IFNAR1^{del} macrophages (*$p < 0.05$).

(C) Adhesion of ctrl- or IFNβ-treated wild-type or *STAT1*$^{-/-}$ macrophages (**$p < 0.01$).

(D) Adhesion of ctrl- or IFNβ-treated CCR wild-type, *CCR1*$^{-/-}$, *CCR2*$^{-/-}$, or *CCR5*$^{-/-}$ macrophages, all on an *apoe*$^{-/-}$ background (*$p < 0.05$, **$p < 0.01$). All bars for in vitro adhesion represent mean of triplicate wells ± SEM; stars indicate significant differences compared to ctrl-treated cells of the same genotype.

(E) Leukocyte arrest in the carotid artery of *apoe*$^{-/-}$ mice (ctrl), after treatment with Met-Rantes (ctrl+Met-Rantes), after 4 days IFNβ treatment (IFNβ), or after 4 days IFNβ treatment combined with Met-RANTES treatment (IFNβ+Met-Rantes). n = 8, 4, 7, and 5 for the four groups, respectively.

Error bars indicate SEM. *$p < 0.05$ or **$p < 0.01$ compared to ctrl; #$p < 0.01$ compared to IFNβ.

macrophage-derived foam cells with a fibrotic cap and some necrosis. IFNAR1^{del} mice showed reduced numbers of advanced lesions and increased numbers of moderate and early lesions (Figure 4E).

Plasma cholesterol levels did not differ between groups just before the start of the diet and after high-fat feeding (Figure 4F). In addition, total leukocyte counts (Figure 4G) and blood levels of leukocyte subsets (Figure 4H) did not differ between IFNAR1^{WT} and IFNAR1^{del} mice. Plasma cytokine levels were reduced, with a strong reduction (−70%) of circulating IL-6 (Figure S3) and a borderline ($p = 0.05$) reduction of the chemokine CCL2 (MCP-1) in IFNAR1^{del} mice.

Further examination of the lesions showed the neutrophil content to be decreased in atherosclerotic lesions of IFNAR1^{del} mice (Figures 5A and 5B), whereas T cell content did not differ (Figure 5C). Because type I IFNs have been implicated in the regulation of cell survival and proliferation (Borden et al., 2007), cell proliferation and apoptosis were assessed by Ki-67 and TUNEL staining, but no differences were observed between the two groups (Figures 5D and 5E). We did, however, find a strong (>70%) reduction of necrosis in the lesions of IFNAR1^{del} mice (Figures 5F and 5G), which is often considered a plaque-destabilizing consequence of impaired efferocytosis in the lesions (Tabas, 2005). Thus, endogenously produced type I IFNs activate myeloid cells, increase lesional accumulation of macrophages and neutrophils, promote necrotic core formation, and thereby promote atherosclerosis development.

IFNβ Induces Chemotactic Factors in Human Macrophages, and IFN Signaling Is Upregulated in Ruptured Human Atherosclerotic Lesions

To investigate whether type I IFNs also play a role in the pathogenesis of human atherosclerosis, we first analyzed the effect of IFNβ on primary human macrophages. Although IFNβ did not induce CCR2 (Figure 6A), it did increase expression of both CCR5 and CCL5 (Figures 6B and 6C) and induced secretion of CCL5 as analyzed by ELISA (Figure S4), confirming our mouse data. Next, Ingenuity Pathway Analysis was performed on an expression database of human atherosclerotic lesions. We compared data from stable carotid endarterectomy specimens and ruptured specimens, which had been classified according to the Virmani classification (Virmani et al., 2000). Of interest, the type I IFN pathway showed a highly significant upregulation in ruptured lesions when compared to stable lesions (Figure 6D). In addition to the upregulation of the type I IFN pathway, several chemotactic factors including CCR5 and CCL5 were also upregulated in ruptured atherosclerotic lesions (Table S1). These data show that upregulation of type I IFN signaling correlates with plaque rupture in human atherosclerosis.

DISCUSSION

In this paper, we show that treatment with IFNβ enhances atherogenesis in different models and that myeloid-specific inhibition of type I IFN signaling reduces atherosclerosis

