Tumor Necrosis Factor Receptor-2 Signaling Attenuates Vein Graft Neointima Formation by Promoting Endothelial Recovery


Objective—Inflammation appears intricately linked to vein graft arterialization. We have previously shown that tumor necrosis factor (TNF) receptor-1 (TNFR1, p55) signaling augments vein graft neointimal hyperplasia (NH) and remodeling through its effects on vascular smooth muscle cells (SMCs). In this study we examined the role of TNFR2 (p75) signaling in vein graft arterialization.

Methods and Results—Inferior vena cava-to-carotid artery interposition grafting was performed between p75−/− and congenic (C57BI/6J) wild-type (WT) mice. Six weeks postoperatively, neointimal and medial dimensions were greater in p75−/− grafts placed into p75−/− recipients (by 42% or 60%, respectively; P<0.05), when compared with WT veins grafted into WT recipients. Relative to WT vein grafts, p75 deficiency augmented early (2-week-old) graft vascular cell adhesion molecule (VCAM)-1 expression (by 2.4-fold, P<0.05), increased endothelial cell apoptosis (2-fold), and delayed graft re-endothelialization. Both cellular proliferation in early, and collagen I content of mature (6-week-old) vein grafts were increased (by 70% and 50%, respectively) in p75−/− grafts. P75 deficiency augmented TNF-induced apoptosis of cultured endothelial cells, but did not affect TNF-stimulated SMC proliferation or migration induced by co-cultured macrophages.


Key Words: TNF ■ inflammation ■ vascular remodeling ■ mouse models ■ endothelial cells

Vein graft failure occurs largely because an atherosclerosis-prone neointima develops in the grafted vein consequent to the hyperdistension and barotrauma that accompanies placement into the arterial circuit. Recent evidence points to injury-induced inflammation, including macrophage infiltration and medial smooth muscle cell activation, as promoting the development of vein graft neointimal hyperplasia (NH). Among many different cytokines and growth factors secreted by macrophages, tumor necrosis factor (TNF) is expressed in vein grafts and appears to play a major role in neointimal hyperplasia of not only vein grafts but also arteries. TNF promotes expression of adhesion molecules on both vascular smooth muscle cells (SMCs) and endothelial cells (ECs), stimulates expression of cell-recruiting chemokines, and induces EC apoptosis, while simultaneously promoting SMC proliferation and migration. It signals via two different receptors, p55 (CD120a, TNFR1) and p75 (CD120b, TNFR2) that share ligand-binding domain homology while possessing divergent intracellular signal transduction moieties.

TNF is expressed in early vein grafts at the peak of the remodeling response in vivo and by activated SMCs in vitro. Although vein grafts lacking p55 demonstrate reduced NH, the role of p75 signaling has not been examined in this setting. In the present study we used cross transplantation of veins from C57Bl/6 and congenic p75−/− mice to examine the consequences of p75 signaling on vein graft arterialization.

Methods

For Supplemental Methods please see http://atvb.ahajournals.org.

Mice

Adult male C57Bl/6 wild-type (WT) and congenic p75-deficient (p75−/−) or p55-deficient (p55−/−) mice were purchased from Jackson Labs. All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals.

Vein Graft Surgery

Interposition vein graft surgery was performed as described previously. Inferior vena cavae from WT or congenic p75−/− or p55−/− donor mice were anastomosed to the right common carotid artery of

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284
recipient mice, in 5 different donor/recipient combinations as follows: (1) WT/WT; (2) p75\textsuperscript{+/+}/WT; (3) p75\textsuperscript{+-}/p75\textsuperscript{+-}; (4) p55\textsuperscript{+-}/WT; and (5) p55\textsuperscript{+-}/p55\textsuperscript{+-}. All graft donors and recipients were age-matched and between 12 and 20 weeks old at the time of surgery. We did not place WT IVC grafts into p75\textsuperscript{-/-} mice because expression of p75 on the surface of vein graft wall cells would almost certainly elicit a mild allograft rejection in p75\textsuperscript{-/-} recipients, as we observed when we placed WT grafts into p55\textsuperscript{-/-} recipients,\textsuperscript{4} or WT arterial grafts into p55\textsuperscript{-/-} recipients.\textsuperscript{5}

**Statistical Analysis**

One-way ANOVA with Tukey post test for multiple comparisons was used to analyze migration, proliferation, cytotoxicity, and morphometric data as well as protein expression for VCAM-1 and factor VIII. T-test was used to analyze expression levels of cleaved caspase-3, Ki-67, and collagen. Data are presented ±SD in the text and ±SE in the figures.

