Effects of Long-Term Type I Interferon on the Arterial Wall and Smooth Muscle Progenitor Cells Differentiation

Yanpeng Diao,* Rajesh Mohandas,* Pui Lee, Zhiyu Liu, Larysa Sautina, Wei Mu, Shiyu Li, Xuerong Wen, Byron Croker, Mark S. Segal

Objective—Patients with systemic lupus erythematosus are at risk for premature atherosclerosis and half of the patients with systemic lupus erythematosus have elevated type I interferon (IFN-I) levels. We hypothesized that IFN-I would induce premature atherosclerosis by increasing the number of smooth muscle progenitor cells (SMPC) in the bloodstream and promoting atherosclerotic lesions within the vasculature.

Approach and Results—SMPC isolated from wild-type and IFN receptor knockout animals were cultured in medium±IFN-I. In vivo, we used electroporation to generate stable IFN-I expression for as long as 4 months. The number of SMPC was determined in mice that expressed IFN-I and in control mice and sections from the bifurcation of the abdominal aorta were analyzed 3 months after electroporation of an IFN-I expression plasmid or a control plasmid. Adding IFN-I to the media increased the number of cultured wild-type SMPC and increased mRNA for SM22, but had no effect on SMPC isolated from IFN receptor knockout mice. Our in vivo results demonstrated a positive relationship between the preatherosclerotic-like lesions and endothelial damage. Although, there were no significant differences in smooth muscle cell density or thickness of the medial layer between groups, the IFN-I–expressing mice had a significant increase in preatherosclerotic-like lesions and immature smooth muscle cells, cells that expressed CD34 and smooth muscle α-actin; but lacked smooth muscle myosin heavy chain.

Conclusions—IFN-I seems to enhance SMPC number in vitro. In vivo IFN-I expression may maintain SMPC in an immature state. These immature smooth muscle cells could give rise to macrophages and eventually foam cells.

Key Words: actin □ atherosclerosis □ foam cells □ interferons □ macrophages
source of SMPC. Adequate understanding the role of SMPC during the atherosclerotic progress is critical in developing new avenues for potential therapy.

In this study, we tested the hypothesis that IFN-I would increase the number of SMPC and maintain the SMPC in an immature phenotype both in vitro and in vivo. In vitro, to test our hypothesis, we cultured SMPC in different culture media. In vivo, we developed a novel, mouse model of chronic expression of IFN-I. In our model, electroporation of a plasmid encoding IFN-I into the posterior thigh muscle of a mouse led to IFN-I expression for >3 months. Increased SMPC colony numbers were observed from peripheral blood of a mouse led to IFN-I expression for >3 months. Increased SMPC colony numbers when compared with vehicle (1.80±0.22 versus 1.00±0.13

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

We sought to study the in vitro effect of IFN-I on cultured SMPC colony number. After 14 days in culture, we routinely observed the appearance of spindle-shaped cells, SMPC colonies (Figure 1A). When peripheral blood mononuclear cells, isolated from 4-month-old wild-type (WT) mice, were exposed to IFN-I during the culturing of the colonies, the number of SMPC colonies significantly increased by 54% when compared with colonies cultured in vehicle (1.54±0.16 versus 1.00±0.10 P=0.01). However, when peripheral blood mononuclear cells, isolated from IFN receptor knockout animals, were exposed to IFN-I, there was no difference in the number of colonies when compared with vehicle (P=0.33; Figure 1B). Similar results were observed when the blood of older, WT mice (10 months old) were treated with IFN-I or vehicle (1.80±0.22 versus 1.00±0.13 P=0.006).

To demonstrate the long-term expression activity of the IFN-I plasmid after electroporation, we measured surface expression of the interferon-inducible protein stem cell antigen-1 (also known as Ly6A) on B lymphocytes. Compared with control plasmid (β-galactosidase), electroporation of a plasmid encoding IFN-α led to a significant increase in stem cell antigen-1 levels as early as 1 day after injection (Figure 2A). Marked elevation of stem cell antigen-1 persisted for at least 4 months after electroporation (Figure 2B). Meanwhile, X-gal staining indicating the β-galactosidase plasmid was transduced successfully can be seen in the muscle at the time of euthanization (Figure 2C).

To evaluate the effect of IFN-I on SMPC numbers in vivo, we cultured peripheral blood mononuclear cells for SMPC colonies from WT animals 3 weeks after electroporation with either an IFN-I or the β-galactosidase, control plasmid. The colony number was significantly higher in the peripheral blood mononuclear cells isolated from mice electroporated with the IFN-I plasmid when compared with the control plasmid (14.4±2.3 versus 5.3±1.7, P=0.007; Figure 2D).