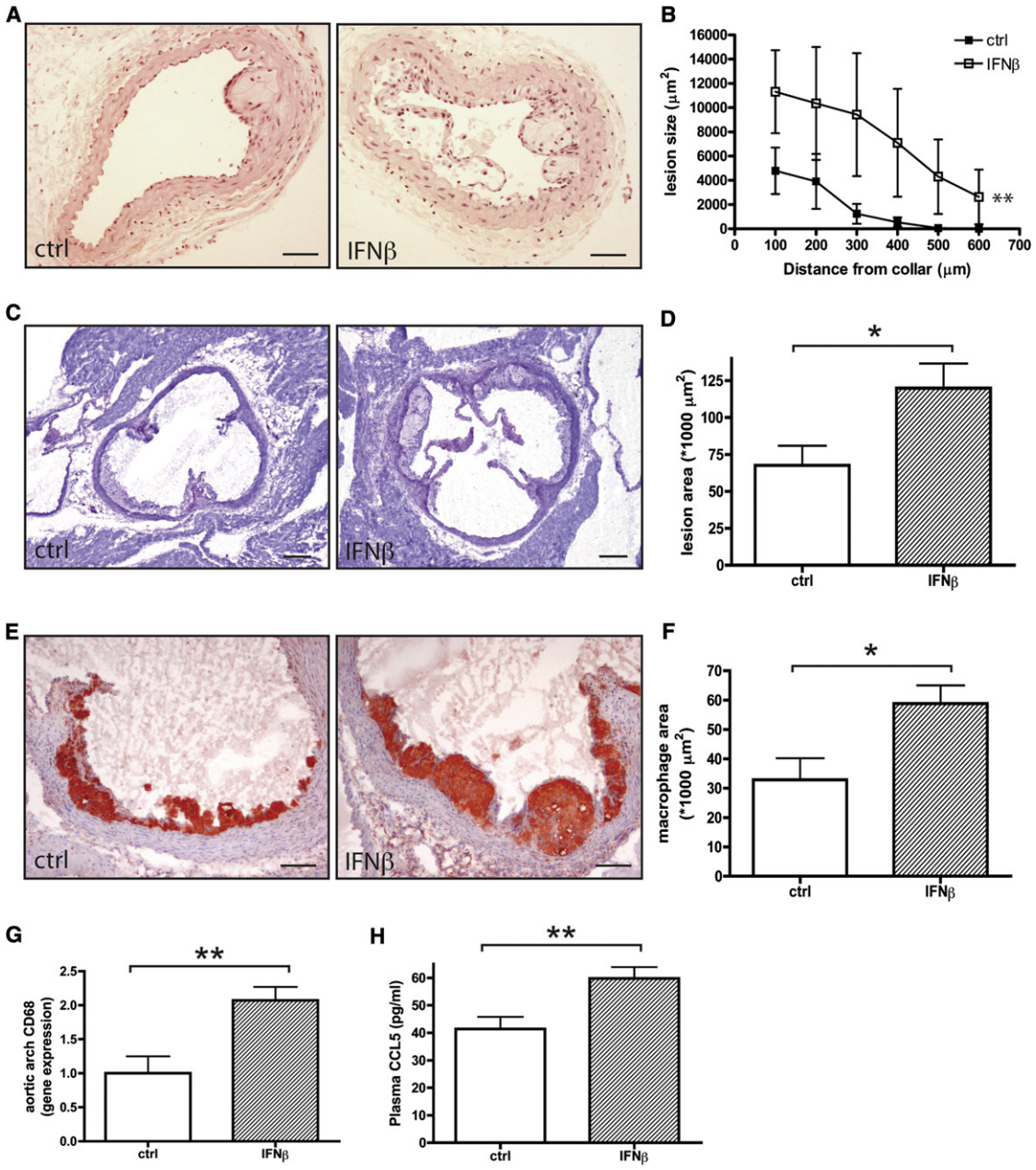

Figure 3. IFNβ Treatment Accelerates Atherosclerosis in *apoe*$^{-/-}$ and *ldlr*$^{-/-}$ Mice

(A) Representative lesions of ctrl- or IFNβ treated mice of collar-induced atherosclerosis in *apoe*$^{-/-}$ mice. Scale bar, 50 μm.

(B) Lesion area measured at six sequential locations proximal from the collar in *apoe*$^{-/-}$ mice that were ctrl or IFNβ treated. **p < 0.01 by two-way ANOVA; n = 9/12.

(C) Representative lesions in the aortic root of ctrl- or IFNβ-treated *ldlr*$^{-/-}$ mice. Scale bar, 200 μm.

(D) Lesion area at the aortic root of ctrl- or IFNβ-treated *ldlr*$^{-/-}$ mice. *p < 0.05; n = 12/14.

(E) Representative MOMA-2-stained lesions from ctrl- and IFNβ-treated *ldlr*$^{-/-}$ mice. Scale bar, 100 μm.

(F) Absolute macrophage area in lesions from ctrl- or IFNβ-treated *ldlr*$^{-/-}$ mice. *p < 0.05; n = 12/14.

(G) CD68 expression in aortic arches from ctrl- or IFNβ-treated *ldlr*$^{-/-}$ mice. **p < 0.01; n = 11/14.

(H) CCL5 levels in plasma from ctrl- or IFNβ-treated *ldlr*$^{-/-}$ mice. **p < 0.01; n = 9/11.

Shown are mean ± SEM.

development. We demonstrate that IFNβ treatment induces chemotactic factors and thereby promotes leukocyte attraction to atherosclerosis-prone sites. Accordingly, absence of endogenous myeloid type I IFN signaling reduced accumulation of cells from the myeloid lineage in the lesions. Thus, type I IFNs are proatherosclerotic cytokines that act by promoting chemokine-dependent leukocyte recruitment to atherosclerotic lesions. Of interest, upregulation of type I IFN signaling is

