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Short Communication

Reduced Transplant Arteriosclerosis in Murine Cardiac Allografts Placed in Interferon-γ Knockout Recipients

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To investigate the functional role of interferon (IFN)-γ in transplant arteriosclerosis, BALB/c hearts were transplanted in immunosuppressed C57BL/6J recipients with (n = 10) or without (n = 10) targeted IFN-γ gene deletion. In 55-day heart allografts, IFN-γ deficiency resulted in a significant decrease in vascular thickening. The severity of intimal thickening measured as the percentage of luminal occlusion (mean ± SEM) in all elastin stained vessels (n = 410) decreased from 37 ± 5% in wild-type recipients to 18 ± 5% in IFN-γ −/− recipients (P < 0.005). In the few diseased vessels in grafts from IFN-γ −/− recipients, the neointima was more cellular with a 90% increase in the nuclear density. This finding correlated with a 50% reduction in fibrosis estimated by α-smooth muscle actin cell accumulation in the neointima. The reduction in severity and altered composition of vascular thickening in grafts from IFN-γ −/− recipients shows that IFN-γ contributes to arteriosclerotic development following transplantation. ***(Am J Pathol 1998, 152:359–365)***

Interferon (IFN)-γ has potential proarteriosclerotic functions including activation of vascular endothelial cells, mononuclear cells, macrophages, and neutrophils.[1] By activating these cells, IFN-γ promotes cytoadhesion to sites of injury and potentiates inflammatory cytokine and growth factor production.[1] IFN-γ also stimulates cytolytic activity of NK cells and promotes B- and T-cell differentiation.[1] In contrast, IFN-γ also has potential antiarteriosclerotic functions in that it has been shown to inhibit smooth muscle cell (SMC) activation and proliferation both *in vitro* and *in vivo*.[2–4] These opposing arteriosclerotic forces have raised questions about the cumulative influence of IFN-γ in vessel remodeling. In transplant arteriosclerosis, IFN-γ may be proarteriosclerotic through its immune effects or antiarteriosclerotic through direct SMC effects.

Mice with targeted gene deletion of IFN-γ (IFN-γ −/−)[5] and IFN-γ receptor (IFN-γ R −/−)[6] have provided new tools to study functional role(s) of IFN-γ *in vitro* and, more importantly, pathophysiological states *in vivo*. The original reports described knockout mice with neither gross abnormalities or perturbations in immune cell distribution (CD3, B220, CD4, and CD8). However, in response to various immunological stimuli (lipopolysaccharide, oxazolone, or allogeneic tumor cells), both major histocompatibility complex (MHC) class I and class II expression on the vascular endothelium were absent in IFN-γ −/− and IFN-γ R −/− mice.[7] When challenged with *Mycobacterium bovis*,[5] *Listeria monocytogenes*, or vaccinia virus,[6] knockout mice had higher morbidity and mortality than wild-type controls. Furthermore, in collagen-induced arthritic models, disease was accelerated in IFN-γ R −/− mice.[8,9] The first report using these mice in transplantation showed accelerated cardiac allograft rejection in untreated IFN-γ −/− recipients.[10] In these two disease states, the absence of IFN-γ surprisingly worsened rather than reduced the disease process. Taken together, the studies using knockout mice have shown that IFN-γ can alter immune responses to specific challenges and change pathological outcomes.

Previously, we and others have demonstrated that high IFN-γ expression correlated with development of transplant arteriosclerosis in rat and mouse cardiac allografts.[11–13] However, the precise roles of IFN-γ in arteriosclerotic syndromes remain to be defined. To study the functional role of IFN-γ, we have established an immuno-

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suppressed, MHC class I and II mismatched mouse heart transplant model. Murine cardiac grafts have preserved ventricular contraction, mild degrees of mononuclear infiltration, and measurable degrees of vascular occlusion. Thus, this model reproduces many of the features of chronic rejection observed in human heart transplants. The purpose of the current study was to investigate whether recipient IFN-γ has a proarteriosclerotic or antiarteriosclerotic effect on immune-mediated transplant arteriosclerosis. Analysis of the severity, distribution, and cellular features of vascular thickening was compared between allografts placed in IFN-γ $-/-$ recipients and in wild-type recipients.