Twenty 7-month-old mice were electroporated with the IFN-I or control plasmid and followed for 3 months. One mouse died from uncertain cause (no obvious abnormality was observed at autopsy) in the group electroporated with IFN-I plasmid, and no mice died in the control plasmid group. In mice injected with the control plasmid, only 45% had any thickening of the abdominal, aortic bifurcation (Figure 3A and 3B). However, 78% of mice in the IFN-I plasmid–injected group had thickening of the abdominal aortic bifurcation, and the size of the lesions were significantly larger than the mice injected with the control plasmid (230.4±69.0 μm² versus 46.4±17.5 μm², P=0.011; Figure 3C). More interestingly, preatherosclerotic-like lesions only were found in the IFN-I plasmid–injected mice, although the total length of most lesions was less than 500 μm. These preatherosclerotic-like lesions were located at the original internal elastic lamina and they were composed of several layers of SMC separated by a duplicated elastica (Figure 3D–3G). However, there was no significant difference in media thickness (13.7±0.8 μm versus 11.8±0.6 μm, P=0.104) and cell

Nonstandard Abbreviations and Acronyms

<table>
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<th>Acronym</th>
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<tr>
<td>BM</td>
<td>bone marrow</td>
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<tr>
<td>IFN-I</td>
<td>type I interferon</td>
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<td>IFNR-KO</td>
<td>interferon receptor knockout</td>
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<td>MHC</td>
<td>myosin heavy chain</td>
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<td>SLE</td>
<td>systemic lupus erythematosus</td>
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<td>SMC</td>
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<td>SM-MHC</td>
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<td>SMPC</td>
<td>smooth muscle progenitor cells</td>
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<td>TGF-β</td>
<td>transforming growth factor-β</td>
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<td>WT</td>
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Arteriosclerosis, Thrombosis, and Vascular Biology

First Published Online

February 2016
density (342.3±34.5/0.01 mm$^2$ versus 288.3±16.3/0.01 mm$^2$, $P=0.193$) between the IFN-I plasmid–injected mice and the control mice.

Within the lesions found both in the IFN-I and control plasmid–treated animals, the majority of the cells were mature SMC and expressed SM-$\alpha$-actin and SM-MHC. Importantly, cells expressing both CD34 and SM-$\alpha$-actin positive, but not SM-MHC, were detected only in the IFN-I–treated animals (Figure 4A–4E). Expression of CD34 and lack of expression of SM-MHC suggests that these cells are more immature. In

Figure 1. The effect of type I interferon (IFN-I) in vitro treatment of peripheral blood mononuclear cells isolated from wild-type (WT) and interferon receptor knockout (IFNR-KO) mice on smooth muscle progenitor cells (SMPC) colony numbers. A, Light microscopy image of a typical WT SMPC colony with spindle-shaped cells after treatment with IFN-I (magnification: ×100). B, The number of SMPC colonies is significantly increased after peripheral blood mononuclear cells isolated from WT animals are treated with IFN-I (black bars) when compared with vehicle (white bars; $^*P=0.01$), whereas there is no effect of IFN-I on the number of SMPC colonies derived from peripheral blood mononuclear cells isolated from IFNR-KO animals ($P=0.33$). The number of IFN-I–treated SMPC colonies were expressed as a fold change of vehicle-treated cells. C–H, Dual immunohistochemical staining of smooth muscle-$\alpha$-actin (red, C and F) and smooth muscle myosin heavy chain (green, D and G) in IFN-I–treated (top) and control (bottom) SMPC after 14 days of culture. E and H, Merged images of C and D and F and G, respectively. Blue is 4′,6-diamidino-2-phenylindole (DAPI) nuclear stain. Bar, 100 μm. CFU indicates colony-forming units; and MHC, myosin heavy chain.

Figure 2. Evaluation of transduction of control β-galactosidase plasmid, pcDNA-LACz, and type I interferon (IFN-I) plasmid, pcDNA-IFN$\alpha$. The level of stem cell antigen-1 (Sca-1) as determined by flow cytometry significantly increased within B cells (A) and could be detected as long as 4 months after electroporation (B). X-gal staining indicates β-galactosidase–expressing gastrocnemius/soleus muscle after electroporation of pcDNA-lacZ (C). The smooth muscle progenitor cells colony-forming units (CFU) was significantly higher in the peripheral blood mononuclear cells isolated from mice electroporated with the IFN-I plasmid than cells isolated from mice electroporated with control plasmid (D; 5.6±1.8 vs 13.3±2.3 $P=0.007$).
the control plasmid–treated animals, no CD34-positive cells were detected in the lesions (Figure 4F–4I).