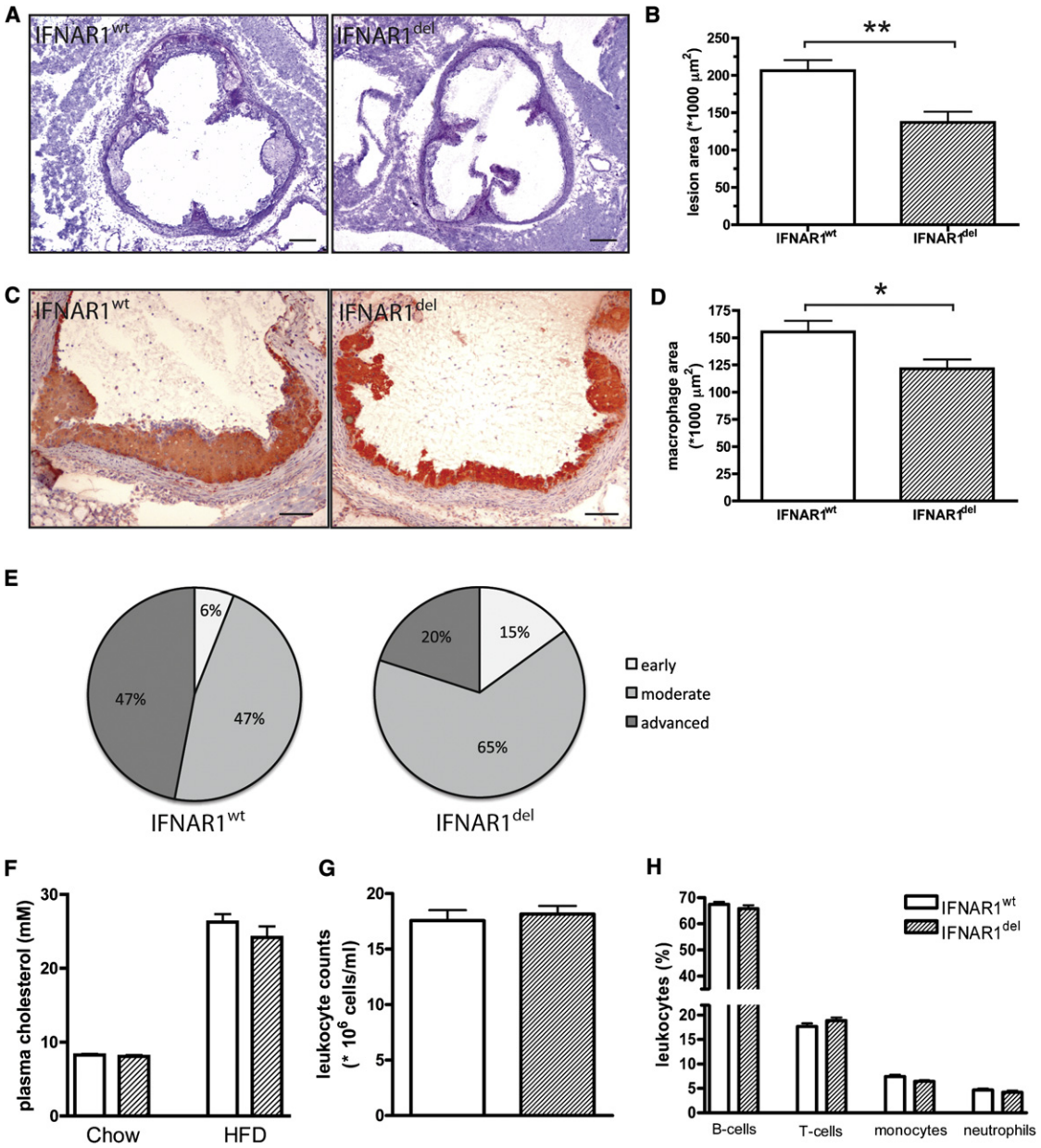

Figure 4. Absence of Myeloid *IFNAR1* Reduces Atherosclerosis Development

Bone marrow from conditional mice devoid of *IFNAR1* only on myeloid cells was transplanted to *ldlr*$^{-/-}$ mice to yield atherosclerosis-susceptible mice that were either wild-type (IFNAR1^{WT}) or deleted (IFNAR1^{del}) for *IFNAR1* in their myeloid cells.

(A) Representative toluidin blue-stained lesions from IFNAR1^{WT} and IFNAR1^{del} mice. Scale bar, 200 μm.

(B) Lesion area at the aortic root of IFNAR1^{WT} and IFNAR1^{del} mice. **$p < 0.01$; n = 19/15.

(C) Representative MOMA-2-stained lesions from IFNAR1^{WT} and IFNAR1^{del} mice. Scale bar, 100 μm.

(D) Absolute macrophage area in lesions from IFNAR1^{WT} and IFNAR1^{del} mice. *$p < 0.05$; n = 17/14.

(E) Lesion severity in IFNAR1^{WT} and IFNAR1^{del} mice. $p < 0.05$ by Chi-square test; n = 19/15.

(F) Plasma cholesterol levels before (chow) and after 8 weeks of high-fat diet (HFD) in IFNAR1^{WT} and IFNAR1^{del} transplanted mice.

(G and H) Total leukocyte counts and relative levels of leukocyte subsets.

Shown are mean ± SEM.

also associated with plaque rupture in human atherosclerotic lesions.

The family of type I IFNs consists of many members, with IFNα and IFNβ being the most abundant and best studied. It was previously shown that IFNα treatment promotes atherosclerosis in *ldlr*$^{-/-}$ mice. However, this effect was accompanied by elevated plasma levels of triglycerides and cholesterol (Levy et al., 2003), which complicates the interpretation of these data. Therefore, we chose to use IFNβ in our treatment studies, although we found that IFNα had similar in vitro effects on