**Results**

**p75 Signaling Attenuates Vein Graft Neointimal Hyperplasia**

In our murine carotid interposition vein graft model, WT vein grafts attain their final wall thickness (∼14 cell layers) 4 weeks after implantation, and develop a neointima comprising predominantly SMC-like cells.\textsuperscript{1,11} Two different cell populations participate in the arterialization of vein grafts: (1) graft-intrinsic cells (cells that are part of the graft at the time of implantation), and (2) graft-extrinsic cells (cells that are recruited into the graft from the recipient). Graft-extrinsic cells ultimately make up the majority of the vein graft neointima and a significant portion of the vein graft endothelium.\textsuperscript{2} As in the preatherosclerotic early stage of human vein graft disease, these murine grafts do not show significant luminal stenosis.\textsuperscript{11}

To determine whether p75 TNF receptor signaling affects vein graft remodeling, we placed inferior vena cava (IVC) grafts from WT or congenic p75\textsuperscript{-/-} mice into the common carotid artery of WT or p75\textsuperscript{-/-} recipients. In the case of p75\textsuperscript{-/-} IVCs grafted into WT recipients, we thus created chimeric mice that were p75-deficient only in cells of the vein graft wall; this surgically created chimera facilitated examining the role of p75 signaling in both vein graft-extrinsic and -intrinsic cells. Compared with WT vein grafts placed in WT mice (WT/WT, “control” grafts), these p75\textsuperscript{-/-} vein grafts placed into WT mice (p75\textsuperscript{-/-}/WT grafts) demonstrated 27±15% greater neointimal thickness but comparable medial thickness (Figure 1). When we placed p75\textsuperscript{-/-} vein grafts in p75\textsuperscript{-/-} mice (p75\textsuperscript{-/-}/p75\textsuperscript{-/-} grafts), we observed a 42±19% increase in average neointimal thickness and a 60±26% increase in medial thickness, compared with control WT/WT grafts (P<0.05, Figure 1). Lumen area was not significantly different among the groups (data not shown). Thus, p75 expression in both vein graft-intrinsic and -extrinsic cells reduced neointimal hyperplasia, such that the total level of systemic p75 (the sum of graft-intrinsic and -extrinsic cell expression) correlated inversely with average neointimal thickness. Intriguingly, the effect of graft-intrinsic cell p75 deficiency on graft neointima formation was opposite in direction from what we found with p55 deficiency.\textsuperscript{4} In addition, vein graft medial hypertrophy was not affected by p55 deficiency in graft-intrinsic or -extrinsic cells,\textsuperscript{4} but it was enhanced significantly by p75 deficiency in graft-extrinsic cells (Figure 1B).

Increased neointimal and medial volume can result from enhanced hyperplasia of graft-intrinsic cells, recruitment or hyperplasia of graft-extrinsic cells, or a combination of these processes. To explore these possibilities, we examined 2-week-old vein grafts, which have not completed the process of arterialization.\textsuperscript{11} In grafts that lacked p75 in both graft-intrinsic and -extrinsic cells, we found evidence for both enhanced cell proliferation and cell recruitment: a 70% increase in the prevalence of proliferating cells, assessed by staining for the proliferation marker Ki-67 (supplemental Figure IA; P<0.05), and a 2.5-fold increase in the expression of VCAM-1 (P<0.05, Figure 2). In contrast, p55 deficiency substantially reduced vein graft VCAM-1 expression.