Meanwhile, interrupted CD31 staining was observed within 77.8% IFN-I–treated mice and 37.5% control mice (Figure 4J and 4K). In addition, the percentage of interrupted endothelium in IFN-I–treated animals was significantly greater than in control animals (1.64±0.38% versus 0.38±0.22%, P=0.015). Although Pearson correlation coefficient and subsequent significance testing indicated a relationship between endothelial cell damage and preatherosclerotic-like lesion in

![Image](image1.png)

**Figure 3.** Increased frequency and size of preatherosclerotic-like lesions in mice electroporated with type I interferon (IFN-I) gastrocnemius muscle were transduced with an IFN-I-expressing plasmid or control plasmid were examined by hematoxylin and eosin (H&E) histological analysis 3 months after transduction. In mice injected with the control plasmid, there was only minor thickening of the vessel wall (A and B), indicated by the black arrow within A. However, the size of intima thickening and preatherosclerotic-like lesions were significantly increased in mice injected with IFN-I plasmid (C). Low (×20, D and F) and high (×40, E and G) magnifications of the H&E stain of cross sections of the aortic bifurcation from mice injected with IFN-I. Intima thickening (D and E) and preatherosclerotic-like lesions (F and G), indicated by black boxes in D and F, respectively, were significantly larger in IFN-I plasmid–injected mice (D–G) when compared with mice injected with control plasmid (A and B). P=0.011. Bar, 50 μm (A, D, and F) and 10 μm (B, E, and G).

![Image](image2.png)

**Figure 4.** The effect of prolonged type I interferon (IFN-I) expression on the vasculature. Immunohistochemical stain (with antismooth muscle-α-actin [red], anti-CD34 [green], and antisMOOTH muscle myosin heavy chain [purple]) of an adjacent section to Figure 3B was examined under low (×20, A) and high (×63, B–E) magnification by confocal analysis. A yellow, immature smooth muscle cell (SMC), a cell that expresses smooth muscle-α-actin (red) and CD34 (green) only, but does not express smooth muscle myosin heavy chain (purple), is indicated by the red arrow in E. Meanwhile, there was no CD34 cells detected in animals injected with control plasmid though there was minimal, intimal thickening (F–I). Within the intimal thickening, seen in animals injected with control plasmid, SMCs expressed both smooth muscle-α-actin (red, F) and smooth muscle myosin heavy chain (purple, H), but, no CD34 cells were detected (G). The merged image of F–G is shown in I (×63). Although endothelial damage (interrupted CD31 [red] stain) was observed in animals expressing IFN-I and control animals, indicated by an orange arrowhead in J and K, the percentage of endothelium damage was significantly worse in IFN-I–treated animals (1.64±0.38% vs 0.38±0.22%, P=0.015). Bar, 25 μm (E and I) and 100 μm (A and J–K).
all mice, there was no significant difference between IFN-I–treated animals and control animals. There was also no significant lesions in IFN receptor knockout animals that underwent electroporation with either IFN-I or control plasmid (Figure I in the online-only Data Supplement).

Unexpectedly, immunohistochemical staining did not detect any signal for F4/80, CD45, CD3, and tenasin-C within any of the preatherosclerotic-like lesions (data not shown).

Discussion

After the identification of BM progenitor angiogenic cells in human peripheral blood in 1997, the first report of the ability of circulating progenitor cells to differentiate into SMC was published in 2002. The role of circulating SMPC on vascular diseases, such as atherosclerosis and neointima formation after injury still remains controversial. Although BM-derived SMPC have been detected in human atherosclerotic tissue and animal models of vascular disease, it is still unknown whether the SMPC serve to accelerate or decelerate the development of vascular lesions.

Elevated serum levels of IFN-I in patients with SLE were first reported over 3 decades ago, and SLE is associated with a significant increase in atherosclerotic, cardiovascular complications. Several studies suggest that BM progenitor angiogenic cells become dysfunctional on exposure to IFN-I, which may exacerbate the development of atherosclerosis in SLE. However, the effect of IFN-I on SMPC and its subsequent effect on premature atherosclerosis in SLE is unknown.