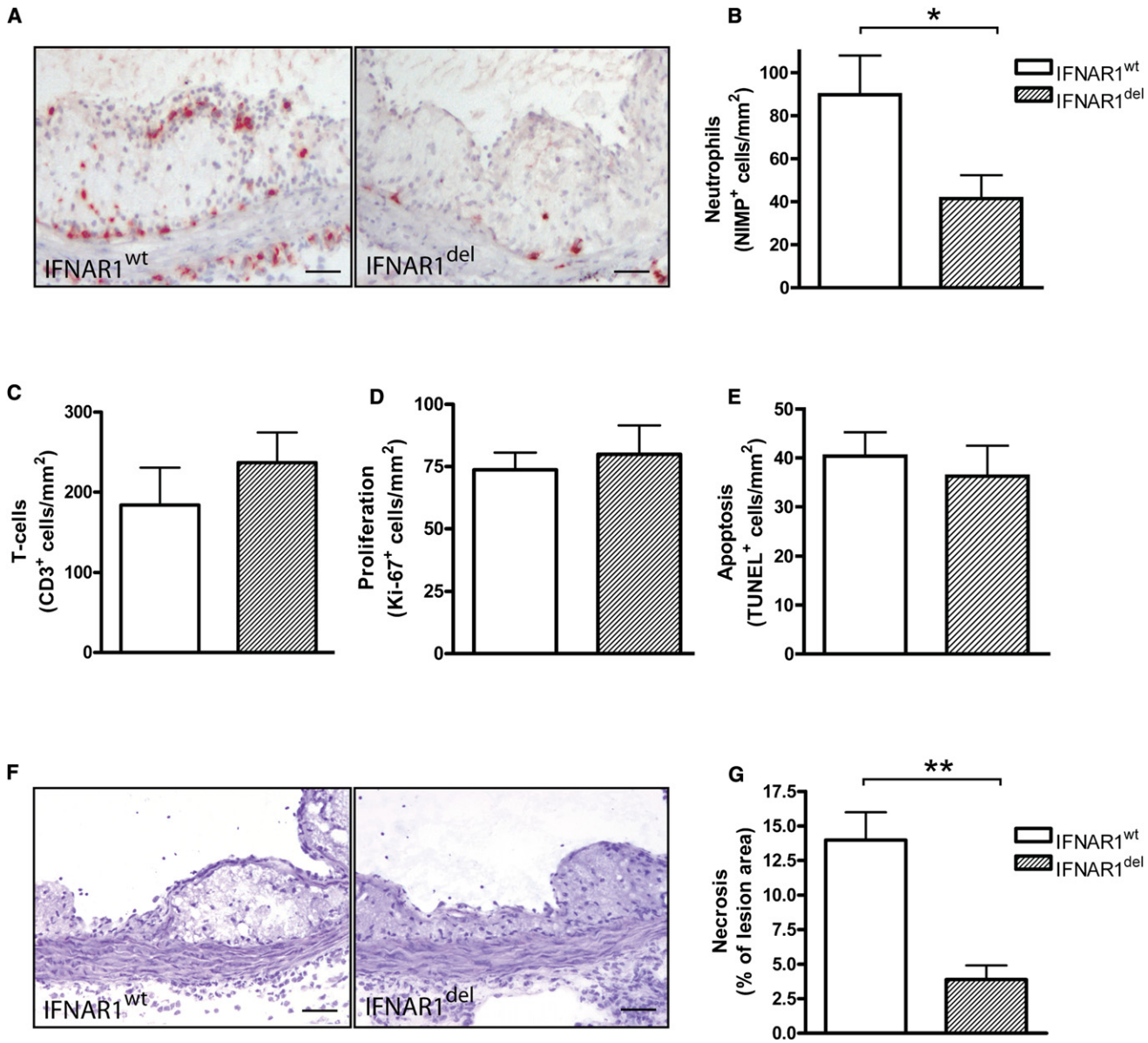

Figure 5. Absence of Myeloid IFNAR1 Influences the Phenotype of Atherosclerotic Lesions

(A) Representative NIMP stainings of lesions from IFNAR1^{WT} and IFNAR1^{del} mice. Scale bar, 50 μm.

(B) Neutrophil influx in lesions from IFNAR1^{WT} and IFNAR1^{del} mice. *$p < 0.05$.

(C–E) T cells, proliferation, and apoptosis in lesions from IFNAR1^{WT} and IFNAR1^{del} mice.

(F) Representative examples of necrosis in lesions from IFNAR1^{WT} and IFNAR1^{del} mice. Scale bar, 50 μm.

(G) Necrosis in lesions from IFNAR1^{WT} and IFNAR1^{del} mice. **$p < 0.001$. For all analyses, n = 19 and 15 for IFNAR1^{WT} and IFNAR1^{del} mice, respectively. Shown are mean ± SEM.

chemotactic factors. Zhang et al. describe that IFNβ administration attenuates lesion formation induced by carotid artery ligation in angiotensin II-infused *apoe*$^{-/-}$ mice (Zhang et al., 2008), which is in contrast to our findings. Thus, angiotensin acceleration of atherosclerosis depends on other inflammatory factors than our models. We now show, using two different models of atherosclerosis, that IFNβ treatment promotes atherosclerosis in high fat-fed mice. In both a collar-accelerated model (in the *apoe*$^{-/-}$ mice) and a solely hyperlipidemia-driven model (in the *ldlr*$^{-/-}$ mice), atherogenesis was enhanced by IFNβ without influencing plasma lipid parameters.

We found that IFNβ treatment of isolated primary macrophages induced IL-10. In addition, IFNβ treatment of mice led to increased levels of circulating IL-10, as is similarly observed in MS patients and mouse models for MS (Rudick et al., 1998). IL-10 is a classical anti-inflammatory cytokine, and the beneficial effects of IFNβ treatment in relapsing remitting MS patients is at least partly attributed to anti-inflammatory factors that it induces