In mature (6-week-old) vein grafts, greater neointimal and medial volume in p75\textsuperscript{-/-}/p75\textsuperscript{-/-} specimens corresponded to a greater number of cells in these graft layers. Nonetheless, when normalized to cell density, the prevalence of SMC-like cells among neointimal and medial cells was indistinguishable between p75\textsuperscript{-/-}/p75\textsuperscript{-/-} and WT/WT vein grafts, as assessed by immunofluorescence for SMC-actin. Also, we were unable to detect any change in macrophage infiltration (as assessed by staining for the F4/80 macrophage antigen,
data not shown). We then quantitated the collagen I content of the vein grafts to determine whether differences in extracellular matrix or fibroblast-like cell density distinguished p75-deficient grafts from WT/WT specimens. Normalized to cellular density, the collagen content of the media was, indeed, 50% greater in p75-deficient grafts than in WT/WT vein grafts (supplemental Figure I; *P<0.05).

p75 Signaling Augments Vein Graft re-endothelialization
Exposing veins to arterial pressure engenders significant graft endothelial dysfunction and cell loss,2,12,13 whereas the reestablishment of an intact endothelial cell layer limits further neointimal hyperplasia.4,14 TNF has been shown to induce EC apoptosis9 in a manner that may be attenuated by EC p75.15 If p75 signaling indeed contributes to EC survival then, by facilitating vein graft re-endothelialization, p75 could attenuate neointimal hyperplasia. To test this possibility, we examined endothelial resurfacing in vein grafts harvested at both 2 and 6 weeks postoperatively. At 2 weeks post-op, p75 deficiency in both graft-intrinsic and -extrinsic cells caused a 67% relative reduction in graft re-endothelialization (*P<0.05), whereas p75 deficiency in just graft-intrinsic cells caused a much smaller, and not statistically significant, reduction in endothelialization (Figure 3). In contrast, p55 deficiency in graft-intrinsic or -extrinsic cells enhanced vein graft endothelialization. Even 6 weeks postoperatively, when endothelial resurfacing of WT or p55-deficient grafts is essentially complete, p75-deficient donor veins manifested incomplete re-endothelialization (Figure 3).

To explore mechanisms by which p75 signaling contributes to endothelial recovery after vein graft placement, we examined induction of apoptosis in vein graft endothelium by immunostaining for the active (cleaved) form of the apoptosis effector caspase 3 (Figure 4). At 2 weeks postop, p75 deficiency more than doubled the percentage of vein graft endothelium that stained positive for cleaved caspase-3 (*P<0.05, Figure 4B), indicating that p75 expression in vein graft endothelial cells greatly reduces their susceptibility to apoptosis following vein grafting.

Role of p75 Signaling on EC and SMC Physiology
Previous work with virally transduced ECs has suggested that p75 activity protects ECs against TNF-induced apoptosis.15 To determine whether physiological expression of p75 affects EC responses to TNF, we isolated aortic ECs from WT and p75−/− mice and verified their p75 expression status (Figure 5A). In contrast, p55 deficiency in graft-intrinsic or -extrinsic cells enhanced vein graft endothelialization. Even 6 weeks postoperatively, when endothelial resurfacing of WT or p55−/− grafts is essentially complete, p75−/− donor veins manifested incomplete re-endothelialization (Figure 3).

To explore mechanisms by which p75 signaling contributes to endothelial recovery after vein graft placement, we

![Figure 2. VCAM-1 expression is augmented by p75 deficiency and attenuated by lack of p55 signaling. Two-week-old vein grafts harvested from the indicated donor/recipient combinations were immunofluorescently stained for VCAM-1 expression (green, upper panels) and DNA (blue, lower panels). Representative fluorescence photomicrographs of each vein graft type are shown. Scale bar=50 μm (Original magnification ×1100). Arrows indicate the luminal surface of the vein graft. Control specimens stained with nonimmune goat IgG yielded no signal (data not shown). VCAM-1 fluorescence was normalized to DNA fluorescence intensity for each microscopic field; resulting ratios were normalized within each group to those of WT donor/WT recipient samples to yield "% of control". The means±SE of n=3 specimens in each group are graphed. *P<0.05 compared with WT/WT control.](http://atvb.ahajournals.org/issue/2/8/286)

![Figure 3. re-endothelialization is attenuated in p75−/− vein grafts. Vein grafts of the indicated donor/recipient combinations were harvested 2 or 6 weeks postoperation and fixed in formalin. A, Specimen cross-sections were simultaneously stained for Factor VIII expression (green) to assess endothelial coverage and for DNA (blue) as described in the Methods section. B, Endothelial coverage was quantitated using ImageJ software. Scale bar=200 μm (Original magnification ×110). Photomicrographs are representative of n=3 performed for each donor/recipient combination. *P<0.05 compared with WT/WT control.](http://atvb.ahajournals.org/issue/2/8/286)
p75-mediated EC antiapoptotic effect could help to explain why vein graft re-endothelialization correlates directly with the expression of p75 in both graft-intrinsic ECs and graft-extrinsic endothelial progenitor cells.

