Interferon-γ deficiency reduces neointimal formation in a model of endoluminal endothelial injury combined with atherogenic diet

NASIM NIK TAVAKOLI[1], ANGIE K. HARRIS[1], DAVID R. SULLIVAN[2], BRETT D. HAMBLY[1] and SHISAN BAO[1]

[1]Discipline of Pathology, School of Medical Sciences and Bosch Institute, The University of Sydney;
[2]Department of Clinical Biochemistry, Royal Prince Alfred Hospital, Sydney, New South Wales, Australia

Received March 20, 2012; Accepted May 3, 2012

DOI: 10.3892/ijmm.2012.1034

Abstract. Interferon (IFN)-γ has been implicated in restenosis, however its precise role in the pathophysiology of neointimal formation following angioplasty is unclear, as it has been shown to both promote and inhibit neointimal formation. Dietary-induced hypercholesterolemia enhances injury-mediated neointimal formation, associated with increased systemic inflammation and serum IFN-γ. This study examined the effect of IFN-γ gene deficiency ($^{-/-}$) on neointimal formation in a mouse model of endothelial injury combined with an atherogenic diet. Neointimal formation was induced via endoluminal endothelial injury of the common iliac arteries of IFN-$\gamma^{-/-}$ and wild-type (WT) C57Bl/6 mice. Histopathological analysis of the arteries was performed at 3 and 6 weeks post-surgery. IFN-$\gamma^{-/-}$ mice demonstrated a significant reduction in neointimal formation at the 3-week time point, compared to their WT counterpart. No significant differences in plasma lipid profile and the extent of re-endothelialization were detected between IFN-$\gamma^{-/-}$ and WT mice, suggesting that the effect of IFN-γ on neointimal formation is due to injury-mediated vessel neointimal responses. In support of the histopathological findings, immunohistochemical analysis revealed a significant reduction in vessel infiltrating macrophages, and neointimal PDGF-B expression, vascular smooth muscle cell composition and cellular proliferation in the IFN-$\gamma^{-/-}$ mice, in comparison to their corresponding WT group at the 3-week time point. In conclusion, the IFN-γ-mediated pathway plays an important role in inflammatory responses and proliferative effects following injury, suggesting that modulation of the IFN-γ pathway would be beneficial in controlling neointimal formation and restenosis.

Correspondence to: Dr S. Bao, Discipline of Pathology, School of Medical Sciences, The University of Sydney, Blackburn Building D06, Sydney, New South Wales 2006, Australia
E-mail: bob.bao@sydney.edu.au

Key words: IFN-γ, restenosis, endothelial injury, neointimal formation

Introduction

Restenosis is a common and serious complication associated with re-vascularisation by percutaneous coronary angioplasty, with considerable clinical implications (1). Inflammatory-mediated mechanisms following endothelial injury and/or stent placement are thought to play a pivotal role in restenosis (1). Moreover, injury-induced proliferation and/migration of vascular smooth muscle cells (VSMCs) is an important contributor to neointimal hyperplasia and in-stent restenosis (2,3). However, the precise underlying molecular mechanism(s) of restenosis are not entirely understood.

The pro-inflammatory cytokine interferon (IFN)-γ exhibits many biological functions including; mediation of inflammatory responses and immunomodulatory effects (4,5). IFN-γ is secreted from a variety of cells such as T helper cell type 1 (Th1) lymphocytes, activated macrophages, VSMCs and natural killer cells, and its expression has been demonstrated in atherosclerotic lesions (4,6). IFN-γ affects major cell types within the lesion and displays a complex role in vascular lesion development and progression (4). The biological effects of IFN-γ include; stimulating the differentiation of monocytes to macrophages, activation of macrophages and T-lymphocytes (4), and increasing the expression of platelet-derived growth factor (PDGF) and PDGF-β receptor on VSMCs, thereby mediating proliferation (7). In contrast, IFN-γ also exhibits protective effects against lesion formation; including inhibition of cultured VSMC proliferation (8,9), and induction of anti-inflammatory molecules; interleukin (IL)-1 receptor antagonist and IL-18 binding protein (10).

IFN-γ has been implicated in restenosis in both human and animal studies (11-13). Activation of IFN-γ signalling in neointimal SMCs was indicated in 37 of the 223 differentially expressed genes in atherectomy specimens of patients with restenosis, determined by a transcriptome study (13). Furthermore, elevated circulating IFN-γ has been detected within 15 min following stent implantation, in both chronic and acute coronary syndrome patients undergoing angioplasty, suggesting that modulation of IFN-γ release plays a role in inflammatory complications of angioplasty during/following stenting (12). Animal studies concerning the role of IFN-γ on injury-mediated models of neointimal formation have yielded

variable results. Both administration of IFN-γ inhibitory protein (11), and exogenous IFN-γ treatment (14) reduced injury-induced neointimal formation in rats. Thus, the role of IFN-γ in injury-mediated neointimal formation is complex, and warrants further investigation.

Of note, a study demonstrated a pathogenic role for hypercholesterolemia in enhancing the degree of neointimal formation and VSMC proliferation, using a newly developed model of porcine vascular injury combined with a high cholesterol diet (15). Moreover, hypercholesterolemia induced systemic inflammation, with an increase in serum IFN-γ and TNF-α, and a local inflammatory response with higher T cell infiltrates (15).

Therefore, the aim of the current study was to determine the role of IFN-γ on injury-mediated neointimal formation, using a combination of a murine endoluminal endothelial-injury model with dietary-induced hypercholesterolemia. Our data demonstrated that IFN-$\gamma^{-/-}$ mice exhibit significantly reduced neointimal formation. These findings may provide useful insight for future design of therapeutic options for restenosis.