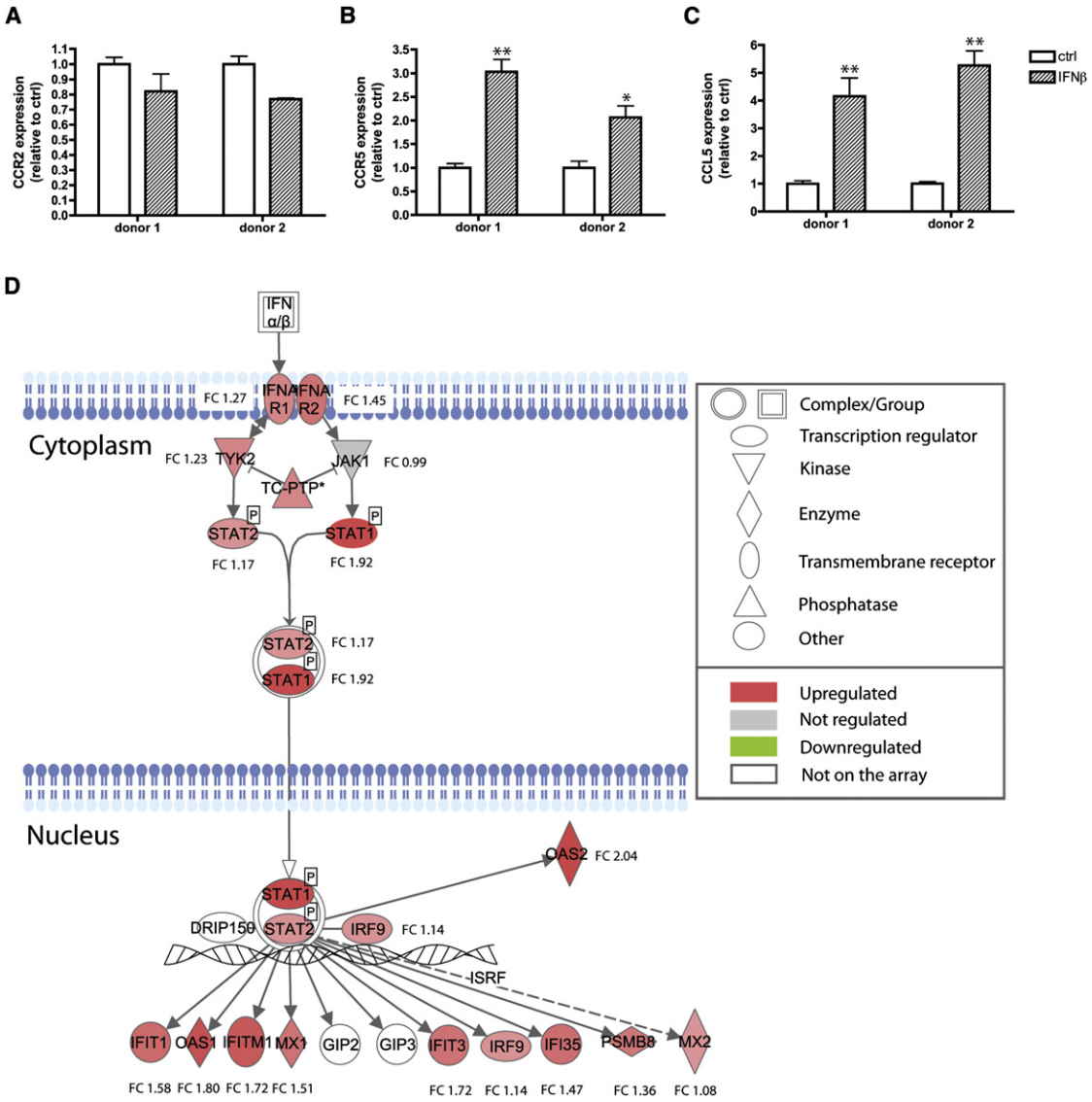

Figure 6. IFNβ Induces Chemotactic Factors in Human Primary Macrophages, and Type I IFN Signaling Is Upregulated in Ruptured Human Atherosclerotic Lesions

(A) CCR2 expression in macrophages from two independent donors after IFNβ treatment.

(B) CCR5 expression in macrophages from two independent donors after IFNβ treatment. *$p < 0.05$; **$p < 0.01$.

(C) CCL5 expression in macrophages from two independent donors after IFNβ treatment. **$p < 0.01$. Error bars indicate mean of triplicate wells ± SEM.

(D) Ingenuity Pathway Analysis of the differentially expressed genes in stable compared to ruptured carotid endarterectomy specimens. Red signals indicate upregulation and the pathway showed a strongly significant ($p = 2.36 \times 10^{-6}$; ratio 16/23 [0.696]) upregulation of type I IFN signaling. Indicated are the fold changes (FC) of the respective genes.

(Billiau, 2006). The induction of this anti-atherogenic (Mallat et al., 1999) cytokine contrasts with the atherosclerosis-promoting effect that we observe for IFNβ. However, we did find IFNβ to specifically elicit chemotactic factors with a reported proatherogenic function. CCL2 (MCP-1) and CCL5 (RANTES) and their receptors (CCR2 and CCR5, respectively) are important in regulating attraction of cells to atherosclerotic lesions and thereby control atherosclerosis development (Boring et al., 1998; Braunersreuther et al., 2007; Veillard et al., 2004). We found that especially CCL5 was strongly upregulated after IFNβ treatment of macrophages, coinciding with a modest induction of CCR5 and CCR2. CCL5 expression after IFNβ was reduced in macrophages lacking either IFNAR1 or STAT1, indicating that CCL5 is either a direct target of IFNAR1-STAT1 signaling or is regulated by interferon regulatory factors (IRFs) induced by STAT1 activation (Cremer et al., 2002; Melchjorsen et al., 2003; Taniguchi and Takaoka, 2001). We could also show that IFNβ increased static adhesion of macrophages to endothelial cells, again in an IFNAR1- and STAT1-dependent manner, which was abolished by macrophage *CCR5* deficiency, but not by deficiency for *CCR1*, the other major receptor for CCL5. *CCR2* deficiency had a modest effect on IFNβ-induced

macrophage adhesion. Furthermore, enhanced leukocyte arrest induced by IFNβ in *apoe*$^{-/-}$ mice could be completely blocked by cotreatment with the CCL5 receptor inhibitor Met-Rantes. In line with these findings, we found an induction of circulating CCL5 in IFNβ-treated mice. Moreover, plasma CCL5 analysis in a small number of mice from the IFNAR1 bone marrow transplantation showed a trend toward a reduction in IFNAR1^{del} transplanted *ldlr*$^{-/-}$ mice (95.0 ± 6.9 and 73.5 ± 5.2 pg/ml for IFNAR1^{WT} and IFNAR1^{del} mice; p = 0.07). Therefore, we think that, despite the induction of IL-10 by IFNβ, the atherosclerotic process is actually enhanced through the induction of especially the CCL5-CCR5 axis, thereby promoting attraction of leukocytes to lesions.

Type I interferons are massively produced by various cell types in response to viruses and other microbial stimuli through engagement of toll-like receptors such as TLR3, TLR4, and TLR9. In search of endogenous triggers that stimulate the inflammatory response accompanying atherosclerosis, it was recently demonstrated that cholesterol crystals can activate the NLRP3 inflammasome and thereby significantly contribute to atherogenesis (Duewell et al., 2010). Moreover, oxidized LDL was shown to activate the TRIF pathway by binding to a TLR4/6-CD36 complex, potentially leading to type I IFN production by macrophages in lesions (Stewart et al., 2010). Additional major in vivo sources of type I IFN are plasmacytoid dendritic cells (pDCs). Of interest, DCs and, more specifically, pDCs have been identified in human atherosclerotic lesions and have been associated with rupture-prone areas of the lesions (Bobryshev and Lord, 1995; Niessner et al., 2006; Yilmaz et al., 2004). Using data sets from stable and ruptured human endarterectomy segments, we now show that segments of ruptured plaque have an upregulation of the type I IFN signaling pathway when compared to stable segments. Thus, ruptured atherosclerotic lesions show induction of type I IFN signaling. This may consequently increase attraction of inflammatory cells, further contributing to matrix degradation and plaque destabilization.

