Macrophages Control Vascular Stem/Progenitor Cell Plasticity Through Tumor Necrosis Factor-α–Mediated Nuclear Factor-κB Activation

Mei Mei Wong, Yikuan Chen, Andriani Margariti, Bernhard Winkler, Paola Campagnolo, Claire Potter, Yanhua Hu, Qingbo Xu

Objective—Vascular lineage differentiation of stem/progenitor cells can contribute to both tissue repair and exacerbation of vascular diseases such as in vein grafts. The role of macrophages in controlling vascular progenitor differentiation is largely unknown and may play an important role in graft development. This study aims to identify the role of macrophages in vascular stem/progenitor cell differentiation and thereby elucidate the mechanisms that are involved in the macrophage-mediated process.

Approach and Results—We provide in vitro evidence that macrophages can induce endothelial cell (EC) differentiation of the stem/progenitor cells while simultaneously inhibiting their smooth muscle cell differentiation. Mechanistically, both effects were mediated by macrophage-derived tumor necrosis factor-α (TNF-α) via TNF-α receptor 1 and canonical nuclear factor-κB activation. Although the overexpression of p65 enhanced EC (or attenuated smooth muscle cell) differentiation, p65 or TNF-α receptor 1 knockdown using lentiviral short hairpin RNA inhibited EC (or rescued smooth muscle cell) differentiation in response to TNF-α. Furthermore, TNF-α–mediated EC differentiation was driven by direct binding of nuclear factor-κB (p65) to specific VE-cadherin promoter sequences. Subsequent experiments using an ex vivo decellularized vessel scaffold confirmed an increase in the number of ECs and reduction in smooth muscle cell marker expression in the presence of TNF-α. The lack of TNF-α in a knockout mouse model of vein graft decreased endothelialization and significantly increased thrombosis formation.

Conclusions—Our study highlights the role of macrophages in directing vascular stem/progenitor cell lineage commitment through TNF-α–mediated TNF-α receptor 1 and nuclear factor-κB activation that is likely required for endothelial repair in vascular diseases such as vein graft. (Arterioscler Thromb Vasc Biol. 2014;34:635-643.)

Key Words: endothelial cells  ▪  macrophages  ▪  stem cells  ▪  tumor necrosis factor-alpha  ▪  vascular smooth muscle

Vein grafts have predominantly been used in surgical interventions of vascular diseases, but their patency rate is often limited by graft failure attributable to the development of atherosclerosis-like lesions in the intima. The occurrence of the lesions is mainly fueled by endothelial damage and death that subsequently leads to the infiltration of mononuclear cells such as macrophages from the blood into the neointima, thereby propagating an inflammatory milieu. Activation of macrophages in the intima leads to the secretion of proinflammatory molecules such as tumor necrosis factor-α (TNF-α) and interleukin-1. At present, little is known about the role of macrophages in vascular regeneration and neointimal formation of vein grafts.

Recently, there has been considerable interest in a population of stem/progenitor cells within vessel compartments that can play crucial roles in vascular diseases. Using a mouse decellularized vessel graft model, Tsai et al found a marked accumulation of cells that were positive for progenitor markers (Sca-1, c-kit, and CD34) within neointimal lesions. Interestingly, the progenitor cells could differentiate into either endothelial cells (ECs) or smooth muscle cells (SMCs) under specific stimuli. Furthermore, Sca-1+ resident progenitor cells were also found to be present within the adventitia. Contrary to the notion that stem/progenitor cells can contribute to the pathogenesis of atherosclerosis, there is also substantial evidence that these cells can simultaneously play reparative and atheroprotective roles. It is therefore vital to understand the effect of the inflammatory microenvironment on the differentiation of progenitor cells for subsequent designs of more effective therapeutic interventions using vein grafts.

Considering that macrophages are largely involved in vascular inflammation and are critical for the development of vascular diseases, we aimed to address their role in the plasticity of stem/progenitor cells into a predominant vascular lineage and to identify the mechanisms that are involved in this process. We provide novel evidence that macrophages are able to
control stem/progenitor cell vascular lineage commitment by inducing EC differentiation while simultaneously inhibiting SMC differentiation. Furthermore, we show that the control of stem/progenitor cell differentiation by macrophages is fundamentally driven by TNF-α via TNF-α receptor 1 (TNF-R1) and canonical nuclear factor-κB (NF-κB) signaling pathways.

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

Results
Macrophages Induce Progenitor and Stem Cell–Endothelial Differentiation Through TNF-α
To investigate the potential effects of macrophages on vascular progenitor cell differentiation, peritoneal macrophages were cocultured with Sca-1⁺ Lin⁻ progenitor cells that were obtained and characterized using previously defined methods. The coculture was performed in the presence of a 0.4-μm membrane insert to prevent physical contact between the 2 cell types. Data revealed that the presence of macrophages caused a significant induction in the transcriptional expression of a panel of endothelial markers such as PECAM1, VE-cadherin, and Flk-1 (Figure 1A). Consistently, an increase of the endothelial NO synthase cell surface expression was also detected (Figure 1B). Previous work from our laboratory has established the capacity of murine embryonic stem cell–derived Sca-1⁺ progenitors to differentiate into both SMC and EC lineages under specific culture conditions. Therefore, we also sought to use these stem cells in parallel with progenitor cells to elucidate the role(s) of macrophages in vascular lineage differentiation. Interestingly, our data showed significant upregulation of endothelial markers in stem cells cultured with macrophages, both at mRNA (Figure 1C) and protein levels (Figure 1D).

To identify macrophage-derived secretory factors that are responsible for induction of stem/progenitor EC differentiation, a Multi-Analyte ELISAArray was performed using conditioned media (CM) from peritoneal macrophages. We observed markedly high levels of TNF-α within the macrophage CM (Figure 1E). Subsequent assays using TNF-α ELISA confirmed that macrophage CM contained 1113.29±5.136 pg/mL of TNF-α, whereas complete culture media contained 1.94±0.156 pg/mL. To this point, we performed experiments in parallel using a macrophage cell line J774.1 and found that the results were indistinguishable from those using peritoneal macrophages, including J774.1 macrophage-mediated induction of endothelial expression by both progenitor and stem cells (Figure 1 in the online-only Data Supplement). Therefore, because of ease of macrophage culture and propagation, we performed subsequent experiments using J774.1 macrophages.

To evaluate whether TNF-α may directly induce stem/progenitor cell differentiation, a neutralizing antibody to TNF-α was added into the stem cell/macrophage cocultures. Data showed that macrophage-mediated EC differentiation was abrogated in the presence of the anti–TNF-α antibody, but not with an anti-IgG1 control antibody (Figure 1F). Furthermore, the addition of exogenous TNF-α (1, 10, or 100 ng/mL) to stem cells alone caused a dose-dependent increment of EC markers at transcriptional (Figure 1G) and protein levels (Figure 1H). The increment of EC markers in response to TNF-α was specific to endothelial differentiation and was not a result of stem cell proliferation (Figure II in the online-only Data Supplement). Notably, although interleukin-6 was also detected in CM of macrophages at a lower level (Figure 1E), experiments using recombinant interleukin-6 or interleukin-6 neutralizing antibodies showed that the cytokine was not involved in EC differentiation (Figure III in the online-only Data Supplement).