Methods and methods

Animals and diet. Male C57Bl/6 IFN-$\gamma^{-/-}$ (16) and WT mice were bred in the Blackburn Animal House, The University of Sydney. All experiments were performed in accordance with the guidelines of The University of Sydney Animal Care and Ethics Committee. Mice were housed under standard conditions, and allowed *ad libitum* access to water and standard mouse chow until ~ 8 weeks of age. Subsequently, 1 week prior to probe-mediated endothelial injury of the iliac artery, mice were placed on an atherogenic diet containing 16% fat, 1% cholesterol and 0.5% cholate, obtained from Specialty Feeds (Cat. no. SF00-245, Glen Forrest, WA, Australia). The diet was continued after arterial injury until euthanisation.

Murine iliac artery endothelial injury model. Neointimal formation was induced in the left iliac artery via a standard procedure involving endoluminal mechanical disruption of the vessel wall (17,18). The resultant endothelial damage provides a primary stimulus for neointimal formation. Mice were anaesthetised via an intraperitoneal injection of Avertin, prior to surgery (19). Using a dissection microscope, the femoral artery, vein and nerve were identified and a custom-made probe (0.8-mm) with a roughened surface was passed via the femoral into the iliac artery. The insertion followed by withdrawing of the probe led to endothelial injury of the iliac artery. The femoral artery was then ligated proximal to the probe access point to stop the bleeding, but distal to the origin of the profunda femoris artery, allowing for continued blood flow through the iliac artery and collateral circulation to the limb. Following the surgical procedure, mice were allowed to recover in a sterile environment. The circulation and activity of the lower limb was monitored daily.

Plasma lipid analysis. Blood samples were collected via cardiac puncture at the time of sacrifice. Plasma was obtained and immediately frozen, and later analysed. Plasma TC and triglyceride levels were measured by specific enzymatic methods on a Cobas Modular autoanalyser (Roche Diagnostics Mannheim, Germany) and high-density lipoprotein cholesterol was measured using a direct enzymatic method on the same instrument. LDL-C was calculated using the Friedewald equation.

Excision of the iliac arteries. To evaluate the temporal development of neointima formation, iliac arteries were excised at 3 and 6 weeks post-surgery with each group consisting of five mice. The left (injured) and right (non-injured, control) iliac arteries were examined for the temporal development of neointima formation and normal intimal thickness, respectively. The iliac arteries and the surrounding muscle tissue were excised from the junction of the abdominal aorta to the ligated proximal segment of the artery, fixed overnight in 95% ethanol and embedded in paraffin wax as previously described (18).

Histopathology of the iliac arteries. Neointima formation was evaluated in 18 alternate serial sections (5 μm) taken through the centre third of the injured, and the corresponding non-injured (control) arteries. Sections were stained with hematoxylin and eosin (H&E), and photographed with an Olympus BX40 microscope attached to a DP71 digital camera (Olympus, Australia). The area of intima and media were evaluated using the computer image analysis software ImagePro Plus 4.5 (Diagnostic Instruments, USA), as previously described (18). Neointimal formation was quantified using two indices: i) %I/(I+M), and ii) %I/M. The counts from all sections of each artery were averaged to represent one mouse.

Immunohistochemistry. Representative paraffin sections (5 μm) were subjected to immunohistochemistry staining. Sections were incubated with rat anti-mouse F4/80 (WEHI, Melbourne, Australia) and CD31 (Abcam, Cambridge, UK) antibodies followed by a Horseradish Peroxidase (HRP)-conjugated rabbit anti-rat antibody (Dako, Sydney, Australia). Sections incubated with rabbit anti-mouse Ki-67 (Abcam) and TNF-α (Abcam) antibodies, and rabbit anti-human VWF (Dako), and PDGF-B (Abcam) antibodies with cross-reactivity with mouse VWF and PDGF-B, respectively, and further incubated with HRP-conjugated goat anti-rabbit antibody (Dako). Immunohistochemical staining for α-SMA and PCNA were performed using mouse anti-human antibodies that cross-react with mouse α-SMA (Dako) and PCNA (Abcam), respectively, and utilizing ARK™ (Animal Research kit) (Dako), according to the manufacturer's instructions. All sections were then visualized with 3,3' diaminobenzidine (DAB) (Dako), and lightly counterstained with hematoxylin.

Immunohistochemical quantitative evaluation. Immunohistochemical quantifications were performed on digitized images using image analysis software ImagePro Plus 4.5 (Diagnostic Instruments). Endothelialization was morphologically assessed in the immunohistochemical staining with anti-CD31 and anti-VWF antibodies. Re-endothelialization was calculated as the percentage of luminal surface covered by CD31 and VWF-positive cells to the total luminal surface. Proliferative activity was determined in the immunohistochemical staining with anti-PCNA and Ki-67 antibodies. The total number of brown-labelled positive nuclei within the neointima was counted, and a PCNA/Ki-67 labelling index was calculated [100% x (number of positive nuclei/total number of nuclei)].

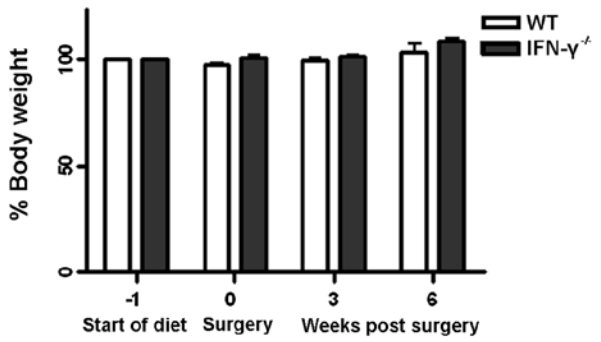

Figure 1. Time-course of relative body weight of wild-type (WT) and interferon (IFN)-$\gamma^{-/-}$ mice. Body weight was examined at the start of the atherogenic diet, at surgery and post-surgery. WT and IFN-$\gamma^{-/-}$ mice showed increased body weight, with no significant differences detected between the two groups at any time point. Data are presented as mean ± SEM.