Materials and Methods

Transplant Model of Graft Arteriosclerosis

BALB/cByJ (H-2^d) donors were transplanted into C57BL/6J (H-2^b) recipients, which were either wild-type (n = 10) or had targeted gene deletion in IFN-γ (IFN-γ $-/-$, n = 10).[5] Isografts (n = 7) were performed from IFN-γ $-/-$ to IFN-γ $-/-$. All mice were purchased from Jackson Laboratories (Bar Harbor, ME). IFN-γ $-/-$ mice had been backcrossed 8 times unto C57BL/6J background by Jackson Laboratories. The targeted gene disruption was confirmed using multiplex polymerase chain reaction assays that amplify a portion of the neomycin cassette and a portion of the targeted exon as recommended by Jackson Laboratories.

Heterotopic cardiac transplantation, monitoring of graft function by palpation (scale of 0 (absent) to 4 (maximal)), and graft harvest was performed as described at 55 days after transplantation.[13,14] For immunosuppression, mice received monoclonal antibodies (mAb) against CD4 (clone GK1.5, rat IgG2b; American Type Culture Collection, Rockville, MD) and CD8 (clone 2.43, rat IgG2b; American Type Culture Collection) at the dose of 500 μg for each mAb per injection for days 1 to 4 after transplantation and weekly thereafter for 30 days. As previously demonstrated by flow cytometry of splenocytes, this program reduces $CD4^+$ and $CD8^+$ cells by >94% during the treatment.[13] Twenty-five days after cessation of the treatment (at day 55 after transplantation), splenic $CD4^+$ cells were 48% of the control level and $CD8^+$ cells were 15% of the control level indicating low-level immunosuppression.

Analysis of Vascular Thickening

The severity of disease (percentage of luminal occlusion) was analyzed in Verhoeff's elastin-stained transverse paraffin sections. All elastin positive vessels of varying sizes were analyzed. Microscopic images were captured and analyzed by two independent observers. The percentage of luminal occlusion was tabulated by tracing the internal elastic lamina and the lumen for each elastin positive vessel cross section in one transverse section per graft with the ScionImage 1.60 software (National Institutes of Health, Baltimore, MD).[12] The mean value for each individual graft was tabulated by two observers and used to derive mean ± SEM per group. To evaluate the distribution of vessels by occlusive severity, vessels were divided into three categories; mild (<20%), moderate (20 to 50%), and severe (>50%). The frequency of diseased vessels was assessed as the percentage of diseased vessels with occlusion >0% out of the total number of vessels in each graft and reported as the mean ± SEM per group. A total of 410-vessel cross sections were used to assess the severity and frequency of vessel thickening with a mean of 15.1 vessel cross sections per graft.

Cellularity within the expanded neointima in severely diseased vessels was assessed by calculating the number of nuclei in the area of the neointima to derive neointimal nuclear density (number of nuclei/μm^2). Because of inherent resolution limits on the video capture system used for image analysis, only a subset of vessels was studied. Larger vessels that had areas delineated by internal elastic lamina >350 μm^2 and that had >40% luminal occlusion were included. In the IFN-γ $-/-$ group, 10 vessel cross sections and, in wild-type, 33 vessel cross sections fulfilled these criteria.

Immunostaining

Immunostaining for CD45 (clone 30F11.1, leukocyte common antigen, Ly-5; Pharmingen, San Diego, CA) was used to identify leukocytes within the vessel as previously described.[15] Myointimal fibrosis, indicative of more advanced arteriosclerotic stages, was assessed by immunostaining for α-smooth muscle actin. Ten grafts from each group were stained using the previously described protocol with minor modifications.[16] Briefly, Verhoeff's elastin-stained paraffin sections were blocked with 10% normal goat sera and then stained with α-smooth muscle actin antibody (clone 1A4, dilution 1:20000, Sigma, St. Louis, MO) overnight at 4°C. This antibody (mouse IgG2a) reacts specifically with a single isoform of α-smooth muscle actin.[17] It has wide reactivity with smooth muscle cells from many species including mouse.[15] Primary antibody incubation was followed by biotinylated secondary antibody (goat-anti-mouse IgG2a, Amersham, Life Science) and detection with avidin-biotin-complex and 3-amino-g-ethylcarbazole (AEC) substrate (Vectrastain ABC kit, AEC-kit; Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions. Replacement of primary antibody with phosphate-buffered saline in 3% goat serum served as a negative control indicating that the secondary anti-mouse IgG2a selectively recognized the primary antibody and did not react nonspecifically in mouse tissue.