In our study, the numbers of SMPC colonies were significantly increased after in vitro treatment of peripheral blood mononuclear cells with IFN-I. In addition, in vitro IFN-I treatment decreased the expression ratio of SM-MHC/SMα-actin, perhaps via the upregulation of SMα-actin mRNA. SM22 is one of the earliest markers of differentiated SMC, and in our study was surprisingly upregulated after treatment with IFN-I. Furthermore, the level of calponin was downregulated, which suggests that SMPC might be maintained in a more immature state and possess a higher ability to proliferate. However, calponin and SM-MHC were downregulated. This suggests that IFN-I induces a different expression pattern in immature SMC when compared with mature SMC.

IFN-I is a superfamil and has dozens of subtypes. However, all of the subtypes do not fully diverge and they bind to the same receptor. Interestingly, some IFN-I subtypes (IFN-α4, IFN-α11, IFN-α12, IFN-β, and limitin) demonstrate a higher biological activity levels when compared with others, whereas IFN-α7 and IFN-α10 exhibit a lower activity. We chose IFN-α5 in our study as an intermediary subtype and speculated the other subtypes might possess a similar capability. The exact molecular mechanism for the increased SMPC colony formation by IFN-I is still under investigation.

However, a recent study provided mechanistic evidence that IFN-I, via the IFN-I receptor, upregulates the production of endothelin-1. Endothelin-1 has multiple and complex effects on SMC and the development of atherosclerosis. IFN-I also has been shown to increase the expression of chemotactic receptors and ligands (CCR5 and CCL5) on BM macrophages.

Eccentric lesions of intimal thickening are an early lesion in the pathogenesis of atherosclerosis. Although intimal thickening is observed in primate models, it is difficult to induce them in commonly used strains of WT rodent models. Only 8%−3% of the aortas of WT mice have lesions even after 9 months on a Western diet. In this study, we demonstrated that after 3 months exposure to IFN-I on regular mice chow, preatherosclerotic-like lesions were clearly visible at atherosclerosis-prone sites within the vasculature, like the bifurcation of the abdominal aorta. Compared with 129sv genetic background mice, C57BL mice are more resistant to the formation of atherosclerosis lesions and the aortic bifurcation is the anatomical location that is less sensitive to atherosclerosis development when compared with the aortic root/arch.

IFN-I is likely to produce more aggressive lesions in locations that are more sensitive to develop atherosclerosis. A recent report demonstrated that IFN-I modulates atherosclerosis lesion progression in lupus-prone and dual knockout of apo-lipoprotein E and IFN-I receptor murine models on a Western diet. Our model provides further insight into the relationship of elevated IFN-I levels and atherosclerosis even in WT animals fed normal chow, particularly, in female animals because lupus is more common in women.

Macrophage infiltration is thought to be another critical step in the development of classic atherosclerosis. However, we could not detect any F4/80 and CD45 cells within the preatherosclerotic-like lesions. Interestingly, we found cells expressing both SMα-actin and CD34, but not SM-MHC. The literature suggests these might be SMC-like macrophages or macrophage-like SMCs within atherosclerotic lesions. Unfortunately, there is no specific marker to precisely differentiate an immature SMC from a macrophage. Our previous study demonstrated that IFN-I modulated the recruitment of monocytes and contributed to chronic inflammation in atherosclerosis, which has been replicated. These monocytes/macrophage may be able to mature into SMC-like macrophages or macrophage-like SMCs and contribute to the
development of atherosclerosis, particularly, in lupus patients with elevated levels of IFN-I.

Because of the technical limitations, we were unable to follow >4 labels within a single-tissue section and the size of lesions limited sufficient slices for further immunohistochemical analysis. We hypothesize that IFN-I may promote SMPC differentiation and in the absence of other atherosclerosis-promoting factors, these SMPC, under the influence of IFN-I, maintain a progenitor cell state. However, if these SMPC are exposed to other atherosclerosis-promoting factors, such as a Western diet or an apolipoprotein E disturbance, these SMPC may have the ability to differentiate along a macrophage lineage (and subsequent foam cells), as occurred in apolipoprotein E mice exposed to IFN-I.36 Further study in atherosclerosis-prone animals, like the apolipoprotein E knockout mice, would allow the development of larger lesions, which would permit a more detailed analysis of the cellular components of the lesions.