Macrophage-Derived TNF-α Induces Differentiation of Sca-1⁺ Vascular Progenitors Into Functional ECs
Subsequent experiments were performed to ascertain the functionality of progenitor-derived ECs in response to TNF-α. Data showed that TNF-α-treated progenitor cells were able to form significantly higher numbers of tubes in vitro (Figure 2A and 2B). Additional in vivo Matrigel assays showed increased formation of tubes and capillaries that expressed PECAM1 and VE-cadherin (Figure 2C and 2D) in plugs that contained TNF-α pretreated progenitor cells. Interestingly, the expression of PECAM1 and VE-cadherin was found to colocalize specifically at cellular junctions of the ECs (Figure 2E), thus postulating that macrophage-derived TNF-α can mediate EC differentiation and vessel formation via junctional stabilization of progenitor ECs. Next, we labeled progenitor cells (+/−TNF-α) with Qdot 625 nanocrystals before injecting the Matrigel plugs into mice to evaluate whether the increase of PECAM1⁺ and VE-cadherin⁺ cells in TNF-α-treated plugs was a result of progenitor cells–EC differentiation, proliferation, or an infiltration of recipient cells into the plug (Figure 2F). Quantification of Qdot⁺DAPI⁺ or Qdot⁺DAPI⁺ cells confirmed that there were no significant changes in proliferation and infiltration of recipient cells into the plug (Figure 2G and 2H). Furthermore, only TNF-α-treated plugs showed positive staining with lectin, a marker of functional blood vessel formation (Figure 2I). Taken together, the results confirm that macrophage-derived TNF-α can induce the differentiation of vascular progenitor cells into functional ECs.

TNF-α–Induced Differentiation Is Mediated by TNF-R1 via Canonical NF-κB (p65) Signaling
Subsequent experiments confirmed that stem cells treated either with macrophages or TNF-α had significantly enhanced TNF-R1 expression (Figure 3A). Interestingly, treatment with macrophages or TNF-α caused a marked increment of

Nonstandard Abbreviations and Acronyms

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<tr>
<td>BM</td>
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<tr>
<td>CM</td>
<td>conditioned media</td>
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<td>EC</td>
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<td>TNF-α</td>
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<td>TNF-R1</td>
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Promoter activation following TNF-R1 or p65 knockdown–mediated α only Data Supplement) and an ablation of TNF-α reporter assay, in response to TNF-α of stem cell VE-cadherin promoter activity using a luciferase EC differentiation. Indeed, we found a dose-dependent increase Data Supplement), we sought to investigate whether p65 trans-tiation. Because TNF-α NF-κ B proteins (Rel-B, NIK, and the levels of noncanonical NF-κB signaling markers (p65 and p50), whereas graphs are shown as means±SEM of 3 independent experiments performed, whereas the levels of noncanonical NF-κB proteins (Rel-B, NIK, and p52/p100) did not change (Figure 3B). To confirm the role of TNF-R1 and canonical NF-κB in the TNF-α-mediated effects, we ablated either TNF-R1 or p65 in stem cells using lentiviral short hairpin RNA. TNF-α short hairpin RNA knockdown led to a marked decrease of both TNF-R1 and p65 expression following stimulation of stem cells with TNF-α; both PECAM1 and VE-cadherin expression were also reduced (Figure 3C). In addition, p65 short hairpin RNA knockdown in TNF-α–treated stem cells resulted in an evident reduction of p65 and VE-cadherin (Figure 3D), and the overexpression of p65 enhanced their expression of EC markers (Figure 3E). These data demonstrate the direct role of TNF-R1 and canonical NF-kB activation in TNF-α–mediated stem cell–EC differentiation. Because TNF-α treatment induced the nuclear translo-cation of p65 from the cytoplasm (Figure IV in the online-only Data Supplement), we sought to investigate whether p65 translocation can directly activate endothelial promoters that lead to EC differentiation. Indeed, we found a dose-dependent increase of stem cell VE-cadherin promoter activity using a luciferase reporter assay, in response to TNF-α (Figure V in the online-only Data Supplement) and an ablation of TNF-α–mediated promoter activation following TNF-R1 or p65 knockdown (Figure 3F). The overexpression of p65 significantly induced the promoter activation (Figure 3G). Using a ChIP assay, we detected specific binding of p65 to the VE-cadherin promoter region between −2504 and −2111 bp (Figure 3H and Figure VI in the online-only Data Supplement). Thus, our study provides first evidence that TNF-α can directly stimulate VE-cadherin gene transcription that leads to endothelial differentiation through direct binding of NF-κB (p65) to the promoter region.

**Macrophage-Derived TNF-α Inhibits SMC Differentiation Through TNF-R1 and NF-κB (p65) Signaling**

To confirm the specificity of macrophage-derived TNF-α in inducing EC differentiation, stem cells were also tested for potential changes in SMC expression. Interestingly, the presence of either peritoneal macrophages or TNF-α resulted in a reduction of SMC marker expression in stem cells (Figure 4A), thus suggesting that macrophages or TNF-α may inhibit SMC differentiation. Using a collagen IV–mediated SMC differentiation protocol established by our laboratory, we found that the presence of peritoneal macrophages and TNF-α can significantly inhibit stem cell–SMC differentiation (Figure 4B and 4C). As before, we confirmed that results from experiments performed using the macrophage cell line J774.1 were undistinguishable from those using peritoneal macrophages, including macrophage-mediated inhibition of SMC differentiation in both stem (Figure VII in the online-only Data Supplement) and progenitor cells (Figure 4D). Subsequent data revealed
that SMC differentiated stem cells had significantly decreased activation of canonical—but not noncanonical—NF-xB signaling genes and protein (Figure 4E and Figure VIII in the online-only Data Supplement). Furthermore, the knockdown of p65 led to the rescue of TNF-α-mediated inhibition of SMC differentiation (Figure 4F), whereas its overexpression caused an evident reduction (Figure 4G). Experiments using TNF-R1 short hairpin RNA also revealed a rescue of SMC expression (Figure 4F). Together, the results demonstrate that macrophage-derived TNF-α can induce stem/progenitor–EC differentiation while inhibiting their SMC lineage through TNF-R1 and NF-xB signaling pathways.