To evaluate the degree of myointimal fibrosis, image analysis using ScionImage 1.60 (National Institutes of Health) was performed to measure the percentage of area of α-smooth muscle actin positivity within the neointima. Again, only larger vessels (area delineated by internal elastic lamina >350 μm^2) with greater than 40% luminal occlusion were studied. The area staining for α-smooth muscle actin was determined by measuring the pixel area displaying the color intensity of immunoposi-

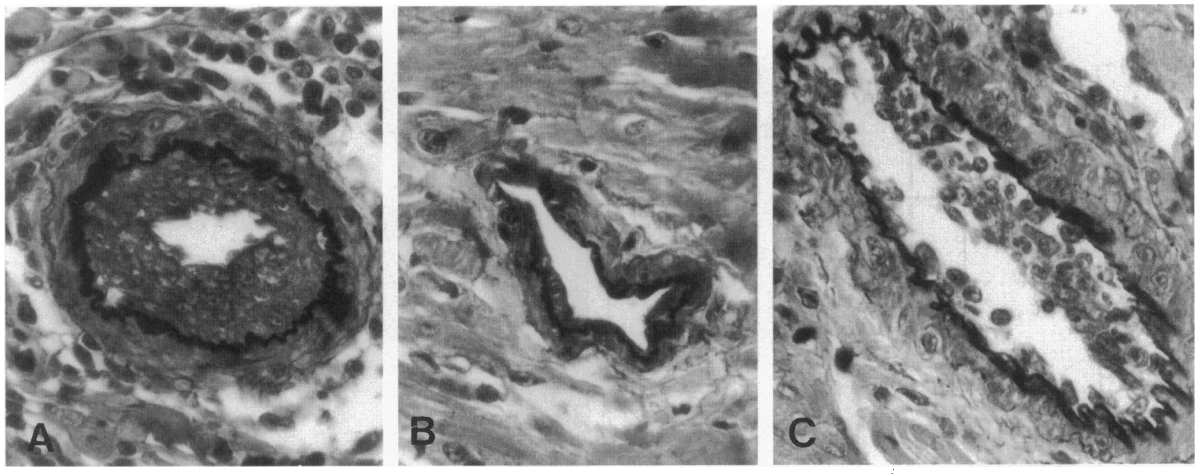

Figure 1. Representative photomicrographs of cardiac allografts placed in IFN-γ −/− or wild-type recipients. Verhoeff's elastin staining showed prominent neointimal thickening in wild-type recipients (**A**) but diminished vascular thickening in IFN-γ −/− recipients (**B**). Those few vessels that were diseased in IFN-γ −/− recipients had more cellular and less fibrotic neointima (**C**). Magnification, ×125.

tive cells. The vessels that had moderate to severe vascular thickening were used for the analysis in wild-type recipients (n = 51, vessel cross sections) and in IFN-γ −/− recipients (n = 20).

Statistical Analysis

All data were given as the mean ± SEM in each subgroup and subjected to multiple analysis of variance without replication (StatView 4.5, Abacus Concepts, Berkeley, CA). If the multiple analysis of variance was significant, individual comparisons were made by the Student's t-test, and the level of significance was corrected by the Bonferroni method.

Results

Cardiac Transplantations

After a 30-day course of anti-CD4/CD8 immunosuppression, all allografts placed in IFN-γ −/− and wild-type recipients survived to the harvest point of 55 days after transplantation. The mean palpation scores (scale 0 to 4) at the time of harvest were comparable between allografts in the IFN-γ −/− recipients (2.8 ± 0.1) and in wild-type recipients (2.9 ± 0.1) indicative of preserved ventricular contraction.