Table I. Plasma lipid profile.

	TC (mmol/l)	LDL-C (mmol/l)
WT - Standard diet	2.28±0.10	0.24±0.02
WT - Atherogenic diet	4.29±0.47[a]	1.99±0.40[a]
IFN-$\gamma^{-/-}$ - Standard diet	2.34±0.08	0.21±0.05
IFN-$\gamma^{-/-}$ - Atherogenic diet	3.27±0.15[a]	0.97±0.07[b]

TC and LDL-C were examined in wild-type (WT) and interferon (IFN)-$\gamma^{-/-}$ mice that were placed on standard diet or 7 weeks of an atherogenic diet, corresponding to 6 weeks post-surgery groups. The atherogenic diet significantly increased TC and LDL-C levels in both groups of mice ([a]P<0.05, [b]P<0.001).

The presence of monocytes/macrophages was assessed using anti-F4/80 antibody and the average number positive cells per cross section of the artery were determined. The neointimal α-SMA composition and TNF-α and PDGF-B expression were examined using image analysis software ImagePro Plus 4.5 (Diagnostic Instruments) to detect and express the positive (brown-labelled) area as a percentage of total neointimal area.

Statistical analysis. All data are expressed as means ± standard error of the mean (SEM). Data analysis was carried out by Student's t-tests and two-way ANOVA using the statistical program in GraphPad Prism Version 4.0 (GraphPad Software, San Diego, CA, USA). A value of P<0.05 was considered statistically significant.

Results

Time-course of relative body weight and plasma lipid profile. All mice recovered well following surgery, and gained normal mobility and function within 24 h. The relative body weight of wild-type (WT) and IFN-$\gamma^{-/-}$ mice increased over the 7-week period, with no significant differences detected between the two groups (Fig. 1).

Placement of mice on the atherogenic diet for 7 weeks (corresponding to 6 weeks post-surgery), significantly elevated total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) levels in both WT and IFN-$\gamma^{-/-}$ mice, in comparison to each group's corresponding standard diet group (P<0.05) (Table I). As expected, IFN-γ deficiency did not alter TC and LDL-C levels in the standard diet groups. However, in the atherogenic diet groups, IFN-γ deficiency resulted in ~24 and 51% reduction in TC and LDL-C levels respectively, although without statistical significance.

Effect of IFN-γ deficiency on neointimal formation following endoluminal injury. The histology of the non-injured (control, right) iliac arteries of WT and IFN-$\gamma^{-/-}$ mice displayed three distinct layers: the intima, media and adventitia (Fig. 2). The arteries were of normal size and thickness, and no histological differences were detected between the two groups. The histopathology of the probe-injured (left) iliac arteries also displayed the three distinct layers (Fig. 2, weeks 3 and 6). However, in both groups, the intimal layer had a high cell density, was substantially thickened and temporal luminal narrowing was observed. A large proportion of the cells in the thickened intima are VSMCs, as confirmed by α-smooth muscle actin (SMA) immunohistochemical staining (Fig. 3).

The effect of IFN-γ deficiency on the extent of neointimal formation was evaluated from two indices using image analysis: i) the intimal area as a percentage of the intimal plus medial area [%I/(I+M)], and ii) the intimal area as a percentage of the medial area (%I/M) (Fig. 2). Both quantifications demonstrated temporal neointimal formation in the probe-injured iliac arteries of WT and IFN-$\gamma^{-/-}$ mice. From the 3-week time point, the WT group displayed near-occlusive neointimal formation. IFN-$\gamma^{-/-}$ mice exhibited significant delay in neointimal formation at the 3-week time point in comparison to their WT counterpart [%I/(I+M): WT, 56.2±2.5 vs. IFN-$\gamma^{-/-}$, 41.1±3.4 , P<0.01; %I/M: WT, 135.8±14.3 vs. IFN-$\gamma^{-/-}$, 72.6±9.9, P<0.01]. By the 6-week time point, no significant differences were observed between the two groups.

The histological analysis of the non-injured (control, right) iliac arteries was carried out to examine baseline values for normal %I/(I+M) and %I/M for WT and IFN-$\gamma^{-/-}$ mice. Morphometry analysis of the non-injured arteries demonstrated consistent values across both time points, with no significant differences between the two groups [at 3 weeks post-surgery; %I/(I+M): WT, 10.8±0.4 vs. IFN-$\gamma^{-/-}$, 10.9±0.6, P>0.05; %I/M: WT, 12.2±0.4 vs IFN-$\gamma^{-/-}$, 12.3±0.8, P>0.05].

Detection of endothelium, neointimal PDGF-B expression, VSMC composition. The extent of endothelial cell coverage in the WT and IFN-$\gamma^{-/-}$ groups was assessed to examine whether differences in neointimal formation was due to differences in the extent of re-endothelialization following injury. A number of markers have been reported for endothelial cells; however many are additionally localized with other cell types (20). Therefore, to achieve a more reliable assessment of re-endothelialization, immunohistochemical staining was performed using both CD31 and von Willebrand factor (VWF) markers for endothelial cells. Near-complete endothelial cell coverage was detected in the injured arteries of WT and IFN-$\gamma^{-/-}$ mice, using both CD31 and VWF markers (Fig. 3). No significant differences in the extent of re-endothelialization were observed between the two groups,