**TNF-α Is Directly Involved in Controlling the Lineage Commitment of Vascular Progenitor Cells**

To confirm our in vitro results in a physiological environment, stem cells were seeded within a decellularized vessel graft bioreactor system in the presence of TNF-α and subsequently evaluated for their differentiation into EC or SMC lineages under the same flow rate. Interestingly, we found markedly increased numbers of cells that lined the luminal area of TNF-α–treated vessels, as visualized by hematoxylin–eosin staining (Figure 5A; black arrows). Next, we performed immunofluorescence staining on the same vessels with EC and SMC markers to identify the cell types within. When compared with the control vessels, we observed significantly higher number of PECAM1-positive cells on the luminal side of TNF-α–treated vessels, as visualized by hematoxylin–eosin or immunofluorescent markers (platelet endothelial cell adhesion molecule 1 [PECAM1] or vascular endothelial [VE]-cadherin, both Alexa488; green; bar, 50 μm). The number of tubes and capillaries that were either PECAM1 or VE-cadherin positive were quantified in 5 random fields of view at ×10 magnification and represented as a graph. E, TNF-α–treated plugs that were stained with both PECAM1 (Alexa 594; red) and VE-cadherin (Alexa 488; green) indicated colocalized expression of the fluorescent markers at cellular junctions (indicated by arrow; bar, 10 μm). F, The number of 4',6-diamidino-2-phenylindole (DAPI)-positive (blue) and Qdot 625 nanocrystal–labeled (red) cells in both control and TNF-α–treated plugs were evaluated (arrows represent the formation of tube-like structures; bar, 50 μm). Qdot 625 nanocrystal–labeled (red) cells in TNF-α–treated plugs also showed positive staining for PECAM1 (Alexa 488; green) and VE-cadherin (Alexa 405; blue). The number of cells that were Qdot 625 and DAPI positive was quantified in 5 random fields of view at ×60 magnification or represented as graphs. Cell proliferation is represented by an increment in the number of Qdot 625–DAPI+ cells (G), whereas the infiltration of recipient cells into the plug is represented by the percentage of Qdot 625–DAPI+ cells (H). I, Tissue sections from Matrigel plugs (either control or TNF-α treated) containing Qdot 625 nanocrystal–labeled (red) cells were stained with VE-cadherin (Alexa 405; blue) and fluorescein–labeled lectin (green) to identify functional blood vessels (indicated by white arrows).

**Macrophage-Derived TNF-α Is Responsible for Endothelial Repair and Neointimal Formation in Vein Grafts**

To validate our rationale in vivo, we performed subsequent vein grafting experiments using TNF-α–deficient mice.
First, we confirmed that TNF-α−/−-derived macrophages had markedly impaired capacity to induce EC (Figure IX in the online-only Data Supplement) and inhibit SMC differentiation (Figure X in the online-only Data Supplement) of stem/progenitor cells when compared with macrophages from wild-type mice in vitro. Next, we found that vein grafts of wild-type mice at 4 weeks showed marked neointimal hyperplasia (Figure 6A; top), whereas vein grafts in TNF-α−/− mice exhibited a pronounced formation of thrombosis (Figure 6A; bottom). Consistently, subsequent quantification of the vessels indicated a significant increase in thrombotic areas and also a reduction in luminal areas of TNF-α−/− versus wild-type vein grafts (Figure 6A; graphs). Interestingly, additional characterization of the vessels revealed the presence of macrophages (Mac-1+), in particular the M1 subtype (Mac-1+CD86+), in wild-type vein grafts, but not in TNF-α−/− vein grafts where thrombus has formed (Figure 6B and 6C and Figure XI in the online-only Data Supplement).

Next, we sought to validate the effects of TNF-α deficiency specifically in bone marrow (BM) cells—a population from which macrophage/monocytic lineages are derived. We created a chimeric mouse model wherein BM cells from either TNF-α−/− or TNF-α+/+ mice were transferred into irradiated wild-type mice. The efficacy of the chimeric model has previously been confirmed, in which we saw >95% of recipient BM cells being replaced by donor-derived BM cells.20 Vein segments from wild-type mice were grafted into chimeric mice with either TNF-α−/− or TNF-α+/+ BM. Consistently, only wild-type animals that received TNF-α−/− BM cells had markedly increased thrombosis and reduced endothelial area 4 weeks after grafting (Figure 6Di, 6Dii, and 6E).

**Figure 3.** Macrophage-derived tumor necrosis factor-α (TNF-α) mediates endothelial differentiation through TNF-α receptor 1 (TNF-R1) activation and direct binding of canonical nuclear factor-κB (NF-κB; p65) to VE-cadherin promoter sequences. Real-time reverse transcription polymerase chain reaction (PCR) was performed for analysis of TNF-R1 gene expression (A), and cell lysates were subjected to Western blotting for detection of canonical NF-κB (p65, p50) and noncanonical NF-κB (Rel-B, NF-κB inducing kinase [NIK], p52/p100) proteins (B). Stem cells were infected either with lentiviral short hairpin RNA (shRNA) for ablation of (C) TNF-R1 and (D) p65 and treated with TNF-α for 48 hours or (E) subjected to the overexpression of p65 gene using plasmid transfection, before analysis of endothelial protein expression using Western blot. F, Stem cells were treated with TNF-α after transfection with a pGL3-VE-cadherin (2.4 kb) promoter reporter gene (0.33 μg/well) after lentiviral shRNA knockdown of TNF-R1 and p65. pRenilla (0.1 μg/5×10⁴ cells) was included as luciferase plasmid control. Luciferase and Renilla activity assays were detected 48 hours after transfection.

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G, Stem cells were also transfected in combination with a pcDNA3-p65 expression plasmid (0.16 μg/well) before detection of luciferase and Renilla activities. H, Chromatin immunoprecipitation assay was performed using an antibody against p65 with an appropriate IgG as a negative control. Aliquots of chromatin before immunoprecipitation served as an input control. The assay revealed that p65 binds directly to the VE-cadherin gene promoter at region (~2504 and ~2111) nucleotides (nt) upstream of the transcription initiation site in TNF-α−/−-treated stem cells. Blots and PCR gel shown are representative of 3 independent experiments, and graphs are shown as means±SEM of 3 independent experiments. *P<0.05 compared with control. NC indicates noncoding; PECAM1, platelet endothelial cell adhesion molecule 1; and RLU, relative light units.
6E), thereby confirming that the effects seen from TNF-α deficiency were specific to the BM cells. Thrombus formation has largely been associated to EC apoptosis during early stages of vein grafts (<1 week postsurgery), suggesting that TNF-α is critical for endothelium healing and prevention of thrombosis during advanced stages of vein grafting.

Figure 4. Inhibition of smooth muscle cell (SMC) differentiation is mediated by tumor necrosis factor-α (TNF-α) via TNF-α receptor 1 (TNF-R1) and nuclear factor-κB (NF-κB) signaling. A, Stem cells were cultured in normal medium in the presence of either peritoneal macrophages or TNF-α for 48 hours and subjected to real-time reverse transcription polymerase chain reaction (RT-PCR) for analysis of SMC gene expression. B, Stem cells were cultured on collagen IV (Coll IV)–coated plates for 5 days to induce SMC differentiation, either in the presence or absence of peritoneal macrophages before the cells were harvested for real-time RT-PCR analysis of SMC gene expression (SM-22α and calponin). C, Stem cells cultured on Coll IV–coated plates were also treated with TNF-α for 5 days before Western blot analysis of SMC protein expression. D, Progenitor cells cultured in Coll IV–coated plates in the presence or absence of J774.1 macrophages were subjected to immunofluorescence staining for SM-22α and calponin (both Alexa 488; green; bar, 30 μm). E, Cell lysates of progenitor cells cultured in Coll IV for 5 days were harvested for real-time RT-PCR and Western blotting to detect canonical NF-κB proteins expression. Stem cell lysates that were either (F) infected with lentiviral short hairpin RNA (shRNA) for ablation of TNF-R1 and p65 or (G) transfected with a p65 plasmid for overexpression were cultured in collagen IV for 5 days before protein analysis using Western blot. Blots shown are representative of 3 independent experiments, and graphs are shown as mean±SEM of 3 independent experiments. *P<0.05; **P<0.01; ***P<0.005 compared with control. NC indicates noncoding.