ECs are exposed to TNF expressed by neointimal SMCs, which are the most abundant cell type in the developing graft neointima. Because both migration and proliferation of SMCs contribute to NH, we sought to investigate the role—if any—of p75 signaling on SMC activities. Cultured SMCs are in an activated state that is characterized by increased proliferation and migration and are thus representative of the SMCs found in vascular lesions. These cells demonstrably express p75 protein, which is lacking in SMCs.
isolated from p75−/− mice (supplemental Figure IIIA). To model the inflammatory milieu of the arterializing vein graft, we examined migration of SMCs toward co-cultured primary macrophages, because macrophages constitute an important fraction of the cells in arterializing vein grafts2,19 and secrete a plethora of cytokines and growth factors that synergistically promote SMC activation.20 In response to these macrophages or to purified TNF or PDGF, p75−/− and WT SMCs demonstrated equivalent migration (supplemental Figure IIIB). Contrastingly, p55−/− SMCs demonstrated no migration to TNF and demonstrated significantly impaired migration to activated macrophages (supplemental Figure IIIB). Just as SMC migration was unaffected by p75 activity, SMC thymidine incorporation in response to TNF, PDGF-BB, or a combination of these 2 agonists was indistinguishable between WT and p75−/− SMCs (supplemental Figure IIIC). In contrast, we have previously shown that TNF-induced proliferation is abolished in p55−/− SMCs.4 Thus, p75 does not appear to mediate changes in SMC physiology that are pertinent to neointimal hyperplasia.

**Discussion**

This study, for the first time, clearly demonstrates the importance of TNF receptor-2 signaling in vein graft arteriolization. We have found that TNF signaling via p75 promotes vein graft re-endothelialization and reduces neointimal hyperplasia—in striking contrast to TNF signaling via p55, which inhibits vein graft reendothelialization and augments neointimal hyperplasia. Although undetectable in uninjured veins and arteries, TNF is expressed in grafted veins as early as 4 days postop.3 TNF expression precedes the onset of neointimal development and remains elevated throughout the duration of graft remodeling. Although predominantly recognized as a growth inhibitory factor produced by macrophages, in vein grafts TNF is also expressed by SMCs and it colocalizes with markers of proliferation in areas of rapid SMC growth.4 This TNF, produced by both SMCs and graft infiltrating macrophages, promotes SMC proliferation and migration via p55.3,4 In addition, TNF enhances recruitment of extrinsic cells into the graft via upregulation of graft chemokines such as MCP-1 and enhanced expression of cell-retaining adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 in SMCs.21 The latter TNF-promoted change in vein graft biology is under opposing control by the 2 TNF receptors, because it is reduced in vein grafts taken from p55−/− and increased in grafts from p75−/− mice.4 Several signaling pathways important to vascular repair appear oppositely regulated in endothelial and vascular smooth muscle cells. A recent report by Chandrasekharan22 has shown ICAM-1 expression to be induced by p75 and attenuated by p55 in endothelial cells, whereas in cultured SMCs, and in proliferative foci of early vein grafts, ICAM-1 is induced by p55.4 Likewise, VCAM-1 expression in early vein grafts is induced by p55 and antagonized by p75, whereas elaboration of this adhesion molecule in ECs appears dependent on p55 and p75 cooperativity.22 Finally, whereas SMCs are stimulated to proliferate by TNF in a p55 dependent manner, this cytokine profoundly retards growth and migration of cultured murine EC,15 induces apoptosis of microvascular,23 cerebrovascular,24 and dermal ECs,25 and is responsible for disseminated endothelial apoptosis observed after administration of lipopolysaccharide (LPS) in vivo.26