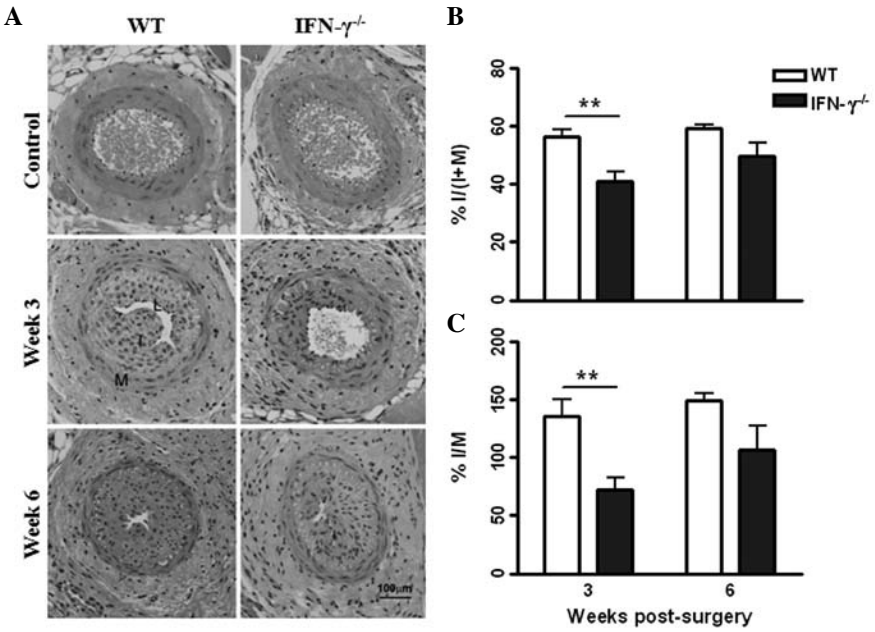

Figure 2. The effect of IFN-γ deficiency on neointimal formation following endoluminal injury. (A) Representative photomicrographs of hematoxylin and eosin (H&E) stained sections of non-injured (control, right), and probe-injured (left) iliac arteries of wild-type (WT) and interferon (IFN)-$\gamma^{-/-}$ mice at 3 and 6 weeks following endoluminal endothelial injury. The control arteries of WT and IFN-$\gamma^{-/-}$ mice displayed normal intimal thickness and histology. The injured arteries exhibited temporal neointimal formation. Lumen (L), intima (I) and media (M) are labelled. Bar represents 100 μm. Bar graphs show temporal neointimal formation in the injured arteries of WT and IFN-$\gamma^{-/-}$ mice, evaluated by the intimal area as a percentage of the intimal plus medial area [%I/(I+M)] (B) and intimal area as a percentage of medial area (%I/M) (C). IFN-$\gamma^{-/-}$ mice displayed significantly reduced neointimal formation at 3 weeks post-surgery using both indices (**P<0.01). Data are expressed as mean ± SEM.

with CD31-positive cells covering 95.8±3.0 and 97.6±2.1%, and VWF-positive cells covering 92.4±2.9 and 85.6±2.9% (Fig. 3) of the arterial circumference at 3 weeks post-surgery in WT and IFN-$\gamma^{-/-}$ mice, respectively.

Immunohistochemical staining was performed for PDGF-B (Fig. 3), an important VSMC mitogen and chemoattractant (21). PDGF-B was detected in both intima and media layers. IFN-$\gamma^{-/-}$ mice exhibited reduced neointimal proportion of PDGF-B-positive area in comparison to their WT counterpart, with a significant difference at 3 weeks post-surgery (WT, 8.6±1.9% vs. IFN-$\gamma^{-/-}$, 2.5±0.4%; P<0.05) (Fig. 3). To examine neointima composition, the presence of VSMCs (α-SMA) (Fig. 3) was examined by immunohistochemistry. Image analysis revealed that VSMCs are a major component of the neointima in this model, consistent with our previous findings (17,18). Similarly, IFN-$\gamma^{-/-}$ mice exhibited significantly reduced proportion of neointimal VSMCs in comparison to their WT counterpart, with a significant difference at 3 weeks post-surgery (WT, 40.6±0.8% vs. IFN-$\gamma^{-/-}$, 22.5±1.5%; P<0.05) (Fig. 3).

Macrophage infiltration, TNF-α expression, and proliferative activity. Immunohistochemical analysis was performed to examine macrophages/monocytes infiltration (F4/80) (Fig. 4) within the vessel. Macrophages were primarily detected in the adventitia layer of the arteries and only a few were present within the neointima, consistent with our previous finding (17). IFN-$\gamma^{-/-}$ mice displayed a significant reduction in the number of vessel macrophages in comparison to their WT counterpart at both 3 (WT, 24.5±3.1% vs. IFN-$\gamma^{-/-}$, 10±0.5%; P<0.05) and 6 weeks post-surgery (WT, 17±2.3% vs. IFN-$\gamma^{-/-}$, 6.5±1.8%; P<0.01) (Fig. 4). Deficiency in both IFN-γ and the pro-inflammatory cytokine TNF-α has been shown to synergistically inhibit injury-mediated neointimal formation (22), and therefore TNF-α expression was assessed by immunohistochemistry (Fig. 4). IFN-$\gamma^{-/-}$ mice displayed lower neointimal TNF-α expression at both time points, although without statistical significance (Fig. 4).

To assess whether the differences in neointimal formation are due to variations in cellular proliferation, immunohistochemistry using the cell proliferation markers; Ki67 and PCNA was performed. Although Ki-67 nuclear antigen is expressed in G1 through M phase and absent from G0 phase, the antibody may not recognise all proliferating cells, such as those with a long, or at the end of, the G1 phase or those entering the S phase of the cell cycle (23). However, proliferating nuclear cell antigen (PCNA) signal increases in G1 phase, with a maximum at S phase, and declines during G2/M phase of the cell cycle (24). Therefore, both PCNA and Ki-67 labelling were assessed to take into account these differences (Fig. 4). Higher Ki-67 labelling index was observed in comparison to PCNA, in both groups of mice. IFN-$\gamma^{-/-}$ mice exhibited a significant reduction in neointimal labelling index for both PCNA (WT, 4.0±0.5% vs. IFN-$\gamma^{-/-}$, 2.5±0.4%; P<0.05) and Ki-67 (WT, 82.1±1.6% vs. IFN-$\gamma^{-/-}$, 75.0±2.0%; P<0.05) (Fig. 4), in comparison to their WT counterpart, at 3 weeks post-surgery.