Figure 5. Tumor necrosis factor-α (TNF-α) can regulate the lineage commitment of vascular progenitor cells ex vivo. Stem cells (5×10⁵) were seeded within a previously decellularized vessel graft in the presence or absence of TNF-α for 12 hours followed by the application of shear stress. A, The grafts were harvested and embedded in mouse liver for liquid nitrogen freezing before being stained with hematoxylin–eosin (left, bar=10 μm; right, bar=100 μm). The frozen sections were also subjected to immunofluorescence staining and quantification of both endothelial cell– and smooth muscle cell–positive cells. B, Platelet endothelial cell adhesion molecule 1 (PECAM1) (Alexa 488; green) and calponin (Alexa 546; red). C, Vascular endothelial (VE)-cadherin (Alexa 488; green) and smooth muscle α-actin (SM-αA; Alexa 546; red). Arrows indicate either PECAM1- or VE-cadherin–positive cells (bar=10 μm). Images shown are representative of ≥3 separate grafts. *P<0.05; ***P<0.005. DAPI indicates 4′,6-diamidino-2-phenylindole.
Indeed, luminal cells in vein grafts of wild-type animals that received TNF-α+/+ BM cells were PECAM1 positive (green), whereas grafts of wild-type animals that received TNF-α−/− BM cells showed significantly less, if any, ECs (Figure 6E). Furthermore, vessels from vein grafts performed in TNF-α−/− mice that received TNF-α+/+ BM cells showed a rescue of vascularization and prevention of thrombosis (Figure 6Diii, 6Div, and 6F). Interestingly, hematoxylin–eosin and SM-22α fluorescent staining revealed marked reductions in neointimal areas of vein grafts from mice that lacked TNF-α or received TNF-α−/− BM cells (Figure 6A and 6D and Figure XII in the online-only Data Supplement; SM-22α). The occurrence of thrombosis in the vein grafts resulted in a decrease in blood flow and cellular gradient within the vessels and is therefore likely the cause of decrease in neointimal formation.

These data strongly suggest that during later stages of vein grafting, TNF-α is increasingly secreted by inflammatory mediators such as macrophages; the release of this proinflammatory cytokine is critical for the prevention of thrombosis. Concomitantly, it is likely that the prevention of thrombosis by TNF-α is mediated through the maintenance of endothelium integrity by controlling the lineage differentiation of stem/progenitor cells that are present within the neointima of vein grafts into ECs.

**Discussion**

The establishment of vein graft pathogenesis is fueled by a time-dependent change of cellular composition within the grafts, which includes a progressive increment of >20 layers of cells and matrix protein deposition (Figure XIIIA in the online-only Data Supplement). Within growing neointimal lesions, a high number of macrophages and also a consistent, albeit small, population of Sca-1+ progenitor cells have been identified (Figure XIIIB and XIIIC in the online-only Data Supplement). To date, the putative interaction between macrophages and vascular progenitor cells and its potential role in vein graft atherosclerosis are not known. In the present study, we provide first evidence that macrophages can control vascular stem/progenitor cells for specific differentiation into ECs, independent of physical contact. Furthermore, our study highlights the potential of macrophages to simultaneously inhibit progenitor/stem cell differentiation into SMCs. We demonstrate that the remarkable dual effect of macrophages is directly mediated through the secretion of TNF-α. Consistent with our results, several studies have previously demonstrated the role of macrophage-derived TNF-α in mediating angiogenesis following injury23 or in tumorigenic conditions,24 likely mediated through the secretion of angiogenic factors such as vascular endothelial growth factor (VEGF), angiopoietin-1, hepatocyte growth factor, and bone

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**Figure 6.** The effect of tumor necrosis factor-α (TNF-α) deficiency on neointimal and thrombus formation in vein grafts. Vena cava segments were surgically removed from C57BL/6 mice under anesthesia and subsequently grafted into carotid arteries of recipient mice. Animals were euthanized at various time points after surgery, and the grafted tissue fragments were fixed in 4% phosphate-buffered (pH, 7.2) formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin–eosin. A, Vein grafts from either TNF-α−/− (i and ii) or TNF-α+/− (iii and iv) mice were performed on animals of the same background. (i and iii, bar=50 μm; ii and iv, bar=10 μm). Thrombosis and luminal areas were quantified as described in detail in Materials and Methods in the online-only Data Supplement. Macrophage subsets in vein grafts from either TNF-α−/− (B) or TNF-α−/− (C) mice were identified using immunofluorescence staining with Mac-1 (Alexa 594, red), CD206 (M2 type; Alexa 405, blue), and CD86 (M1 type; Alexa 488, green; bars, 10 μm). D, Bone marrow (BM) cells (1 x 10⁷) from either wild-type (i) or TNF-α−/− (ii) mice were transplanted into wild-type irradiated recipients (x-ray; 950 Rads) via tail vein injection, and vein grafts from wild-type mice were subsequently performed on the chimeric recipients 4 weeks later. Similarly, BM cells from either (iii) TNF-α−/− or (iv) wild-type mice were transplanted into TNF-α−/− irradiated recipients before vein grafting from wild-type mice on chimeric recipients 4 weeks later. Hematoxylin–eosin staining indicates the presence of neointimal or thrombus formation in vein grafts 4 weeks after grafting (bars, 50 μm). E and F, Quantification of thrombosis and endothelial area was quantified and represented as graphs shown as means±SEM. *P<0.05.
morphogenetic protein-2. In light of these data, we found that macrophage CM contained a panel of angiogenic factors and chemokines including VEGF-A, VEGF-C, monocyte chemotactic protein-1, macrophage inflammatory protein-1α, and macrophage inflammatory protein-1β (Figure XIV in the online-only Data Supplement). Nevertheless, subsequent experiments showed that most of the angiogenic factors (ie, VEGF-A and VEGF-C) were not involved in macrophage-mediated EC differentiation (Figure XV in the online-only Data Supplement). Notably, stem/progenitor–EC differentiation and interactions with macrophages may also be facilitated by their migration and chemotactic responses toward TNF-α.