Histological Features of Cardiac Allografts

To evaluate the role of ongoing recipient IFN-γ deficiency in transplant arteriosclerosis, we compared donor hearts from IFN-γ −/− with wild-type recipients. All allografts showed mild, patchy mononuclear infiltration with little to no myocyte necrosis consistent with low-grade inflammation. In grafts from wild-type recipients, there was prominent dense vascular thickening (Figure 1A). In contrast, heart allografts placed in IFN-γ −/− recipients had much less striking vascular thickening (Figure 1B). Most vessels were disease-free, and only a few vessels appeared to be affected with prominent intimal thickening (Figure 1C). In allografts placed in IFN-γ −/− recipients, the expanded neointima in the few diseased vessels appeared more cellular (Figure 1C). CD45 (leukocyte common antigen) positive leukocytes were identified scattered within the parenchyma and expanded neointima in allografts from both recipient groups. Of course, the majority of vessels in grafts from IFN-γ −/− recipients were negative as they had no thickening. However, in those few vessels with expansion of vascular neointima, the CD45 positivity was more striking than in grafts from wild-type recipients. IFN-γ −/− isografts had no infiltrating cells, vascular thickening, or tissue destruction (data not shown).

Quantitative evaluation of each elastin-stained vessel was performed using a computer assisted system. The severity of vascular thickening, measured as the percentage of luminal occlusion (Figure 2A) was significantly lower in heart grafts from IFN-γ −/− recipients (n = 10) compared with wild-type recipients (n = 10) (18 ± 5% *versus* 37 ± 5%, respectively, P = 0.0042). Vascular thickening plotted as the distribution of vessels by occlusive severity is shown in Figure 2B. In allografts from IFN-γ −/− recipients, the majority (76%) of vessels (n = 132) had mild (<20%) to no luminal occlusion, 11% had moderate (20 to 50%) luminal occlusion, and 13% had severe (>50%) luminal occlusion. In contrast, in grafts from wild-type recipients, 40% of all vessels (n = 219) were severely affected, 21% were moderately diseased, and 39% had mild to no luminal occlusion. The overall frequency of vessel thickening in grafts was significantly lower in grafts from IFN-γ −/− compared with wild-type recipients (39 ± 8% *versus* 71 ± 7%, $P < 0.01$). IFN-γ −/− isografts (animal n = 7, vessel n = 59) had significantly lower severity and frequency of vascular disease than either of the allograft groups (9 ± 2% and 24 ± 6%, respectively, $P < 0.0001$ for both). Taken together, recipient deficiency of IFN-γ decreased severity and frequency of graft vascular thickening and shifted the distribution of occlusive severity toward milder forms.