Discussion

The present study demonstrates that in this model of endoluminal injury combined with dietary-induced hypercholesterolemia, the overall effect of IFN-γ is to enhance neointimal formation.

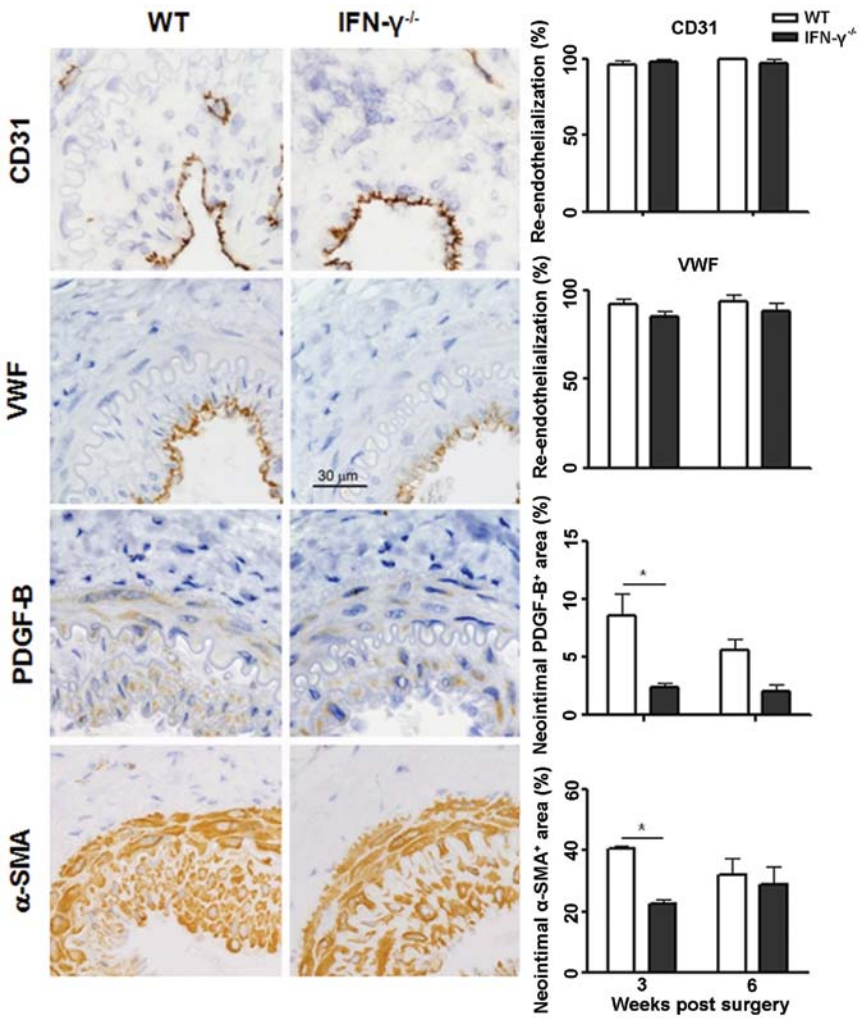

Figure 3. Detection of endothelium, neointimal PDGF-B expression, VSMC composition. Probe-mediated injury was induced in the iliac arteries of wild-type (WT) and interferon (IFN)-$\gamma^{-/-}$ mice, and the arteries were examined at 3 and 6 weeks post-surgery. Immunohistochemical staining for endothelial cells (CD31 and VWF), PDGF-B expression and VSMC (α-SMA) were performed. Representative photomicrographs at 3 weeks post-surgery are displayed. Bar graphs display percentage re-endothelialization assessed by CD31 and VWF, and PDGF-B expression and VSMC, at 3 and 6 weeks post-surgery. There were no significant differences in the extent of re-endothelialization between WT and IFN-$\gamma^{-/-}$ mice using both markers. IFN-$\gamma^{-/-}$ mice displayed significantly reduced neointimal proportion of PDGF-B- and α-SMA-positive area at 3 weeks post-surgery ($^{*}P<0.05$). Data are expressed as mean ± SEM.

IFN-γ deficiency led to a significant reduction in endothelial injury-induced neointimal formation at the 3-week time point. In WT mice, endoluminal injury of the iliac artery resulted in rapid neointimal responses at the initial time point (3 weeks) and appeared to stabilize by the final time point (6 weeks). IFN-$\gamma^{-/-}$ mice exhibited delayed injury-induced neointimal formation at the earlier time point, although achieved a similar extent of neointimal formation by the final time-point, in comparison to their WT counterpart. The significant reduction in neointimal formation in the IFN-$\gamma^{-/-}$ group is consistent with decreased macrophage infiltration, and reduced neointimal VSMC composition, PDGF-B expression and cellular proliferation, with no differences in re-endothelialization between the two groups of mice.

Restenosis is multifactorial, and has been attributed to elastic recoil, neointimal proliferation induced by vessel wall injury, and negative remodelling (1). Inflammatory mechanisms have been indicated to play a pivotal role in neointimal formation and restenosis. The pleiotropic pro-inflammatory cytokine IFN-γ has been implicated in the pathogenic processes of restenosis (11-13). However, IFN-γ has been shown to both promote (11) and inhibit (14) injury-mediated neointimal formation, and therefore warrants further investigation.