Although previous studies have shown that the occurrence of thrombosis could be attenuated at later stages of vascular grafts in which an intact endothelium can also be found,1,2 the precise cellular responses responsible for the observations remain unclear. The present findings showed that either vein grafts in TNF-α−/− mice or vein grafts with TNF-α−/− BM cells resulted in the absence of endothelial regeneration that led to severe thrombosis. During this time point, other investigators including ourselves have demonstrated the presence of a large number of infiltrated macrophages and high levels of TNF-α in the media/neointima of vein grafts.6,27 Therefore, macrophages are likely to promote endothelial repair via TNF-α that also lead to reduced thrombotic events. It is however noteworthy that the direct administration of TNF-α in a mouse model of ferric chloride–induced arterial injury was also found to induce antithrombotic effects that were independent of any cellular differentiation.28 In the present study, we also showed that TNF-α was required for neointimal formation because its deficiency markedly ablates the presence of neointima. This was not surprising because TNF-α deficiency was likely to inhibit the migration and proliferation of other key drivers of neointimal hyperplasia, namely mature SMC.

Contrary to traditional belief that macrophages are key promoters of atherosclerosis, our results showed that macrophages and its secreted effectors may be required to initiate a reparative response by inducing EC differentiation from a population of stem/progenitor cells during later stages of vein grafts. This concept is of increasing relevance given recent accruing evidence demonstrating that different populations of macrophages with defined phenotypes and functions exist in neointimal lesions.17,26 Indeed, we found that Mac-1+ macrophages that reside in neointimal lesions of 4-week vein grafts predominantly consist of the proinflammatory M1 subtype (CD86+) and not the anti-inflammatory M2 subtype (CD206+; Figure 6B and 6C and Figure XI in the online-only Data Supplement). Although it is tempting to postulate that M1 macrophages are largely responsible for inducing EC repair in late-stage vein grafts, further work is still required to confirm the intriguing findings.

Torres and Watt previously demonstrated that the withdrawal of the pluripotency growth factor, leukemia inhibitory factor, resulted in an upregulation of NF-κB signaling that is concomitant with mouse stem cell differentiation. Although the lineage specificity of stem cell differentiation was not clarified by the authors, we show that the switching on of canonical NF-κB via TNF-R1 activation results in definitive differentiation toward the endothelial route by direct binding of p65 to specific elements of the VE-cadherin promoter. Furthermore, we identified that the induction of endothelial and inhibition of SMC differentiation by macrophage-derived TNF-α were directly regulated by canonical NF-κB signaling. We could not detect significant changes in the activities of noncanonical NF-κB signaling proteins, potentially because they are mainly involved in maintaining stem cell pluripotency.31 Furthermore, although studies also indicate a putative role of TNF-α/TNF-R1/NF-κB in regulating the survival of other cell types,32 our in vitro and in vivo data did not show any significant rescue of stem/progenitor apoptosis after TNF-α treatment (Figure XVI in the online-only Data Supplement).

Collectively, data from our study provide novel evidence that canonical NF-κB can act as a genetic switch that is responsible for controlling the specific lineage commitment (plasticity) of a vascular progenitor cell. Nevertheless, the field still remains in its infancy because suitable mouse models that enable lineage tracing of the Sca-1+ progenitor cells are still required to fully comprehend the roles of progenitor cell plasticity/differentiation during specific stages of pathology.

In summary, the present study provides evidence of the coexistence of macrophages and progenitor cells in the neointima during pathological conditions of vein grafts. We demonstrated that macrophage-derived TNF-α can activate TNF-R1/canonical NF-κB signaling pathways that lead to the simultaneous induction of EC differentiation and SMC suppression of stem/progenitor cells. Using a TNF-α−/− vein graft mouse model, we demonstrated a role of TNF-α in promoting vein graft endothelial repair, while preventing thrombus formation. Together, our data provide crucial evidence that will increase the understanding of the mechanisms by which vascular progenitor plasticity can be controlled. This can, therefore, expedite the identification of cellular/molecular targets that will lead to more efficient strategies for vascular interventions for vein graft failure.

Sources of Funding
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Disclosures
None.

References
At present, the cointeraction between macrophages and stem or progenitor cells in the vascular system is poorly understood. Furthermore, the role of macrophages in controlling vascular progenitor differentiation is largely unknown and may play an important role in vascular diseases such as in vein grafts. In the present article, we provide novel evidence that macrophages can act as master switches that control the lineage commitment of vascular stem/progenitor cells through tumor necrosis factor-α-mediated tumor necrosis factor receptor 1/nuclear factor-κB signaling pathways. We demonstrate that the control of vascular stem/progenitor cell lineage commitment is potentially required for endothelial repair and prevention of thrombosis in a vein graft model. We think that our findings provide further understanding of key cellular interactions within pathophysiological conditions and can lead to the identification of novel approaches that can potentially be exploited to improve stem cell therapy for vascular repair and regeneration in the future.

**Significance**
Macrophages Control Vascular Stem/Progenitor Cell Plasticity Through Tumor Necrosis Factor-α-Mediated Nuclear Factor-κB Activation
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Supplemental Figure I. J774.1 macrophage cell line can induce progenitor and stem cell differentiation into the endothelial lineage. (A) Vascular progenitor cells or (B) stem cells were cultured in the presence or absence of J774.1 macrophage cell line for 48 hours and subjected to real-time RT-PCR for analysis of endothelial gene expression. Graphs are shown as mean ± SEM of three independent experiments. *P<0.05, **P<0.01 compared with control.
Supplemental Figure II. TNF-α inhibits stem cell proliferation. (A) Total number of stem cells were quantified before and after 48 hours of TNF-α treatment. Stem cells cultured in complete media were also quantified as a control. (B) Evaluation of stem cell proliferation in response to TNF-α treatment (or control) was confirmed using a BrdU incorporation assay. Graphs are shown as mean ± SEM of three independent experiments. *P<0.05, **P<0.01 compared with control.
Supplemental Figure III. IL-6 is not involved in macrophage-mediated endothelial differentiation of stem cells. (A) ES cells were co-cultured with J774.1 macrophages in the presence an anti-IL-6 mAb (10µg/ml) prior to gene expression analysis of endothelial markers. An anti-IgG1 mAb (10µg/ml) was used as a control. (B) ES cells were also cultured with an increasing concentration of recombinant murine IL-6 for 48 hours. Total RNA was collected for real time PCR analysis of PECAM1 and VE-cadherin. Graphs are shown as mean ± SEM of three independent experiments. n.s. $P>0.05$ compared with control.
Supplemental Figure IV. TNF-α induced stem cell endothelial differentiation through TNF-R1 is mediated by p65 nuclear translocation. Nuclear and cytoplasmic fractions from TNF-α-treated stem cells infected with lentiviral shRNA for ablation of (A) TNF-R1 or (B) p65 genes were harvested for western blotting to detect p65 protein expression. Nuclear translocation of p65 was only observed in non-coding (NC) shRNA infected TNF-α- treated cells (controls). Blots shown are representative of three independent experiments performed.
Supplemental Figure V. TNF-α induced endothelial differentiation is mediated by VE-cadherin promoter activation. VE-cadherin promoter activation was analysed using a luciferase reporter assay by transfecting stem cells with a pGL3-VE-cadherin (2.4kb) promoter reporter gene (0.33μg/well). pRenilla (0.1 μg/5 × 10⁴cells) was included as luciferase plasmid control. Luciferase activity (RLU) of stem cells was detected 48 hours after transfection and dose treatment with TNF-α. Graph is shown as mean ± SEM and is representative of three independent experiments. ***P<0.005 compared with control.
Supplemental Figure VI. 