Because a significant fraction of the endothelium undergoes apoptosis after vein grafting, and is subsequently being replaced by recipient-derived endothelial progenitor cells,13 we found that the TNF receptor status of the recipient greatly influences the speed of endothelial recovery. Thus endothelial progenitor cells that lack p75 are increasingly likely to undergo apoptosis when adherent to the TNF-expressing SMCs of early vein graft lesions.4 In contrast, vein graft re-endothelialization proceeds faster in p55−/− recipients. To understand reciprocal effects of TNF receptors on vein graft re-endothelialization, it is important to consider TNF receptor-specific signaling pathways. Even though it elicits proliferative signals in SMCs, the death domain-containing p55 can induce apoptosis in ECs through activation of the RIP1-ASK1-JNK/p38 pathway.27 In WT ECs, however, pro-apoptotic p55 activity is mollified by p75-mediated activation of antiapoptotic signaling: the EC-specific Etk/BMX kinase pathway,28,29 and possibly the cIAP-induced ASK1 ubiquitination and degradation.30 Thus, in the context of vein graft hyperdistension injury and TNF elaboration from graft infiltrating leukocytes and activated SMCs, either lack of proapoptotic p55 signaling or activation of antiapoptotic p75 signaling will accelerate EC recovery and limit graft NH.

The contribution of p75 signaling to vascular lesion development appears to be specific to the type of vascular injury induced and may depend on the balance between p55- and p75-evoked signaling in ECs. This balance may be shifted toward p75 under certain pathological conditions such as ischemia15 or inflammation,31 which increase EC p75 expression relative to that of p55.

Thus, results obtained in our vein graft model accord with those generated in the mouse hind limb ischemia model of angiogenesis.15,32 In that model, p75 activity accelerates angiogenesis, which, like vein graft re-endothelialization,2 involves proliferation and migration of local ECs as well as immigration of bone marrow–derived endothelial progenitor cells. In contrast, whereas p75 signaling protects against neointimal hyperplasia in our vein graft model, it appears to have no affect on neointimal hyperplasia induced by wire injury to the carotid artery.33 The apparent discord may result from the relative importance of SMC and EC hyperplasia in these 2 different injury models, or may be attributable to the variable but generally small neointimal hyperplastic response produced by carotid wire injury in the C57BL/6 mouse.34,35

In contrast to neointimal hyperplasia, medial dimension is increased only in p75−/− graft recipients (Figure 1). We have demonstrated significant infiltration of bone marrow–derived cells of the monocyte/macrophage lineage into the media of arterIALIZING vein grafts.3 However, there was no change in the overall cellular density in the vein graft media of p75−/− recipients and we were unable to detect an altered ratio of macrophage to non-macrophage cells. Possibly, an increase in the retention of graft infiltrating macrophages might be accompanied by an increase in the proliferation of nonmacrophage-like cells.
In the course of vein graft arterialization, we have observed that the balance between what is regarded as beneficial medial hypertension and deleterious neointimal hyperplasia appears to be controlled substantially by the extent of vein graft re-endothelialization. This complex process, in turn, appears to be regulated significantly by TNF, through opposing actions of its p55 and p75 receptors. Whether p75 can be targeted productively to treat nonatherosclerotic vein graft neointimal hyperplasia remains to be determined.

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Disclosures

None.

References

14. Zhang et al. TNFR2 Mitigates Neointimal Hyperplasia
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Methods Supplement

Histology

Grafts were harvested 2 to 6 weeks postoperatively and prepared for histochemical and immunofluorescent analysis as we described previously.\textsuperscript{1} Morphometry was performed on vein grafts as described with average neointimal and medial thicknesses calculated from measurements of layer areas.\textsuperscript{2} Immunofluorescence employed rabbit IgG’s against factor VIII (DAKO), VCAM-1, Ki-67, collagen I (all from Santa Cruz Biotech, Inc.), and cleaved caspase-3 (Cell Signaling). Negative control sections were incubated with non-immune rabbit IgG in lieu of primary antibody. Macrophages were identified with rat monoclonal anti-F4/80 IgG (clone A3:1, Abcam), anti-rat IgG/biotin, and streptavidin/FITC; negative controls lacked A3:1, and all sections were pre-treated with unconjugated streptavidin to reduce nonspecific fluorescence.