Of note, mild hypercholesterolemia has been demonstrated to enhance the degree of stenosis and VSMC proliferation, and significantly elevate systemic IFN-γ and TNF-α levels, in a newly developed model of porcine vascular injury combined with a high cholesterol diet (15). Additionally, hypercholesterolemia has been shown to induce macrophage infiltration following balloon injury via NF-κB activation and increase ICAM-1 expression in rabbits (25), and increase PDGF-A and -B mRNA expression in circulating mononuclear cells in patients, in comparison to normocholesterolemic individuals (26). The effect of hypercholesterolemic state on the role of IFN-γ in the acute injury model remains to be explored. Therefore, the current study utilised an injury-induced model of restenosis combined with the atherogenic diet to examine the role of IFN-γ on neointimal formation in a hypercholesterolemic state, in an attempt to recapitulate the processes that occur in humans following angioplasty. Notably, the Th1

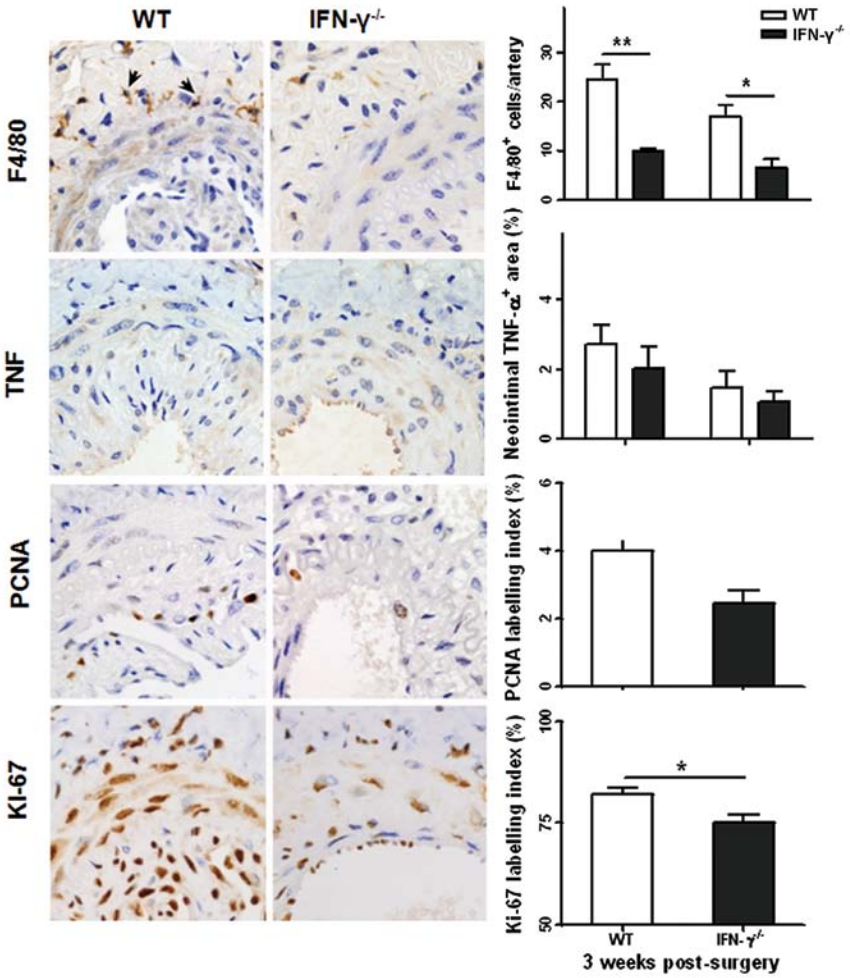

Figure 4. Macrophage infiltration, TNF-α expression, and proliferative activity. Endoluminal endothelial injury was induced in the iliac arteries of wild-type (WT) and interferon (IFN)-$\gamma^{-/-}$ mice, and the arteries were examined at 3 and 6 weeks post-surgery. Immunohistochemical staining for macrophages (F4/80), TNF-α, PCNA and Ki-67 expression were performed. Representative photomicrographs at 3 weeks post-surgery are displayed. Arrows indicate macrophages. Bar graphs display the average number of F4/80-positive cells per cross section of the artery, percentage neointimal TNF-α-positive area, and the neointimal percentage labelling index of PCNA- or Ki-67-positive cells. IFN-$\gamma^{-/-}$ mice displayed significantly reduced F4/80-positive cells at both 3 and 6 weeks post-surgery ($^{*}P<0.05$, $^{**}P<0.01$). There was no significant difference in TNF-α expression between WT and IFN-$\gamma^{-/-}$ mice. IFN-$\gamma^{-/-}$ mice displayed significantly reduced PCNA and Ki-67 percentage labelling index in comparison to their WT counterpart ($^{*}P<0.05$). Data are expressed as mean ± SEM.

pattern of cellular immune responses is predominant in human atheroma and in animal studies of atherosclerosis (27). Thus, in the present study C57Bl/6 strain of mice was utilized, as this strain is thought to be amongst the most Th1-responsive strains, and therefore more responsive to deficiency in Th1 polarising cytokines, principally IFN-γ (28).

In the present study, the inclusion of the atherogenic diet led to elevated TC and LDL-C levels, as previously documented (29), whilst IFN-γ deficiency did not significantly alter TC and LDL-C levels, in line with the findings of non-injury atherosclerotic model in LDLR$^{-/-}$ mice (30), and ApoE$^{-/-}$ mice (28). Furthermore, the inclusion of the atherogenic diet accelerated neointimal formation in WT mice, in comparison to our previous study of the endoluminal injury model in normocholesterolemic WT mice (17), indicating that dietary-induced hypercholesterolemia promotes injury-mediated neointimal formation.

Endothelial injury is an important contributing mechanism to restenosis, and endothelial regeneration has inhibitory effects on neointimal formation and development of restenosis (31). In the present study, re-endothelialization was near complete in both WT and IFN-$\gamma^{-/-}$ mice at the 3-week time point, indicating that IFN-γ deficiency did not alter the extent of re-endothelialization, consistent with the findings in BALB/c mice on standard diet (22). Such data suggest that the differences in neointimal formation between WT and IFN-$\gamma^{-/-}$ mice are due to injury-induced neointimal responses, rather than variations in regulation of endothelial growth.