Our study also raises the question as to the source of the cells expressing SM-α-actin and CD34 within the atherosclerotic-like lesions. These cells may be derived from dedifferentiated cells from the vasculature or may be derived from circulating SMPC; these circulating cells can penetrate the vessel wall at sites of endothelial injury at atherosclerosis-prone areas. Interestingly, Tang et al39 reported that there were SM-α-actin-positive only cells in the vessel media, which might contribute to the development of atherosclerosis. Hu et al also demonstrated that adventitia origin of progenitor cells might play an important role in atherogenesis. Although the media thickness and cell density were not significantly changed after preliminary analysis in our current study, we could not rule out the potential effect of IFN-I on local SMPC from the vessel wall (particularly, after the endothelium was damaged because of IFN-I) and their contribution to the development of atherosclerosis. Further studies are needed to differentiate between these 2 possibilities, like within BM chimeric animal. Whatever the source, a recent study demonstrated that targeting SM-α-actin and CD34-expressing cells could inhibit intimal hyperplasia and promote regenerative repair of the artery.41 Thus, targeting these SM-α-actin and CD34-positive cells may be a potential treatment to prevent atherosclerosis, this may be especially relevant in patients with lupus with elevated levels of IFN-I.

In our study, control animals had minor levels of intima thickening and damage to the integrity of endothelium. We think that this is an age-related lesion because the animals were 10 months old when they were euthanized. However, significantly larger intima thickening and atherosclerotic-like lesions were observed in IFN-I–treated animals accompanied by more severe damage of the endothelium. Interestingly, further Pearson correlation coefficients analysis indicated that the relationship between endothelium damage and atherosclerosis-like lesion was not significantly different in IFN-I–treated animals and control animals, although there was positive relationship between the atherosclerotic-like lesions and endothelium damage in all mice. It suggests that if we consider that IFN-I essentially accelerated natural occurring age-related atherosclerosis, under specific circumstances, atherosclerotic-like lesion can occur before overt endothelial injury or the repair ability of endothelial progenitor cells still could compensate the damage at early stage of the development of IFN-I–related atherosclerosis. Our findings with regard to the damage of endothelial cells because of IFN-I was in accordance with our10 and others42,43 previous reports.

Other key components in the development of atherosclerosis are T cells and tenasin-C.44,45 None of which we detected in the IFN-I–induced preatherosclerotic-like lesions. The lack of these elements suggests that these lesions might be early, stage in the development of lupus-related atherosclerosis, before the stage when T cells are recruited or tenasin-C is expressed.

In summary, we have examined the effect of IFN-I exposure on SMPC in vivo and in vitro. Using our novel model, we describe the effect of IFN-I on the development of early stage atherosclerotic-like lesion at atherosclerosis-prone sites within the WT animal vasculature. Although circulating SMPC was considered a protective role in atherogenesis,46 our data suggest that IFN-I may prevent the full maturation of SMPC into smooth muscle cells. Furthermore, this immature state may be more prone to differentiation along the macrophage line and results in a less matured SMC within fibrous cap. Both could lead to the development of a plaque that is more likely to undergo rupture. Our data in combination with other published studies suggest a complex interplay between IFN-I and other atherosclerotic factors in SMC maturation, which contributes to the accelerated atherosclerosis in patients with SLE.

Acknowledgments

We thank Dr Dong-Er Zhang for the IFN receptor knockout animals. We thank the Molecular Pathology Core at the UF for performing histological stain.

Sources of Funding

This work was partially supported by a grant from the Alliance of Lupus Research (Dr Segal) and Division of Nephrology, Hypertension, and Renal Transplantation Gatorade Research Funds.

Disclosures

None.

References


To investigate whether the high level of type I interferon, expressed in some lupus patients, is a risk factor in the development of atherosclerosis, we developed a mice model that leads to chronic, long-term expression of type I interferon. Our study, using this mice model suggests that type I interferon promotes impairment of endothelial cells and an increase in the number of immature smooth muscle cells. These immature smooth muscle cells may differentiate into macrophages and foam cells eventually under the influence of atherosclerosis-promoting factors. Our study suggests that type I interferon may be a clinical target to decrease the incidence of atherosclerosis in some lupus patients.

Significance
Effects of Long-Term Type I Interferon on the Arterial Wall and Smooth Muscle Progenitor Cells Differentiation
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Arterioscler Thromb Vasc Biol. published online December 3, 2015;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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MATERIALS AND METHODS

Mice
Adult female, wild-type (WT), C57BL/6J mice (4, 7 and 10-months-old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous IFN receptor knock-out (IFNR-KO) animals with C57BL/6J were a generous gift from Dr. Dong-Er Zhang at Moores UCSD Cancer Center in University of California San Diego\(^1\). The animals were maintained in a light/dark (12-h/12-h) cycle at 24°C and received food and water ad-libitum during the experiments. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at the University of Florida and complied with the Guide for the Care and Use of Laboratory Animals.