Cyanine 3-conjugated 1A4 IgG (Sigma) was used to detect SMC actin, as described.\textsuperscript{3} The DNA-binding dye Hoechst 33342 (10 µg/ml) was added to the secondary antibody incubations to visualize nuclei. Single microscopic fields were imaged for multiple fluorophores, as described.\textsuperscript{3}

To quantitate VCAM-1, factor VIII, Ki-67 or collagen I protein expression within the vein grafts, specimens from all vein graft groups were stained and imaged simultaneously, batch-wise. Identical exposure times and incident light intensities were used to visualize each specimen. Images were captured with a SPOT RT CCD camera (Diagnostic Instruments). Four orthogonal clock hours of each specimen were analyzed using ImageJ software (NIH) and averaged. Specific staining was obtained by subtracting mean fluorescence values of negative control specimens from those incubated with the relevant primary antibody. Specific immunofluorescence was normalized to cognate nuclear fluorescence in the same microscopic field. These ratios were averaged among all vein grafts within each group. Thus, VCAM-1, Ki-
67 or collagen immunofluorescence was compared in a manner that accounted for the cellularity of each specimen. Endothelialization was quantitated as the fraction of the vein graft lumen perimeter occupied by cells positive for factor VIII. All quantitation was performed by observers blinded to specimen identity. Apoptosis of vein graft endothelium was calculated as the fraction of the vein graft luminal cells double positive for factor VIII and cleaved caspase 3, divided by the luminal cells positive for factor VIII only. All quantitation was performed by observers blinded to specimen identity.

**SMC Migration and [³H]Thymidine Incorporation Assay**

Primary murine SMCs were derived from aortas of C57Bl/6 or congenic p75<sup>−/−</sup> mice by explant outgrowth and rendered quiescent by 36 h culture in serum free medium (SFM) as described. Each SMC experiment was performed with at least 2 independently isolated SMC lines of each genotype. Migration of quiescent murine SMCs towards murine TNF, human PDGF-BB or mouse peritoneal macrophages was assessed in collagen-coated, 24 well plate culture inserts with 8 μm pores (Falcon) as described. Mouse peritoneal macrophages were isolated as described. For thymidine incorporation, primary murine SMCs were rendered quiescent by 48 h culture in SFM. Cells were subsequently incubated with 10 ng/ml murine TNF, 5 ng/ml human PDGF-BB, or both, and incorporation of [³H]thymidine was assessed as described.
Endothelial Cell Cytotoxicity

Mouse endothelial cells were isolated from p75\(^{-/-}\) and congenic WT C57B1/6 mice as described.\(^5\) Briefly, the thoracic aorta was isolated in situ and perfused with PBS containing 1000 U/ml heparin. Following removal of adventita, the aorta was excised, cannulated, filled with collagenase type II solution (2 mg/ml in DMEM, Sigma) and tied on both ends. After a 45-minute incubation at 37\(^\circ\) C, the lumen was flushed with 5 ml of 20% FBS-DMEM and ECs were collected by centrifugation. Cells were plated onto collagen type I-coated dishes and cultured in 20% FBS-DMEM. Non-adherent cells were removed after two hours and the ECs were further incubated in DMEM containing 20% FBS (heat-inactivated), 1× L-glutamate, non-essential amino acids, and sodium pyruvate (Invitrogen), 25 mM HEPES (pH 7.0-7.6), 100 µg/ml heparin, 100 µg/ml EC growth supplement (Sigma), 1× antibiotic/antimycotic (Invitrogen), and 10 µg/ml ciprofloxacin (Sigma). Amphotericin and ciprofloxacin were removed from the medium 1 wk after EC isolation.\(^5\) Purity of endothelial cell preparations was assessed by factor VIII immunofluorescence, and was ≥ 90%.