TNF-α induced endothelial differentiation is mediated by NF-κB binding to the VE-cadherin promoter. Schematic of the VE-cadherin promoter containing the specific location of NF-κB binding site based on chromatin immunoprecipitation (ChIP) assays carried out.
Supplemental Figure VII. J774.1 macrophages are potent inhibitors of stem cell differentiation into smooth muscle cells. Stem cells were cultured on collagen IV-coated plates for 5 days to induce SMC differentiation, during which they were cultured in the presence of J774.1 macrophages with 0.4µm inserts to prevent physical contact. Cells were harvested for (A, B) real time RT-PCR or (C) western blotting for analysis of smooth muscle cell marker (SM-22α and Calponin) expression. Graphs are shown as mean ± SEM of three independent experiments. *P<0.05 **P<0.01 ***P<0.005 compared with control.
Supplemental Figure VIII. Non-canonical NF-κB signaling is not involved in SMC differentiation of stem cells. Stem cells were cultured on collagen IV-coated plates for 5 days to induce SMC differentiation. The cells were also seeded on gelatin-coated plates as a control. Cell lysates (40µg per condition) were harvested to detect Rel-B, NIK and p52 proteins using western blotting. Anti-α-tubulin was used as a loading control. No obvious changes were observed in the non-canonical NF-κB signaling proteins. Blots shown represent three independent experiments performed.
Supplemental Figure IX. TNF-α/- macrophages have impaired capacity to induce endothelial differentiation. (A) Peritoneal macrophages from either TNF-α/- or TNF-α+/- mice were harvested and subjected to RT-PCR for analysis of TNF-α expression and confirmation of gene knock-out according to protocol described by The Jackson Laboratory. (B) Stem cells cultured with TNF-α/- or TNF-α+/- macrophages for 48 hours in the presence of 0.4µm inserts were harvested for analysis of endothelial markers using real time RT-PCR. (C) Progenitor cells cultured with TNF-α/- or TNF-α+/- macrophages (48 hours with 0.4µm inserts) were detached and subsequently seeded at 2x10⁴ cells per well of Matrigel®-coated chamber slides, in triplicates (Scale bar= 100µm). (D) The number of tubes formed after 6 hours were quantified and represented as a graph. Graphs are shown as mean ± SEM of three independent experiments. *P<0.05  **P<0.01 compared with control.
Supplemental Figure X. TNF-α-/- macrophages have attenuated capacity to inhibit smooth muscle cell differentiation. (A) Stem cells were differentiated into smooth muscle cells in Collagen IV-coated plates for 5 days in the presence of TNF-α-/- or TNF-α+/- macrophages (with 0.4µm inserts) and harvested for analysis of smooth muscle markers using real time RT-PCR. (B) Progenitor cells cultured in Collagen IV-coated plates in the presence of TNF-α-/- or TNF-α+/- macrophages were subjected to immunofluorescence staining for calponin and SM-22α (both Alexa 546; red) (Scale bar= 50µm). The number of (C) calponin or (D) SM-22α positive cells were quantified and calculated as a percentage of DAPI positive cells in 8 random fields of focus. Graphs are shown as mean ± SEM of three independent experiments. *P<0.05  **P<0.01 ***P<0.005 compared with control.
Supplemental Figure XI. Classification of macrophage subsets in 4 week vein grafts. (A) Vein grafts from either TNF-α +/+ or TNF-α -/- mice were performed using animals of the same genotype. Mac-1+ macrophage subsets in the vessel grafts were identified using immunofluorescence staining with Mac-1 (Alexa 594-red), CD206 (M2 type; Alexa 405-blue) and CD86 (M1 type; Alexa 488-green) (Scale bars=100µm). (B) The number of Mac-1+CD206+ and Mac-1+CD86+ cells were quantified in 8 random fields of view at 60x magnification and represented as a graph.
Supplemental Figure XII. The identification of SMCs in vein grafts. The procedure for vein grafting and preparation of HE-stained sections from grafts 4 weeks postoperatively are the same as described for Figure 6D (same images of HE staining). 1x10^7 BM cells from either wild type (i) or TNF-α-/- (ii) mice were transplanted into wild type irradiated recipients (x-ray; 950 Rads) via tail vein injection and vein grafts from wild type mice were subsequently performed on the chimeric recipients 4 weeks later. Similarly, BM cells from either TNF-α-/- (iii) or wild type (iv) mice were transplanted into TNF-α-/- irradiated recipients prior to vein grafting from wild type mice on chimeric recipients 4 weeks later. Immunofluorescence staining of vessels with SM-22α indicates the presence of SMC in vein grafts 4 weeks after grafting. (Scale bars= 50µm). Immunofluorescent images are representative of vein grafts from 4 animals per group.
Supplemental Figure XIII. Identification of macrophages and progenitor cells in neointima of vein grafts. (A) The procedure for vein grafting and preparation of HE-stained sections from grafts 4 weeks postoperatively are the same as described for Figure 6. HE staining showed marked changes of cellular composition within the grafts (Scale bar= 30µm). (B, C) Immunofluorescence staining and semi-quantification of progenitor cells (Sca-1; green as indicated by arrows) and macrophages (Mac-1; red as indicated by arrowheads) in vessel grafts (4 weeks post-operatively). (Scale bar= 30µm). HE and immunoflorescent images are representative of vein grafts from 8 animals per group.
Supplemental Figure XIV. Identification of macrophage-derived soluble factors. Conditioned media from macrophages were harvested and subjected to ELISA arrays for the quantification of secreted (A) Ang-2, VEGF-A, VEGF-C and VEGF-D, and (B) a panel of chemokines. Normal culture media was used as a control for all ELISA arrays that were carried out. Angiopoieting-2; Ang-2, vascular endothelial growth factor; VEGF, regulated on activation normal T cell expressed and secreted; RANTES, monocyte chemotactic protein-1; MCP-1, macrophage inflammatory protein; MIP, stromal cell-derived factor-1; SDF-1, interferon gamma-induced protein 10; IP-10, monokine induced by gamma interferon; MIG, thymus and activation regulated chemokine; TARC, macrophage-derived chemokine; MDC, keratinocyte-derived chemokine; KC.
Supplemental Figure XV. VEGF-A and C are not involved in macrophage-mediated endothelial differentiation of stem cells. (A) ES cells were co-cultured with J774.1 macrophages in the presence an anti-VEGF-A mAb (10µg/ml) prior to gene expression analysis of endothelial markers. An anti-IgG1 mAb (10µg/ml) was used as a control. (B) Total RNA was harvested from ES cells co-cultured with J774.1 macrophages in the presence of an inhibitor of VEGF receptor kinase activity, KRN633 (100nM) and subjected to real time RT-PCR analysis of endothelial markers. DMSO was used as a control in these experiments. Graphs are shown as mean ± SEM of four independent experiments. n.s. P>0.05 compared with control.
Supplemental Figure XVI. TNF-α induces stem cell apoptosis in vitro, but not in vivo.