Isolation and culture of SMPC (colony assay)
Four months old WT animals (n=10) were sacrificed for SMPC culture. Primary murine SMPC were cultured from peripheral whole blood, collected after cardiac perfusion with 0.9% heparin saline (100 IU/ml). Peripheral blood mononuclear cells were separated from red blood cells via density centrifugation over a Ficoll (GE Healthcare) gradient for 30 minutes at x 400g. The “Buffy-coat” fraction of supernatant was collected, washed with 2% FBS/PBS buffer twice, divided into two equal quantities, and plated on collagen (BD-Biosciences) coated 6-well dishes with EGM-2 medium (Lonza Switzerland) supplemented with 50ng/ml PDGF-BB (Bio Legend San-Diego, CA) with or without IFN-I (2,000 IU/ml). After 7 days colonies were counted and the colony number was normalized to the number of mononuclear cells plated, and expressed as a percentage of untreated cells. The colonies were cultured for another week in a medium without PDGF-BB and processed for immunohistochemical staining of SMC markers. The ratio of MHC to SM-α-actin positive cells was evaluated. In addition, SMPC cultured from four-month-old IFNR-KO mice (n=10) and 10-month-old WT mice (n=12) were processed similarly.

To further evaluate the effect of IFN-I on the phenotype of SMPC, after the first week of culture, media containing PDGF was replaced with media containing transforming growth factor-β (TGF-β, 1 ng/ml), TGF-β and IFN-I, IFN alone, or regular culture medium (See Supplement Table 2).

After two weeks culture, FACS of non-SMC markers (CD14, CD36, F4-80 and CD34) were processed to determine the phenotype of the majority of the colonies (n=4); meanwhile, mRNA was processed for real-time PCR determination of SM-MHC, SM-α-actin, SM22 and calponin to determine the SMPC phenotype (n=2).

Real-time PCR
Total RNA was isolated using RNeasy kit (QIAGEN, Germantown, MD), then 0.5 µg of total RNA was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative real-time RT-PCR was performed with SsoFast EvaGreen Supermix (Bio-Rad). Primers were designed using International DNA Technology web tool. Real-time PCR was performed using a CFX96 real-time PCR (Bio-Rad) with the protocol:
50°C for 2 min, then 95°C for 30 sec, then 40 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. Relative gene expression was analyzed by ΔΔC(t) method using the CFX Manager software (v. 1.6; Bio-Rad, Hercules, CA). The sequence of primers: SM MHC: Forward: 5'GACAACCTCTCTCCTTTGG3'; Reverse: 5'GCTCTCCAAAGCAGGTAC'3'; Calponin: Forward: 5'TTGAGAGAGACGAGCACTC3'; Reverse: 5'GTACCCAGTTTTGAGATCAGAG'3'; SM-α-actin: Forward: 5'CCTAACCCTTGGACGCTTGT3'; Reverse: 5'GAAAAGGTGGGATCATAGAG'3'; SM22: Forward: 5'ACACGCTTGT3'; Reverse: 5'GCAAGGCTGGAGACACTAGG'3'; GAPDH: Forward: 5'GGGTGTGAACCACGAGAA3'; Reverse: 5'AGTTGTCATGGATGACCTTG'3';

Plasmid Construction and Electroporation
Complementary DNA for β-galactosidase or murine IFN-α5 were generated by reverse transcription PCR and inserted into pcDNA3.1 vector via restriction digest followed by DNA ligation. DH5α e.coli (Invitrogen, Carlsbad, CA) were transformed with the ligation products, colonies picked, plasmid DNA isolated, and the cDNA sequence verified by DNA sequencing. Large-scale production was performed using Qiagen Endofree GigaPrep Kit (Qiagen, Germantown, MD) following the manufacturer’s instructions.

Animals were anesthetized using isoflurane and the skin on the hind limb was cleansed with 70% alcohol after removal of hair by shaving. One-hundred micrograms of a plasmid encoding IFNα5 or a control plasmid (β-galactosidase) in 50 µL of PBS was injected into the posterior thigh and immediately followed by placement of needle electrodes (Harvard Apparatus, Boston, MA) adjacent to the site of injection. Eight square-wave pulses (180 V, 20 ms) were delivered using Gene Pulser II (Biorad, Hercules, CA). Animals then were returned back to cages after recovering from anesthesia.