Macrophage infiltration plays an important role in the pathogenesis of restenosis, supported by a higher abundance in atherectomy tissue from patients that develop restenosis (32). Moreover, systemic inactivation and depletion of monocytes/macrophages results in reduced neointimal formation in injury models (33). In the present study, the significant reduction in vessel macrophage infiltration detected in the IFN-$\gamma^{-/-}$ mice is in line with the important role of IFN-γ in activation and/or recruitment of monocytes/macrophages (4,5). Thus, it is speculated that the decreased macrophage infiltration in the IFN-$\gamma^{-/-}$ mice may have altered the pool of vessel cytokines and growth factors (34), attenuating injury-mediated vessel

responses and leading to reduced neointimal formation in these mice. In this study, macrophages were predominantly found within the adventitia layer of the artery, consistent with our previous finding (17). This is in support of the increasing evidence that vascular inflammation begins in the adventitia and progresses inward towards the intimal layer (35).

In addition, PDGF-mediated pathway may be responsible for the observed reduction in neointimal formation in the IFN-$\gamma^{-/-}$ mice. PDGF is produced by a number of cells including activated macrophages and SMCs, and PDGF-BB has a well-recognised role in the pathogenesis of restenosis (36). In the present study, reduced infiltrating macrophage in the IFN-$\gamma^{-/-}$ group may have contributed to lower PDGF-B chain expression, corresponding to decreased neointimal formation at the 3-week time point. Moreover, PDGF-BB gene expression has been demonstrated to enhance neointimal formation by stimulating proliferation and migration of SMC into the intima layer, as well as increasing extracellular matrix synthesis (21). Furthermore, exogenous IFN-γ has been shown to potentiate the proliferative effect of PDGF-BB and up-regulate PDGF-β receptor in VSMCs, without having direct mitogenic effects (7). Thus, in the current study, the reduced PDGF-B chain expression may be responsible for the significant reduction in neointimal VSMCs and proliferation in the IFN-$\gamma^{-/-}$ mice, consistent with lower neointimal formation, suggesting that the PDGF-B-mediated pathway plays an important role in neointimal formation in this model.

Within the pro-inflammatory cytokine cascade, there is redundancy and overlap of effects, therefore interpretation of data obtained in relation to gene deficiency is complex (37). In the present study, IFN-$\gamma^{-/-}$ mice reached the same stable neointimal size as the WT group at the 6-week time point, despite the IFN-$\gamma^{-/-}$ group displaying reduced macrophage infiltration. This may be due to inflammatory pathway redundancy or other factors influencing injury-mediated neointimal responses. Interestingly, deficiency in both IFN-γ and TNF-α has been shown to synergistically inhibit neointimal formation (22). In the present study, IFN-$\gamma^{-/-}$ mice displayed reduced neointimal TNF-α expression, although without statistical significance. Further evaluation of other cytokines and growth factors may allow for clarification of this multifaceted biological process.

There are a number of limitations associated with this study. The model used in the present study is injury-mediated; however, the procedure was performed on non-atherosclerotic arteries, and may not accurately resemble the vascular processes that occur in human coronary artery angioplasty. Moreover, whilst the inclusion of the atherogenic diet allowed for induction of hypercholesterolemia in a timely manner, the effects of the diet may not precisely represent human hypercholesterolemia. Thus, further investigations are required to accurately determine the role of IFN-γ in the pathogenesis of restenosis in clinical settings.

In conclusion, these data demonstrated that the absence of IFN-γ markedly reduced neointimal formation, consistent with reduced inflammatory responses and proliferative effects, in a mouse model of endoluminal injury combined with dietary-induced hypercholesterolemia. However, in the absence of IFN-γ, other inflammatory pathway(s) may exhibit compensatory effects with time. This suggests that in combination, modulating the IFN-γ-mediated pathway as well as targeting other inflammatory cytokines and growth factors may prove to be beneficial in controlling neointimal formation and restenosis.

Acknowledgements

We thank Dr Jane Radford for her expert technical immunohistochemical assistance.