(A, B) Number of apoptotic (Annexin-V positive) stem cells were quantified using flow cytometric analysis after 48 hours of TNF-α treatment or co-culture with J774.1 macrophages. Stem cells cultured in complete media were used as a control. (C, D) Evaluation and quantification of stem cell apoptosis following TNF-α treatment (or control) in vivo Matrigel plugs was visualized by in situ TUNEL (red; white arrows) staining. Graphs are shown as mean ± SEM of three independent experiments. n.s. $P>0.05$, **$P<0.01$** compared with control.
Materials and Methods

Reagents
Murine recombinant proteins TNF-α and IL-6 were purchased from Peprotech UK. Mouse TNF-α antibody (polyclonal goat IgG), mouse IL-6 antibody (polyclonal goat IgG), normal goat IgG isotype control antibody (all from R&D Systems), mouse VEGF-A antibody (rat IgG2a) and purified rat IgG2a isotype control antibody (both from Biolegend) were used as neutralizing antibodies in co-cultures. A VEGF receptor kinase activity inhibitor (KRN633) was purchased from Merck, Millipore.

Cell Culture
Sca-1+ mouse adult progenitor cells were isolated from outgrowth of vein graft samples and purified using a microbead kit (Miltenyi Biotec) as previously described. Mouse embryonic stem cells (ES-D3 cell line, ATCC) were cultured on collagen IV-coated flasks (5µg/ml) for 2 days prior to Sca-1+ sorting using the microbead kit as before to obtain Sca-1+ progenitor cells. Both Sca-1+ cell types were cultured in gelatin-coated flasks (Sigma-Aldrich) and maintained in DMEM (ATCC) medium with 10% FBS, leukemia inhibitory factor (10 ng/ml) and 0.1mM 2-mercaptoethanol. Stem/progenitor cells were differentiated into SMC by culturing on collagen IV for 5 days in α-MEM (Gibco) with 10% FBS and 0.5 mM 2-mercaptoethanol. Mouse macrophage cells were either isolated from the peritoneal cavity of untreated C57B/6 mice by flushing the peritoneum with cold DMEM (Gibco) or purchased (J774A.1 cell line, ATCC) and maintained in DMEM (Gibco) with 10% FBS.

Mice
Animal experiments were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animal. C57BL/6 TNF-α-deficient mice were purchased from The Jackson Laboratory (Bar Harbour, Maine, USA). Genotyping for TNF-α +/+ and -/- mice using RT-PCR were performed according protocol provided by The Jackson Laboratory. Total RNA was prepared with absolute RNATM RT-PCR Miniprep Kit (Stratagene). Following primers were used: oIMR4182 (Common) 5’-TAG CCA GGA GGG AGA ACA GA-3’, oIMR4183 (TNF-α +/+; 183bp) 5’- AGT GCC TCT TCT GCC AGT TC-3’ and oIMR7297 (TNF-α -/-; 318bp) 5’-CGT TGG CCC GTG ATA TT-3’.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Real-time RT-PCR
Total RNA was isolated from respective cells using an RNeasy Mini kit (QIAGEN Inc.) according to manufacturer’s instructions. 2µg RNA were reverse transcribed into cDNA with random primers by MMLV reverse transcriptase (RT) (Promega). Real time RT-PCR was performed using 2ng of cDNA per sample with the SYBR Green Master Mix in a 25-µl reaction. Ct values were measured using ABI PRISM 7000 Sequence Detector (Applied Biosystems). The 18 S ribosomal RNA was used as an endogenous control to normalize the amounts of RNA in each sample. Sequences of SMC and EC primer sets used were as previously described by our laboratory². Sequences of other primer sets used in this study are as follows. TNF-R1:5’>ACCAAGTGCCACAAGGAAAC-3’ 5’>CACGCAGTGAAGTGTT CT<3’, p65: 5’>GGCTCATCCACATGAACTT<3’ 5’>ATCTTGAGCTGCGCAGTGT<3’, p50/p105:5’>GCATCCCAACCTGAAAATCGT<3’ 5’>GCATAAGCTTCT GCCGTTC<3’, c-REL: 5’>CCTCAATGTGGTACGTTGTT<3’ 5’TTCACGTCAGGCAGT<3’.

Immunofluorescence Staining
Progenitor cells were seeded in either gelatine- or murine collagen IV-coated chamber slides (BD Biosciences) prior to treatment (+/-TNF-α). The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 10% normal swine serum (Dako). Frozen tissue sections were fixed with cold acetone prior to permeabilization and blocking with the same reagents. Incubation of cell or tissue samples with primary antibodies was performed at 4°C overnight, followed by incubation with secondary antibodies for 45mins at 37°C after extensive washes in between. Cells/tissue samples were counterstained with DAPI (1:1,000 in PBS) for 3mins at
room temperature and mounted with fluorescent mounting media (Dako) before image acquisition using the Axio Imager.M2 microscope and AxioVision Digital Imaging System (Carl Zeiss Ltd.). Primary antibodies used were Sca-1, CD117, CD34, PECAM1, VE-cadherin, p65, SM-22a, Calponin, CD206, CD86 (all purchased from Abcam), eNOS (Sigma Aldrich), Mac-1 (produced in lab) and lectin-fluorescein isothiocyanate (Vector Laboratories). The appropriate fluorescent-conjugated IgG antibodies were used as secondary antibodies (Invitrogen).

**Western Blot Analysis**
ES cells were harvested for lysis with IP-A buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% Triton X-100 plus protease inhibitors) and proteins were measured using the Bradford method. 40 μg of lysate was applied to SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences), followed by a standard western blotting procedure using antibodies against PECAM1, VE-cadherin, SM-22a, Calponin, GAPDH, p65, p50, TNF-R1, Iκ-Bα (all purchased from Abcam, UK), SM-αA, eNOS, α-tubulin (all from Sigma Aldrich), Rel-B, NIK, p52/p100 (all from Cell Signaling Technology, Inc.) and Histone H1 (Santa Cruz Biotechnology, Inc.).

**Mouse Multi-Analyte Array, TNF-α, Angiopoietin-2, VEGF-A, VEGF-C and VEGF-D ELISA**
Conditioned media from macrophages were harvested and subjected either to a Mouse Th1/Th2/Th17 Cytokines or a Mouse Common Chemokines Multi-Analyte ELISArray Kit (both purchased from SA Biosciences, QIAGEN) according to the manufacturer’s protocol. Levels of TNF-α, angiopoietin-2, VEGF-A, VEGF-C and VEGF-D in macrophage conditioned media were measured using a Mouse TNF-α Quantikine kit (R&D Systems), Mouse Angiopoietin-2 Elisa kit (MyBioSource.com), Mouse VEGF-A Platinum ELISA (eBioscience, UK), Mouse VEGF-C and VEGF-D Elisa kit (both purchased from MyBioSource.com), respectively, according to individual manufacturer’s instructions. Normal media was used as controls.