To examine the effect of 3 weeks of in-vivo IFN-1 expression on SMPC, 11 WT mice were electroporated with IFNα5 plasmid, and 9 control mice were electroporated with β-galactosidase plasmid. Three weeks following electroporation, the animals were sacrificed, whole peripheral blood was collected and SMPC were cultured as above.

To study the effect of 3 months of IFN-I in vivo exposure on the vessel wall, 10 WT mice were electroporated with IFN-I plasmid, and 11 mice were electroporated with β-galactosidase plasmid as control animals. Three months after electroporation, animals were sacrificed and abdominal aortas were collected. Experiments were also carried out in fifteen IFNR-KO animals, 8 mice electroporated with IFNα plasmid and 7 mice were used as control.

Flow cytometry and β-galactosidase staining
The level of Sca1+ was measured as early as one day after the electroporation of IFNα5 or control plasmid in WT animals. All antibodies were purchased from BD Bioscience and cell staining was performed as described. Fifty thousand events per sample were acquired using a CYAN ADP flow cytometer (Dako, Fort Collins, CO) and analyzed with FCS Express 3 (De Novo Software, Ontario, Canada).
The antibodies for anti-CD14, CD36, F4-80 and CD34 were purchased from BioLegend (San Diego, CA) and cell staining was performed as above. Fifty thousand events per sample were acquired using a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed with FCS Express 4 (De Novo Software, Ontario, Canada).

β–galactosidase staining was performed on frozen section of posterior thigh muscles 3 days after plasmid electroporation using Beta-gal Assay Kit (Invitrogen).

**Aorta Harvest and Histology**
The vessel segments containing the abdominal aorta bifurcation were harvested from animals and frozen sections were obtained and processed as described previously. The entire section was analyzed in serial 6-micron sections. Adjacent sections were used for hematoxylin and eosin staining and immunohistochemical stain. Quantitative evaluation was performed at 200X magnification using a Zeiss Axiovert Microscope (Carl Zeiss Microlmaging, Thornwood, NY); the images were analyzed with Axiovision 4.1 software (Carl Zeiss Microlmaging). The media thickness of the arterial wall was measured (μm) and cell density of the media was calculated (cell density=total cell number/total media area (0.01 mm²)). Hematoxylin and eosin stained slides were evaluated by a pathologist blinded to the treatment group.

**Dual and Triple Immunohistochemistry and Confocal Laser Scanning Microscopy**
SMPC were identified by dual immunohistochemical staining with anti-smooth muscle-α-actin (Sigma Inc., St. Louis, MO; Clone 1A4) and anti-smooth muscle-myosin heavy chain (SM-MHC, Kamiya Technologies Inc. Seattle, WA) as previously described. To evaluate the cellular components of the pre-atherosclerotic-like lesions, six sections of the lesion were stained by a dual- or triple-immunohistochemistry technique, as previously described. Briefly, the first section was incubated with anti-CD34 antibody (BD Pharmingen, San Jose, California), followed by donkey anti-rat biotin-conjugated Fab (Jackson ImmunoResearch, West Grove, PA) and then streptavidin-conjugated 488 (Invitrogen, Grand Island, NY). The section was then incubated with anti-smooth muscle-α-actin and anti-smooth muscle-MHC antibodies followed by Alexa 594-conjugated anti-mouse antibody and Alexa 647-conjugated anti-rat antibody. The third section was incubated with anti-F4/80 antibody (AbD Serotec, Oxford, UK) followed by anti-rat biotin-conjugated Fab and then streptavidin-conjugated 488. The section was then incubated with anti-smooth muscle-α-actin and anti-smooth muscle-MHC antibodies followed by Alexa 594-conjugated anti-mouse antibody and Alexa 647-conjugated anti-rat antibody. The third section was incubated with anti-CD31 antibody (Santa Cruz Biotechnology, Dallas, Texas) and anti-CD45 (BD Pharmingen, San Jose, California) antibody followed by Alexa 594-conjugated anti-goat antibody and Alexa 647-conjugated anti-rat antibody. The fifth section was incubated with anti-tenascin C antibody (Millipore, Billerica, MA) followed by anti-rabbit biotin-conjugated Fab and then streptavidin-conjugated 488. The section was then incubated with anti-CD3 (Dako, Carpinteria, CA) antibody followed by Alexa 594-conjugated anti-rabbit antibody. The second, forth and sixth sections were used as negative controls and were incubated with isotype-matched IgG substituted for the primary antibody. Some sections were pretreated with high temperatures as an antigen unmasking technique before being stained.
In an attempt to quantify the damage to the endothelium, the length of interrupted endothelium (lack of CD31 stain) was normalized to the calculated whole vessel circumference and expressed as a percentage.