Using conditional knockouts, we demonstrate that myeloid cells are important targets for endogenously produced type I IFN in atherosclerosis. Through induction of chemotactic factors, type I IFN signaling promotes macrophage adhesion to endothelial cells. As such, deletion of IFNAR1 in myeloid cells ultimately leads to reduced accumulation of macrophages in atherosclerotic lesions and thereby reduces atherosclerosis development. We determined deletion efficiency to be ~70% in our bone marrow cultures, indicating some remaining type I IFN signaling. In addition, it was previously shown that circulating monocytes from LysMCre-IFNAR1^{floxed} mice show severely hampered, but not fully absent, type I IFN signaling (Prinz et al., 2008). Thus, the phenotype that we observe is likely the result of reduced, but not completely absent, myeloid type I signaling. Whether this merely means an underestimation of the contribution of endogenous type I signaling in atherosclerosis or whether additional functions are therefore undetectable remains to be discovered.

In addition to affecting macrophages, myeloid *IFNAR1* deficiency also reduces accumulation of neutrophils in the lesions, which have recently been found to contribute to atherosclerosis (van Leeuwen et al., 2008; Zernecke et al., 2008). Disturbed macrophage CCL5 production may impair lesional recruitment of neutrophils, which use CCR1 to respond to CCL5 (Liehn et al., 2008). Alternatively, IFNAR1 deficiency may have unexplored direct effects on neutrophils influencing migration to inflammatory sites. However, in our treatment approach with *ldlr*$^{-/-}$ mice, neutrophil accumulation in the lesions was not affected (data not shown), indicating that IFNβ treatment does not affect neutrophils in these studies. In addition, although we clearly identify that myeloid IFNAR1 signaling highly controls lesion development, we cannot exclude that other targets of type I IFN, such as endothelial cells or fibroblasts, will contribute to the effects that we observe upon treatment of the mice with IFNβ.

We found that inhibition of endogenous myeloid type I IFN signaling reduces necrotic core formation and thus directly affects plaque stability. Reduced necrosis paralleled impaired progression of the lesions. However, upon analysis of a subset of lesions from both groups with similar size (84.5 ± 4.8 and 81.4 ± 4.2 × 1000 μm^2 for the IFNAR1^{WT} and IFNAR1^{del} groups) and severity (moderate lesions), we found that necrotic core formation was still reduced in the IFNAR1^{del} group (12.6% ± 2.4% and 4.0% ± 1.3% for the IFNAR1^{WT} and IFNAR1^{del} groups). Thus, inhibition of myeloid IFNAR1 function directly inhibits necrotic core formation independent of lesion size and severity and without any clear effects on endocytosis or phagocytosis. Depending on the experimental context, both pro- and anti-cell death functions have been attributed to type I IFN. The antitumor effects of type I IFN are at least partly attributed to the induction of cell death by type I IFN (Borden et al., 2007), in which IFNs and interferon-stimulated genes are both sensitizing tumor cells to immune cell-mediated cytotoxicity and augmenting lytic activity of immune cells. In line with this, several groups have reported that endogenous type I interferon signaling sensitizes cells to pathogen-induced cell death (O'Connell et al., 2004; Qiu et al., 2008) or apoptosis induced by serum deprivation (Wei et al., 2006). In addition, treatment of MS patients with IFNβ was shown to prime monocyte-derived macrophages for apoptotic cell death (Van Weyenbergh et al., 2001). We show that inhibition of myeloid type I IFN signaling reduces necrosis, but treatment of *ldlr*$^{-/-}$ mice with IFNβ did not enhance necrotic core formation. Thus, in atherogenesis, the necrosis-promoting effect of endogenous type I IFN is apparently already maximal and not further promoted by exogenously added IFNβ.

Our findings are supported by previous studies demonstrating that type I IFNs are important inducers of cellular migration. The attraction of inflammatory cells is an important feature of type I IFN action in fighting microbial infections (Borden et al., 2007). In addition, it was recently shown that type I IFNs are also essential mediators of TNF-induced lethal inflammatory shock by enhancing cell death and promoting white blood cell influx in tissues through induction of a set of chemokines (Huys et al., 2009). Moreover, in search of IFNβ-related adverse side effects in MS patients, several groups performed gene expression studies on peripheral blood mononuclear cells and demonstrated induction of chemotactic factors in response to IFNβ treatment (Satoh et al., 2006; Wandinger et al., 2001). In line with this, it was recently shown that a group of chemotactic genes, including CCL5 and CCL2, was among those most prominently upregulated in brain macrophages (microglia) treated with IFNβ (Prinz et al., 2008). We focused on the major chemokines and chemokine receptors with a demonstrated

role in atherogenesis and found that especially the CCL5-CCR5 axis may be very important in mediating the proatherogenic function of IFNβ.

In conclusion, we show that type I IFNs promote atherosclerosis development. Contrary to the antiviral effect of type I IFN acting on a multitude of cell types, we report that the specific interaction of endogenous type I IFN with myeloid cells enhances the recruitment of these cells to atherosclerotic lesions. This effect is in accordance with the type I IFN-induced macrophage adhesion mediated by upregulation of specific chemokines and their receptors, mainly involving the CCL5-CCR5 axis. Furthermore, we present plaque destabilizing necrotic core formation as a direct consequence of myeloid type I IFN signaling. Collectively, these data raise the demand for further detailed analyses of cardiovascular risk in patients treated with type I IFN and may even imply that caution should be taken in using type I IFN as a therapeutic option. In contrast, targeting of type I IFN signaling may be an attractive target for prevention and treatment of atherosclerosis.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice and *ldlr*$^{-/-}$ mice on a C57BL/6 background were obtained from Jackson Laboratory (Bar Harbor, ME), and *apoe*$^{-/-}$ mice on a C57BL/6 background were obtained from Iffa Credo (Lyon, France). *IFNAR1*$^{fl/fl}$, *LysM-Cre-IFNAR1*$^{fl/fl}$, *apoe*$^{-/-}$, *apoe*$^{-/-}$*CCR1*$^{-/-}$, *apoe*$^{-/-}$*CCR2*$^{-/-}$, *apoe*$^{-/-}$*CCR5*$^{-/-}$, and *STAT1*$^{-/-}$ mice were all on a C57BL/6 background and have been described before (Durbin et al., 1996; Prinz et al., 2008; Schober et al., 2004; Zernecke et al., 2006). All animal experiments were approved by the Committee for Animal Welfare of the Maastricht University or complied with German animal protection law.