For cytotoxicity assay, ECs were plated in quadruplicate on day 1 at 2 × 10\(^4\) cell/well in collagen-coated 96-well plates, in EC growth medium containing only 10% FBS, and allowed to attach overnight. On day 2, ECs were re-fed with EC growth medium/10% FBS containing cycloheximide (10 µg/ml), without (control) or with murine TNF (10 ng/ml), and incubated at 37 \(^\circ\)C for 7 hours. Apoptotic ECs were removed by aspiration of the medium and a single wash with Dulbecco’s PBS (Invitrogen). ECs were then stained with 0.5% (w/v) crystal violet/5% (v/v) ethanol for 60 min. After extensive washing, ECs were dried completely, and cell-adsorbed crystal violet was dissolved in 40% acetic acid. The absorbance of each well was then read at 562 nm, and corrected for the absorbance obtained in wells in which no ECs had been
plated (~5% of maximal). The relationship between OD$_{562}$ and EC number was linear from 0 to $5 \times 10^4$ cell/well (data not shown).

**Immunoblot Analysis**

Immunoblotting of cell membrane proteins was performed as described previously,$^4,^6$ with rabbit anti-p75 IgG (sc-7862 Santa Cruz) or non-immune rabbit IgG. Nitrocellulose blotted for p75 (or with non-immune IgG) was subsequently re-probed for actin, as a loading control.$^4$

**References:**


**Figure Legends**

**Supplemental Figure I:** Cell proliferation is enhanced in vein grafts from p75<sup>−/−</sup> mice. A, Murine vein grafts of the indicated donor/recipient combination were harvested two weeks post op, fixed in formalin and immunofluorescently stained for the proliferation marker Ki-67 (red) and DNA (blue). B, Ki-67 expression in the vein graft wall was quantitated using ImageJ software and expressed as fold over WT/WT control. Scale bar = 100 µm. Photomicrographs are representative of n ≥ 3 performed for each combination. *, P < 0.05 compared with WT/WT control.

**Supplemental Figure II:** Increased collagen production in p75<sup>−/−</sup> vein grafts. A, Murine vein grafts of the indicated donor/recipient combination were harvested six weeks post op, fixed in formalin and subjected to modified trichrome stain to visualize neointima/media boundary. Serial sections were immunofluorescently stained for collagen I (red) and DNA (blue). B, vein graft wall collagen I expression was quantitated using ImageJ software and normalized to DNA content. The collagen/DNA ratio was then normalized to that obtained for the neointimal layer of WT/WT grafts and plotted as mean ± S.E., *, P < 0.05 compared with WT/WT control.
Supplemental Figure III: SMC migration and proliferation are not regulated by p75 signaling.

A, p75 expression is demonstrated in cultured primary macrophages (Mφ) and vascular smooth muscle cells by SDS-PAGE/Immunoblot analysis (IB) using 20 µg of membrane protein. Blots were sequentially probed with anti-p75 and anti-pan actin antibodies. A single IB is shown, representative of ≥ 3 performed.

B, Migration of quiescent aortic SMCs derived from congenic WT, p75−/−, or p55−/− mice was assessed in response to murine TNF (10 ng/ml), PDGF-BB (50 ng/ml) or co-cultured WT macrophages (Mφ). Within each experiment SMC migration was normalized to that elicited by PDGF-BB in WT SMCs (4.3 ± 0.3 fold/basal) to obtain “% of control”. Results are shown as mean ± SE from ≥ 3 independent experiments performed in triplicate. *, P < 0.05 compared with WT control.

C, SMCs of the indicated genotype were stimulated without (basal) or with PDGF-BB (5 ng/ml), murine TNF (10 ng/ml), or both for 24 h, and assayed for thymidine incorporation as in Methods. Plotted are the mean ± S.E. of two independent experiments performed in triplicate. Basal thymidine incorporation for WT and p75−/− SMCs was 2.3 ± 0.3 and 2.7 ± 0.2 × 10³ CPM, respectively.
A

2-Week old Vein Grafts (Donor / Recipient)

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Red: Ki-67; Blue: DNA

B

2-Week-old Vein Grafts

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B

<table>
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<tr>
<th>Layer:</th>
<th>Neointima</th>
<th>Media</th>
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<tr>
<td></td>
<td>(Collagen / DNA (% of control))</td>
<td></td>
</tr>
<tr>
<td>Vein Graft:</td>
<td>WT</td>
<td>p75(-/-)</td>
</tr>
<tr>
<td>Recipient:</td>
<td>WT</td>
<td>p75(-/-)</td>
</tr>
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</table>