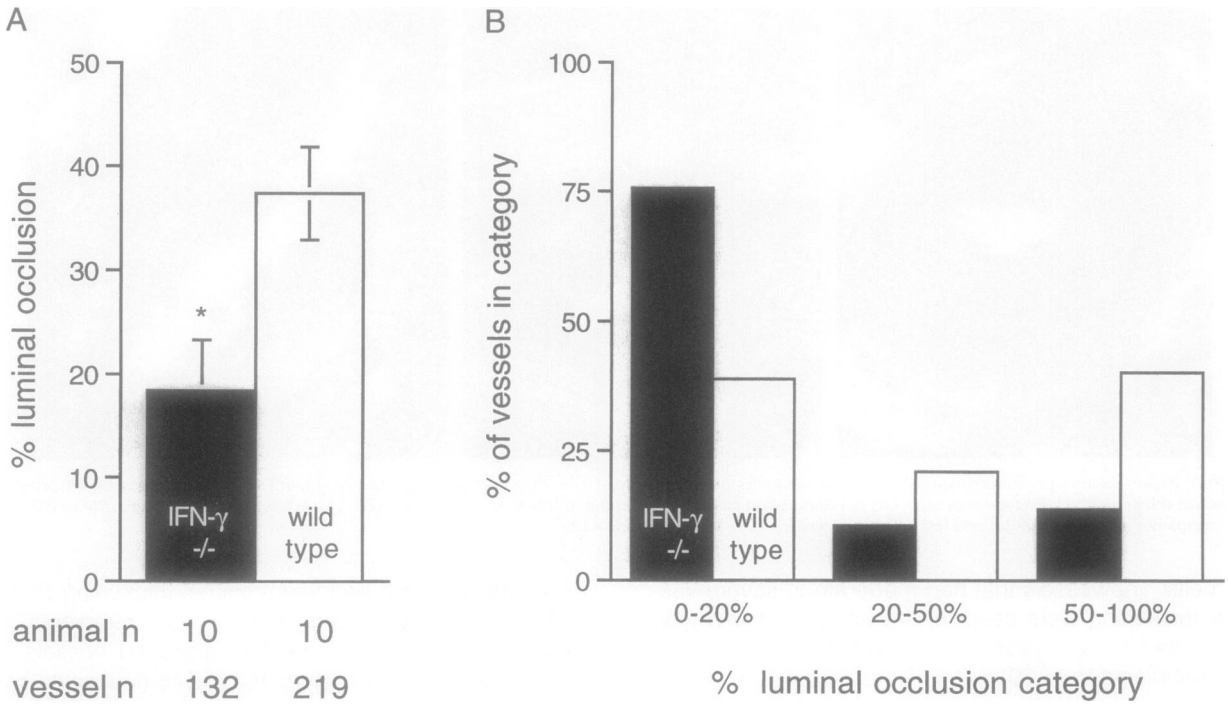

Figure 2. A: The severity of vascular thickening in cardiac allografts was significantly reduced in IFN-γ −/− recipients compared with wild-type recipients. $^{*}P <$ 0.011. Data are given as mean ± SE of all grafts in each group. **B:** The distribution of vessels by occlusive severity from grafts in IFN-γ −/− (**black**) and wild-type (**white**) recipients. In the IFN-γ −/− group, the distribution is shifted toward less severe luminal occlusion.

Deficiency of IFN-γ Alters Neointimal Composition

In most rodent models of transplant arteriosclerosis, vessel thickening occurs in stages with early mononuclear cell adhesion (endothelialitis) and late smooth muscle cell thickening (fibrosis or arteriosclerosis).[16,18,19] To determine which phases were disrupted by IFN-γ deficiency, we evaluated the myointimal composition. In IFN-γ −/− recipients, the few diseased vessels with expanded neointima appeared to be more cellular with many inflammatory mononuclear cells along the lumen (Figure 1C). To evaluate such differences in neointimal composition, we counted nuclei within the neointima of vessels with > 40% luminal occlusion. This subset of diseased vessels from grafts in IFN-γ −/− recipients had 90% higher nuclear density than those in grafts from wild-type recipients (0.017 ± 0.002 nuclei/μm^2 (n = 10) *versus* 0.009 ± 0.001 nuclei/μm^2 (n = 33; P = 0.0001)). Whereas, intimal thickening in grafts from wild-type recipients appeared more fibrotic.

Immunostaining for α-smooth muscle actin was used to identify the later fibrotic stage of arteriosclerosis. In grafts placed in wild-type recipients, α-smooth muscle actin positive cells predominated within the expanded neointima (Figure 3). In contrast, heart allografts in IFN-γ −/− recipients had fewer α-smooth muscle actin positive cells interspersed throughout the neointima. Quantitative image analysis of the more severely diseased vessels in allografts from the IFN-γ −/− recipients (n = 20) and wild-type (n = 51) recipient groups confirmed this pattern. Deficiency of IFN-γ was associated with a decrease in the area of α-smooth muscle actin positivity from 50.6 ± 3.5% in wild-type to 18.4 ± 3.9% in IFN-γ −/− recipients, $P < 0.0001$. Hence, in this mouse cardiac allograft model, IFN-γ appears to act, in part, by promoting the formation of a neointima rich in smooth muscle cells.

Discussion

Our study shows that IFN-γ is an important cytokine in the development of transplant arteriosclerosis in immunosuppressed mouse heart allografts. We demonstrate that targeted gene deletion of recipient IFN-γ reduces both severity and frequency of vascular thickening. In those lesions that do form when recipient IFN-γ is absent, the progression to advanced arteriosclerotic stages is altered. Specifically, in grafts from IFN-γ −/− recipients, there was an increase in the neointimal nuclear density associated with a reduction in α-smooth muscle actin positive cell accumulation. Hence, the neointima was more cellular and less fibrotic in the absence of IFN-γ. Taken together, IFN-γ regulates the progression of vascular thickening in heart allografts toward advanced, fibrotic lesions.