Interferons

Recombinant murine IFNα or IFNβ were obtained from Hycult Biotech (Uden, The Netherlands). Human IFNβ was obtained from Peprotech (Rocky Hill, NJ).

In Vitro Murine Macrophage Culture

Bone marrow cells were isolated from femurs and tibiae of wild-type mice (C57BL/6), *IFNAR1*$^{fl/fl}$, *LysMCre-IFNAR1*$^{fl/fl}$, *apoe*$^{-/-}$, *apoe*$^{-/-}$*CCR1*$^{-/-}$, *apoe*$^{-/-}$*CCR2*$^{-/-}$, *apoe*$^{-/-}$*CCR5*$^{-/-}$, or *STAT1*$^{-/-}$ mice. Cells were cultured in RPMI-1640 (GIBCO Invitrogen, Breda, The Netherlands) with 10% heat-inactivated fetal calf serum (Bodinco B.V., Alkmaar, The Netherlands), penicillin (100 U/ml), streptomycin (100 ug/ml), and L-glutamine 2 mM (all GIBCO Invitrogen, Breda, The Netherlands) (R10) supplemented with 15% L929-conditioned medium (LCM) for 8–9 days to generate bone marrow-derived macrophages (BMM), as described previously (Kanters et al., 2003). Cells were treated for 24 hr with 100U/ml IFNα or IFNβ.

In Vitro Human Macrophage Culture

Human mononuclear cells were obtained by apheresis of 2× the blood volume from healthy volunteers using a Cobe Spectra (CaridianBCT Europe, Zaventhem, Belgium). Monocytes were subsequently enriched by counter flow centrifugation using the Elutra Cell Separation System (CaridianBCT) and were cultured in R10 supplemented with 5 ng/ml M-CSF (Peprotech) for 8 days to generate human macrophages. Cells were treated for 24 hr with 100U/ml human IFNβ.

Gene Expression

RNA was isolated from BMM or human macrophages with the High Pure RNA Isolation Kit (Roche, Basel, Switzerland). 500 ng total RNA was reverse transcribed using the iScript cDNA Synthesis Kit (BioRad, Veenendaal, The Netherlands). Quantitative PCR (Q-PCR) was performed using 10 ng cDNA, 300 nM of each primer, and IQ SYBR Green Supermix (BioRad) in a total volume of 20 μl. Deletion efficiency in BMM of the LysMCre-IFNAR1$^{fl/fl}$ mice was measured by Q-PCR on 25 ng DNA as described before (Kanters et al., 2003), using primers specifically detecting the floxed and not the deleted or wild-type IFNAR1 allele. All gene expression levels were corrected for cyclophilin A as housekeeping gene, and primer sequences are available upon request.

In Vitro oxLDL and Bead Uptake

BMM untreated or treated for 24 hr with 100U/ml IFNβ were incubated for 3 hr in Optimem-1 with fluorescently labeled latex beads or 25 μg/ml DiI-labeled oxLDL and were generated through copper oxidation, as described previously (Kanters et al., 2003). Uptake was assessed by flow cytometry after residual beads or oxLDL were washed away.

Flow Cytometry

BMM (± 100U/ml IFNβ for 24 hr) were stained with antibodies against VLA-4 (PE labeled, Cedarlane, Burlington, Ontario, Canada), Mac1, LFA-1, PSGL1 (all PE labeled, BD PharMingen, Erembodegem, Belgium), CCR2 (Epitomics, Burlingame, CA), or CCR5 (biotinylated, BD PharMingen), and their mean fluorescence was measured by FACS analysis. For the CCR2 staining, cells were permeabilized using Cytoperm Plus Permeabilization Buffer and Perm/Wash Buffer (both BD PharMingen), and CCR2 antibody was detected using anti-rabbit IgG FITC (Sigma-Aldrich). CCR5 antibody was detected with APC-conjugated streptavidin (BD PharMingen).

ELISA

Murine CCL5 secretion from BMM (± 100 U/ml IFNβ for 24 hr) was measured by ELISA using anti-mouse CCL5 (R&D Systems, Abigndon, UK) as coating antibody and biotinylated anti-mouse CCL5 (R&D Systems) as detection antibody with mouse CCL5 (Peprotech) as standard. Human CCL5 secretion in the supernatants from human macrophages (± 100U/ml IFNβ for 24 hr) was measured by commercial ELISA (PBL Interferon Source, New Brunswick, NJ). Absorbance was measured at 450 nm using a microtiterplate reader (BioRad, Hercules, CA).

In Vitro Adhesion Assay

A confluent monolayer of bEND5 endothelial cells was grown in fluorescence 96-well microplates (Greiner Bio-one, Frickenhausen, Germany). Triplicate wells were incubated for 30 min with 10^5 BMM (pretreated ± 100U/ml IFNβ for 24 hr) that had been fluorescently labeled with a PKH dye according to the manufacturer's instructions (Sigma Aldrich, Zwijndrecht, The Netherlands). Subsequently, the wells were washed three times with R10, and adherent cells were measured by fluorometry in a Synergy HT microtiter plate reader (BioTek, Bad Friedrichshall, Germany) at an excitation of 485 nm and an emission of 520 nm.

In Vivo Leukocyte Adhesion

Apoe$^{-/-}$ mice were put on a high-fat diet (0.15% cholesterol, 21% fat, Altromin) for 6 weeks and during the last 4 days were daily injected subcutaneously with either saline or IFNβ (5000 U). Four mice receiving saline injections and five receiving IFNβ injections were cotreated with an intravenous Met-RANTES (50 μg/mouse) injection 30 min before the experiment. After sedation (intraperitoneal Ketamine/Xylazin) and intravenous rhodamin injection, the left carotid artery was exposed and three high-power fields (hpf) near the carotid bifurcation were visualized by epifluorescence microscopy (Zeiss Axiotech, 20× water immersion objective) as described before (Bernhagen et al., 2007). Short movies were recorded for each hpf, in which the cells attaching to the vessel wall were counted by eye.