Sections were examined under a confocal laser scanning microscope (Leica TCS SP2; Leica Microsystems GmbH, Heidelberg, Germany). Images were acquired with a HC PL APO 20× (numerical aperture = 0.70) and HCX PL APO 63× immersion (numerical aperture = 1.4) objectives and evaluated with Leica LCS software (v2.61).

**Statistics**

Quantitative morphological data were presented as mean ± SE and analyzed by the unpaired Student’s t-test. The analyses were performed with SigmaStat 2.0 and SigmaPlot 8.0 software, and a statistical significant difference was defined by p < 0.05.

A one-way Analysis of Variance (ANOVA) was used to examine mean differences of average counts of the expression of CD14, CD36, F4-80 and CD34 between four culture media groups. Pearson’s correlation coefficients and subsequent significance testing were computed for early-stage atherosclerotic-like lesions and the percentage of endothelium broken in IFN-I expressing mice and control. All analyses were conducted using SAS 9.4 (Cary, NC). p-values less than 0.05 were considered statistically significant.

**References:**


Supplement Table I: The expression of non-smooth muscle cell markers by flow cytometry in SMPC cultured in the presence or absence of IFN-I, TGF-β, or both. 50,000 events were included for all conditions. Results are expressed in percentage (mean ± SE). There is no significant difference between groups in one-way analysis of variance.

<table>
<thead>
<tr>
<th>Marker/media</th>
<th>Vehicle (%)</th>
<th>IFN-I (%)</th>
<th>TGF-β (%)</th>
<th>TGF-β &amp; IFN-I (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>0.05 ± 0.01%</td>
<td>0.14% ± 0.08%</td>
<td>0.13% ± 0.06%</td>
<td>0.09% ± 0.02%</td>
</tr>
<tr>
<td>CD36</td>
<td>1.03% ± 0.30%</td>
<td>1.99% ± 0.76%</td>
<td>0.99% ± 0.23%</td>
<td>0.78% ± 0.21%</td>
</tr>
<tr>
<td>CD34</td>
<td>2.37% ± 1.59%</td>
<td>3.60% ± 2.28%</td>
<td>1.81% ± 1.14%</td>
<td>2.33% ± 1.33%</td>
</tr>
<tr>
<td>F4-80</td>
<td>0.70% ± 0.24%</td>
<td>0.92% ± 0.54%</td>
<td>0.64% ± 0.25%</td>
<td>0.56% ± 0.16%</td>
</tr>
</tbody>
</table>
Supplement Table II: The expression of smooth muscle cell markers by real-time PCR in SMPC cultured in the presence or absence of IFN-I, TGF-β, or both. The level of expression of the mRNA for the indicated proteins were expressed as a fold-change as compared to vehicle treated cells.

<table>
<thead>
<tr>
<th>mRNA of SMC marker/media</th>
<th>Vehicle (%)</th>
<th>IFN-I (%)</th>
<th>TGF-β (%)</th>
<th>TGF-β &amp; IFN-I (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-α-actin</td>
<td>1.00 ± 0.007</td>
<td>4.25 ± 0.068</td>
<td>0.018 ± 0.000</td>
<td>2.50 ± 0.099</td>
</tr>
<tr>
<td>Calponin</td>
<td>1.00 ± 0.001</td>
<td>0.33 ± 0.003</td>
<td>0.071 ± 0.002</td>
<td>0.002 ± 0.000</td>
</tr>
<tr>
<td>SM-MHC</td>
<td>1.00 ± 0.001</td>
<td>1.01 ± 0.032</td>
<td>0.011 ± 0.000</td>
<td>0.008 ± 0.000</td>
</tr>
<tr>
<td>SM22</td>
<td>1.00 ± 0.160</td>
<td>75.4 ± 41.98</td>
<td>4.41 ± 0.075</td>
<td>56.8 ± 11.8</td>
</tr>
</tbody>
</table>
Supplement Figure I: No significant lesions were observed from interferon receptor knock-out (IFNR-KO) animals that undergone pcDNA-IFNα (A) or control plasmid electroporation (B, 16.52 ± 8.33 μm² vs 20.64 ± 7.80 μm² p=0.68). Magnification: 200X. Bar=50 μm.