References

1. Li JJ, Nie SP, Zhang CY, Gao Z, Zheng X and Guo YL: Is inflammation a contributor for coronary stent restenosis? Med Hypotheses 68: 945-951, 2007.
2. Bennett MR: In-stent stenosis: pathology and implications for the development of drug eluting stents. Heart 89: 218-224, 2003.
3. Schwartz RS, Holmes DR Jr and Topol EJ: The restenosis paradigm revisited: an alternative proposal for cellular mechanisms. J Am Coll Cardiol 20: 1284-1293, 1992.
4. Harvey EJ and Ramji DP: Interferon-gamma and atherosclerosis: pro- or anti-atherogenic? Cardiovasc Res 67: 11-20, 2005.
5. Schroder K, Hertzog PJ, Ravasi T and Hume DA: Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 75: 163-189, 2004.
6. Leon ML and Zuckerman SH: Gamma interferon: a central mediator in atherosclerosis. Inflamm Res 54: 395-411, 2005.
7. Tellides G, Tereb DA, Kirkiles-Smith NC, *et al*: Interferon-gamma elicits arteriosclerosis in the absence of leukocytes. Nature 403: 207-211, 2000.
8. Nunokawa Y and Tanaka S: Interferon-gamma inhibits proliferation of rat vascular smooth muscle cells by nitric oxide generation. Biochem Biophys Res Commun 188: 409-415, 1992.
9. Hansson GK, Jonasson L, Holm J, Clowes MM and Clowes AW: Gamma-interferon regulates vascular smooth muscle proliferation and Ia antigen expression in vivo and in vitro. Circ Res 63: 712-719, 1988.
10. Muhl H and Pfeilschifter J: Anti-inflammatory properties of pro-inflammatory interferon-gamma. Int Immunopharmacol 3: 1247-1255, 2003.
11. Kusaba K, Kai H, Koga M, *et al*: Inhibition of intrinsic interferon-gamma function prevents neointima formation after balloon injury. Hypertension 49: 909-915, 2007.
12. Paroli M, Mariani P, Accapezzato D, *et al*: Modulation of tachykinin and cytokine release in patients with coronary disease undergoing percutaneous revascularization. Clin Immunol 112: 78-84, 2004.
13. Zohlnhofer D, Richter T, Neumann F, *et al*: Transcriptome analysis reveals a role of interferon-gamma in human neointima formation. Mol Cell 7: 1059-1069, 2001.
14. Hansson GK and Holm J: Interferon-gamma inhibits arterial stenosis after injury. Circulation 84: 1266-1272, 1991.
15. Busnelli M, Froio A, Bacci ML, *et al*: Pathogenetic role of hypercholesterolemia in a novel preclinical model of vascular injury in pigs. Atherosclerosis 207: 384-390, 2009.
16. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A and Stewart TA: Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. Science 259: 1739-1742, 1993.
17. Harris AK, Shen J, Radford J, Bao S and Hambly BD: GM-CSF deficiency delays neointima formation in a normolipidemic mouse model of endoluminal endothelial damage. Immunol Cell Biol 87: 122-130, 2009.
18. Shen J, White M, Husband AJ, Hambly BD and Bao S: Phytoestrogen derivatives differentially inhibit arterial neointimal proliferation in a mouse model. Eur J Pharmacol 548: 123-128, 2006.
19. Bao S, Beagley KW, Murray AM, *et al*: Intestinal IgA plasma cells of the B1 lineage are IL-5 dependent. Immunology 94: 181-188, 1998.
20. Middleton J, Americh L, Gayon R, *et al*: A comparative study of endothelial cell markers expressed in chronically inflamed human tissues: MECA-79, Duffy antigen receptor for chemokines, von Willebrand factor, CD31, CD34, CD105 and CD146. J Pathol 206: 260-268, 2005.
21. Pompili VJ, Gordon D, San H, *et al*: Expression and function of a recombinant PDGF B gene in porcine arteries. Arterioscler Thromb Vasc Biol 15: 2254-2264, 1995.

22. Murayama H, Takahashi M, Takamoto M, *et al*: Deficiency of tumour necrosis factor-alpha and interferon-gamma in bone marrow cells synergistically inhibits neointimal formation following vascular injury. Cardiovasc Res 80: 175-180, 2008.
23. Aoyagi M, Yamamoto M, Wakimoto H, Azuma H, Hirakawa K and Yamamoto K: Immunohistochemical detection of Ki-67 in replicative smooth muscle cells of rabbit carotid arteries after balloon denudation. Stroke 26: 2328-2332, 1995.
24. Kurki P, Ogata K and Tan EM: Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. J Immunol Methods 109: 49-59, 1988.
25. Song JM, Kim HS, Park SJ, *et al*: Effect of hypercholesterolemia on macrophage infiltration after balloon injury to rabbit iliac artery. Jpn Circ J 65: 117-122, 2001.
26. Billett MA, Adbeish IS, Alrokayan SA, Bennett AJ, Marenah CB and White DA: Increased expression of genes for platelet-derived growth factor in circulating mononuclear cells of hypercholesterolemic patients. Arterioscler Thromb Vasc Biol 16: 399-406, 1996.
27. Hansson GK: Inflammatory mechanisms in atherosclerosis. J Thromb Haemost 7 (Suppl 1): 328-331, 2009.
28. Whitman SC, Ravisankar P and Daugherty A: IFN-gamma deficiency exerts gender-specific effects on atherogenesis in apolipoprotein $E^{-/-}$ mice. J Interferon Cytokine Res 22: 661-670, 2002.
29. Vergnes L, Phan J, Strauss M, Tafuri S and Reue K: Cholesterol and cholate components of an atherogenic diet induce distinct stages of hepatic inflammatory gene expression. J Biol Chem 278: 42774-42784, 2003.
30. Buono C, Come CE, Stavrakis G, Maguire GF, Connelly PW and Lichtman AH: Influence of interferon-gamma on the extent and phenotype of diet-induced atherosclerosis in the LDLR-deficient mouse. Arterioscler Thromb Vasc Biol 23: 454-460, 2003.
31. Kipshidze N, Dangas G, Tsapenko M, *et al*: Role of the endothelium in modulating neointimal formation: vasculoprotective approaches to attenuate restenosis after percutaneous coronary interventions. J Am Coll Cardiol 44: 733-739, 2004.
32. Moreno PR, Bernardi VH, Lopez-Cuellar J, *et al*: Macrophage infiltration predicts restenosis after coronary intervention in patients with unstable angina. Circulation 94: 3098-3102, 1996.
33. Danenberg HD, Fishbein I, Gao J, *et al*: Macrophage depletion by clodronate-containing liposomes reduces neointimal formation after balloon injury in rats and rabbits. Circulation 106: 599-605, 2002.
34. Libby P, Schwartz D, Brogi E, Tanaka H and Clinton SK: A cascade model for restenosis. A special case of atherosclerosis progression. Circulation 86: 11147-11152, 1992.
35. Maiellaro K and Taylor WR: The role of the adventitia in vascular inflammation. Cardiovasc Res 75: 640-648, 2007.
36. Heldin CH and Westermark B: Mechanism of action and in vivo role of platelet-derived growth factor. Physiol Rev 79: 1283-1316, 1999.
37. Zhou Z, Lauer MA, Wang K, *et al*: Effect of anti-tumor necrosis factor-alpha polyclonal antibody on restenosis after balloon angioplasty in a rabbit atherosclerotic model. Atherosclerosis 161: 153-159, 2002.