Collar-Induced Atherosclerosis

Seventeen-week-old male *apoe*$^{-/-}$ mice (n = 21) were operated after 3 weeks of high-fat diet (0.25% cholesterol, 16% fat) to introduce a 2 mm long nonconstrictive silastic tube around both carotid arteries, as described before (von der Thüsen et al., 2001). During the 24 days postoperation, the high-fat diet was continued and the mice were treated by daily subcutaneous injection of either IFNβ (5000 U) or saline. Upon sacrifice, the right carotid artery was isolated and embedded in paraffin. Sections of 5 μm were made, and after every 100 μm, sections were stained with hematoxilin/eosin for lesion area analysis.

IFNβ Treatment in *ldlr*$^{-/-}$ Mice

Twelve-week-old male *ldlr*$^{-/-}$ mice were fed a high-fat diet (0.15% cholesterol, 16% fat, Arie Blok, The Netherlands) for 6 weeks in order to induce early atherogenesis. At this point, daily subcutaneous injections of either IFNβ (5000 U) or saline were started and continued for 3 weeks while the high-fat diet was continued. Upon sacrifice, the heart and aorta were taken out. The hearts were cut perpendicular to the heart axis just below the atrial tips. Tissue was frozen in tissue-tec (Shandon, Veldhoven, The Netherlands) and cut into sections of 7 μm as described before (Kanters et al., 2003). Serial cross-sections from every 42 μm were stained with toluidin blue. All lesion areas were quantified using Adobe Photoshop software. The aortas were snap frozen and RNA was isolated using the RNeasy Mini Kit (QIAGEN, Venlo, The Netherlands), including DNase treatment. Gene expression was assessed by Q-PCR, as described above.

Bone Marrow Transplantation

One week before transplantation, female *ldlr*$^{-/-}$ mice were housed in filter-top cages and provided with acidified water containing neomycin (100mg/l; GIBCO, Breda, The Netherlands) and polymyxin B sulfate (6×10^4 U/l; GIBCO). The animals received 2 × 6Gy total body irradiation on two consecutive days. On the second day, bone marrow was isolated from 6 LysMCre-IFNAR1$^{fl/fl}$ mice (IFNAR1^{del}) and 6 IFNAR1$^{fl/fl}$ littermates (IFNAR1^{WT}), and 10^7 cells/mouse were injected intravenously to rescue the hematopoietic system of the irradiated mice. Four weeks after the transplantation, mice were fed a high-fat diet (0.15% cholesterol, 16% fat, Arie Blok, The Netherlands) for 11 weeks. After sacrifice, the hearts from the bone marrow-transplanted mice were taken out, and lesion size in the aortic root was measured as described above. The lesions were also typed according to severity as early, moderate, and advanced, as described before (Kanters et al., 2003).

Mouse Blood Parameters

At several time points during all in vivo atherosclerosis experiments, blood was drawn from the mice. Plasma lipid levels were monitored enzymatically (Sigma Aldrich, Zwijndrecht, The Netherlands), and plasma cytokine levels were measured by flow cytometry using a Cytometric Bead Array kit (BD-PharMingen, San Diego). Plasma CCL5 levels were analyzed by ELISA according to manufacturer's instructions (R&D systems). For the bone marrow-transplanted mice, leukocytes were counted using a Coulter counter, and blood cell distribution was quantified by flow cytometry after antibody staining with either Mac1-PE and Gr1-FITC for macrophages and granulocytes or 6B2-PE and KT3-FITC for B and T cells (BD-PharMingen, Erembodegem, Belgium).

Immunohistochemical Staining

Lesions from the aortic root were fixed in acetone and incubated with antibodies against macrophages (MOMA-2, a gift from G. Kraal), granulocytes (NIMP, directed against Ly6G, a gift from P. Heeringa), T cells (KT3, directed against CD3, a gift from G. Kraal), and proliferating cells (Ki-67, Dako, Glostrup, Denmark). Apoptotic cells in the plaques were stained by the TUNEL staining (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Necrotic areas were analyzed on toluidin blue-stained sections and identified by the presence of pyknosis, karyorrhexis, or complete absence of nuclei.

Human Plaque Transcriptomics

Microarray analysis was performed on RNA isolated from 44 (22 stable and 22 ruptured) human carotid plaque specimens using Illumina Human Sentrix-8 V2.0 BeadChip technology to detect differential expression. For pathway analysis, we used the Ingenuity Pathway Analysis (Ingenuity Systems, http://www.ingenuity.com) system. Further details are given in the Supplemental Experimental Procedures.

Statistical Analysis

The statistical analyses were performed using Graphpad Prism (Graphpad Software). Differences between two groups were evaluated using a t test unless stated otherwise. Values are represented as mean ± SEM. A p value of less than .05 was considered to be statistically significant. All mouse data passed a normality test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at doi:10.1016/j.cmet.2010.06.008.

ACKNOWLEDGMENTS

This work was supported by the Netherlands Heart Foundation (grant no. 2005B175), Netherlands Organization for Scientific Research (ZonMW VIDI 917-66-329 to M.P.J.d.W. and VIDI 016-086-326 to E.L.), the European Union (Marie-Curie MEST-CT-2005-020706/CADRE2), the Humboldt foundation (Sofja Kovalevskaja grant to E.L.), and the European Vascular Genomics Network (EVGN). M.P.J.d.W. and E.L. are established investigators of the Netherlands Heart Foundation (2007T067 and 2009T034). We thank Lauran Stöger for critically reading the manuscript and Drs. Mathieu and Gysemans for providing the STAT1-deficient mice. We thank Chantal Pöttgens and Birgit Senden-Gijsbers for technical support.

Received: September 3, 2009
Revised: January 5, 2010
Accepted: June 2, 2010
Published: August 3, 2010

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