IFN-γ Is Proarteriosclerotic

Our finding that IFN-γ plays an unique role in promoting transplant arteriosclerosis by mediating fibrotic progression extends other studies examining the role of IFN-γ. First, Paul Russell and colleagues[20] showed that after

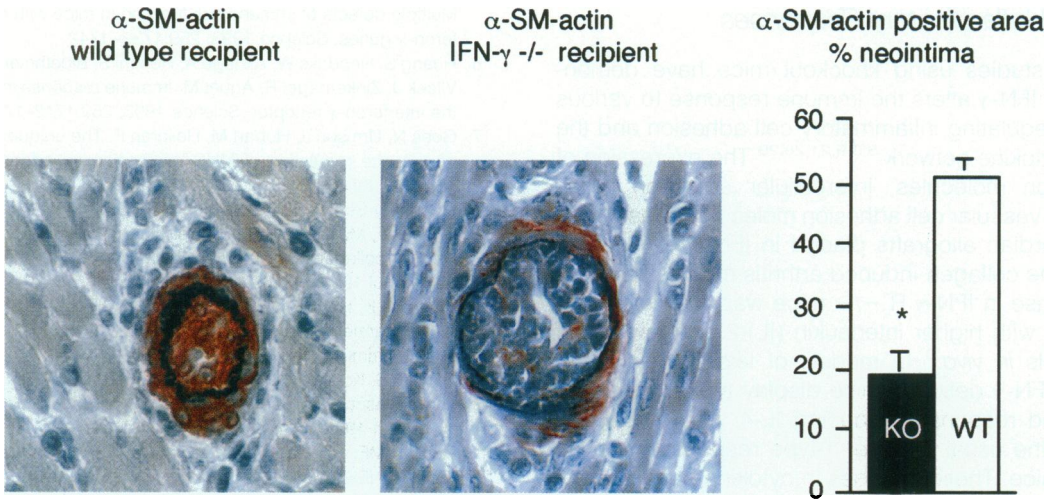

Figure 3. Representative immunostained diseased vessels demonstrated α-smooth muscle (SM) actin rich neointima (**red**) in wild-type recipients with decreased smooth muscle (SM) actin positivity in IFN-γ −/− recipients (counterstained with Verhoeff's elastin stain; magnification, ×125). Quantitative image analysis showed that the neointima in grafts from IFN-γ −/− recipients (KO) had significantly (*$P < 0.0001$) decreased percent area of α-smooth muscle actin positivity in the neointima than the wild-type controls (WT). Data given as mean ± SE.

biweekly treatment of anti-IFN-γ mAb, heart transplants with class I (B10.A to B10.BR) or class II (bm12 to C57BL/6) mismatches had diminished vascular changes. This finding was recently confirmed and extended by Nagano et al[21] using the same bm12 to C57BL/6 strain combination. They showed equivalent inhibition of graft arterial disease (but not parenchymal rejection) when using anti-IFN-γ mAb treatment in wild-type recipients or placing the grafts in IFN-γ −/− recipients. Our study extends these reports by evaluating the high responder mismatch involving both MHC class I and II (BALB/c to C57BL/6). In contrast to the study by Nagano et al,[21] our immunosuppressed model does not have prominent parenchymal rejection superimposed on vascular changes. This histological pattern is similar to that seen in human cardiac transplants with chronic rejection.[22] Despite methodological differences (qualitative *versus* quantitative) in assessing the vascular involvement, it is clear that reducing IFN-γ levels attenuates vascular thickening in response to alloimmune injury in the different strain combinations.

In accordance with our findings, an important role for IFN-γ in ordinary atherosclerotic formation has been recently demonstrated. Apo-E/IFN-γ R−/− double knockout animals were placed on a western diet to induce lipid injury.[23] Double mutated mice had a 59% reduction in atherosclerotic lesion area compared with control Apo-E −/− animals receiving the same lipid-rich diet. IFN-γ receptor deficiency increased the expression of ApoA-IV levels (atheroprotective lipoprotein) in these hyperlipidemic animals, thus pointing to one of the many potential protective mechanisms.

IFN-γ and Neointima

Our findings suggest that IFN-γ may regulate the progression of vascular remodeling. In IFN-γ −/− recipients, nuclei density in the expanded neointima increased in concert with decreases in myointimal fibrosis as shown by quantitating α-smooth muscle actin positive cell accumulation. Hence, recipient IFN-γ deficiency may have either delayed temporal development of vascular thickening and/or disrupted the progression toward myointimal fibrosis in this model.

In this transplant arteriosclerosis model, we demonstrate that deletion of IFN-γ decreased vascular lesion severity and frequency. In those vessels that did develop lesions, they had reduced myointimal fibrosis compared with controls. Antiarteriosclerotic roles for IFN-γ have been suggested by *in vitro* studies showing that addition of IFN-γ to rat aortic smooth muscle cells in culture-reduced proliferation and fibrosis[2,24] and reports that exogenous administration of IFN-γ reduced vessel thickening after carotid balloon denudation.[3,4] Our contrasting finding that the presence of IFN-γ promotes arteriosclerosis could have several possible explanations. First, the model systems may produce different forms of vessel injury. The balloon models involve mechanical injury, whereas the transplantation models have mainly immune-mediated injury.[25] Alternatively, one could speculate that IFN-γ regulates SMC remodeling indirectly in our transplant model through regulation of T-cell and macrophage activation. In fact, macrophages and T cells are known to be a potent source of various growth factors that can modulate SMC differentiation, proliferation, and migration both *in vitro* and *in vivo*.[26] Either way, the findings from *in vitro* SMC studies or in the rat carotid denudation model probably do not mimic the responses to immune-mediated injury following cardiac transplantation. This ongoing injury to the vascular endothelium and underlining structures in allografts involves multiple, superimposed antigen-dependent and antigen-independent factors.[27] Taken together, our *in vivo* studies in transplant arteriosclerosis show that a role for IFN-γ as an inhibitor of SMC proliferation or fibrosis is unlikely.

IFN-γ and Inflammatory Responses

Functional studies using knockout mice have demonstrated that IFN-γ alters the immune response to various stimuli by regulating inflammatory cell adhesion and the complex cytokine network.[5,6,9,21,28,29] The expression of cytoadhesion molecules, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 was diminished in cardiac allografts placed in IFN-γ −/− recipients.[21] In the collagen-induced arthritis model, the onset of the disease in IFN-γ R −/− mice was accelerated in association with higher interleukin (IL)-2 and lower IL-4 serum levels *in vivo*.[9] In models of leishmaniasis and listeriosis, IFN-γ deficient mice display typical T-helper-2-associated response producing IL-4, IL-5, and IL-13 instead of the usual T-helper-1-type responses seen in wild-type mice. These changes in cytokine patterns, isotype switching, and impaired macrophage production of NO_2 on stimulation may account for the higher susceptibility and morbidity to infection in IFN-γ deficient mice.[6,28,30] Our earlier studies, in rejecting mouse cardiac allografts from IFN-γ −/− recipients, have extended these findings showing up-regulation in IL-2 transcripts and selective changes in macrophage markers (down-regulated allograft inflammatory factor-1 and inducible nitric oxide synthase but up-regulated monocyte chemoattractant protein-1).[31] Hence, IFN-γ deficiency alters both the T-cell and macrophage cytokine responses to injury associated with attenuated heart allograft rejection. This modulation of inflammatory cells may have contributed indirectly to reduced lesion formation associated with chronic rejection.

In conclusion, IFN-γ plays an important role in the progression of vascular thickening associated with chronic rejection in this model. IFN-γ deficiency prevented vessel thickening and reduced the development of myointimal fibrosis in the neointima. Additional studies using this model will permit dissection of the molecular mechanisms through which IFN-γ regulates smooth muscle cell accumulation in the vascular wall.

Acknowledgments

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