**In Vitro Matrigel Assay**
A total of $2 \times 10^4$ progenitor cells were cultured either in the presence of macrophages (physical contact prevented using 0.4 μm inserts) or TNF-α for 48 hours before seeding onto Matrigel-coated chamber slides (10 mg/ml; BD Matrigel Basement Membrane Matrix, A6661) in 350μl of complete DM. Progenitor cells that were cultured in DM only were also used as controls. Changes in cellular morphology and tube formation were observed and quantified after 6 hours. Images were assessed with Axioplan 2 imaging microscope with Plan-NEOFLUAR 10×, NA 0.3, objective lenses, AxioCam camera, and Axiovision software (all Carl Zeiss MicroImaging, Inc.) at room temperature, and were processed with Photoshop software (Adobe).

**In Vivo Angiogenesis Assay**
Sca-1+ progenitor cells were cultured in the presence or absence of TNF-α for 48 hours and labeled with Qdot® nanocrystals using a Qtracker® 625 cell labeling kit (Invitrogen), prior to mixing with 50 μl of Matrigel (10 mg/ml; BD, A6661) for subcutaneous injection into the back or flank of C57BL/6 mice. Six injections were conducted for each group. Seven days later, the mice were euthanized and the plugs were harvested, frozen, cryosectioned and fixed with acetone. H/E staining was performed and images were assessed with an Axioplan 2 imaging microscope with Plan-NEOFLUAR 10×, NA 0.3 objective lenses (Carl Zeiss MicroImaging, Inc.) at room temperature.

**Gene Knockdown and Overexpression**
Stem cell knockdown of p65 (NM_009045) and TNF-R1 (NM_011609.2) was carried out using MISSION short hairpin RNA (shRNA) lentiviral plasmids transfer, with non-targeting vector (SHC002) as negative controls (all Sigma Aldrich UK). For transient over-expression of p65, 1.0μg per 1x106 ES cells of pcDNA3-p65 (Addgene plasmid 20012) expression plasmid were introduced into ES cells by nucleofector II (Amaxa, Germany) with a mouse ES cell nucleofection kit (Amaxa, VPH-1001) and using program A-30 according to the manufacturer’s instructions. An empty vector pcDNA3 (Addgene plasmid 10792) was included as a negative control. Total ES cell proteins were harvested after gene ablation or overexpression and subjected to western blot analysis.
Luciferase Reporter Assay
Stem cells were transfected with pGL3-VE-cadherin (2.4kb) promoter reporter gene (0.33μg/well) alone or in combination with pcDNA3-p65 expression plasmid (0.16μg/well) using a Fugene-6 Reagent (Roche). Renilla luciferase (0.1μg/well) was also included as an internal control. Media (+/-TNF-α) change was carried out on the ES cells after the overnight transfection before Luciferase and Renilla activities were detected after 48 hours. Relative luciferase unit (RLU) was defined as the ratio of Firefly versus Renilla with that of the control (set as 1.0).

Chromatin Immunoprecipitation (CHIP) Assay
ChIP assays were carried out using the EZ-ChIP™ (Milipore) according to the manufacturer’s instructions, and as previously described3. Control or TNF-α treated cells were treated with 1% (vol/vol) formaldehyde, quenched with glycine and sonicated for chromatin shearing. Immunoprecipitation was carried out with a p65 antibody (Abcam, UK), with a normal IgG as a control. The immunoprecipitates were eluted in 200 μl of elution buffer (and crosslinks of protein/DNA complexes were reversed to obtain purified DNA. PCR was carried out on the DNA to amplify the promoter region of VE-cadherin gene using primers: VE-cadherin Prom forward 5’-CAACCATGGGCATGCAGT-3’; VE-cadherin Prom reverse 5’-AGGGCTAGGAGGTCAGAGG-3’. Aliquots of chromatin were also analysed before immunoprecipitation and was used as an input control. The PCR products were analyzed on 2% agarose gels and assessed using BioSpectrum AC Imaging System and Vision-WorksLS software.

Bioreactor
As previously described3, 5x10⁵ stem cells were seeded in a previously decellularised bioreactor in the absence or presence of TNF-α for 12 hours followed by shear stress at stepwise rates ranging from 10 to 35 dynes/sqcm² for 48 hours, after which the grafts remained under constant shear stress of 35 dynes/sqcm² for another 72 hours until harvesting. The harvested grafts were embedded in mouse liver and frozen in liquid nitrogen prior to H/E or immunofluorescence staining for the detection of various marker expressions.

Vein Graft
Vein grafts were performed using animals of the same genotype, according to the procedure previously described4,5. The right common carotid artery of recipient mice was mobilized free from the bifurcation at the distal end toward the proximal and cut in the middle. A cuff made using an autoclavable nylon tube (Portex LTD) was placed at the ends. The artery was turned inside out over the cuff and ligated. Donor vein segment was grafted between the two ends of the carotid artery by sleeving the ends of the vein over the artery-cuff and ligating them together with an 8-0 suture. Vein grafts were harvested at different post-operative time points (0, 1, 4 and 8 weeks) and embedded in paraffin before sectioning. Thrombus and neointimal lesions were defined as the region between the lumen and media, which contains 2 to 3 layers of condensed cells without microvessels. For luminal area measurement, sections were analysed using a BX60 microscope (Zeiss, Germany) where images were first scanned, saved and then overlaid by different linings to trace the lumen and media. The lesion area was determined by subtracting the area of the lumen from the area enclosed by the line inside of the media.

Chimeric mouse model
The procedure used for creating chimeric mice was similar to that which has previously been established in our group 6,7. Briefly, donor mice (TNF-α -/- and +/-) were sacrificed and their femurs and tibias were removed aseptically. Marrow cavities were flushed with HBSS (GIBCO-BRL and passed through a nylon mesh filter to obtain single cell suspensions. Six to eight week old mice (either TNF-α +/- or TNF-α -/-) received whole body X-ray irradiation (950 Rads). The irradiated recipients received 1x10⁷ bone-marrow cells in 0.3 ml RPMI 1640 via tail vein injection. Vein grafts from wild type mice were subsequently performed on the irradiated recipients 4 weeks after the bone marrow transfer.
Cell Counting
Stem cells were seeded in a T75cm² flask at 5x10^5 cells and cultured in the presence or absence (DM only) of 100ng/ml of TNF-α. Forty eight hours later, the cells were trypsinized and subjected to cell size calculation using a multisizer 3 coulter counter (Beckman Coulter) according to the manufacturer's instructions.

BrdU Incorporation Assay
Stem cells were seeded in a 96-well plate at 2x10^3 cells/well and cultured in the presence or absence (DM only) of 100ng/ml of TNF-α. Twenty four, 48 or 72 hours later, the cells were labeled with BrdU for at least 2 hours at 37°C before the addition of an anti-BrdU-POD antibody. The BrdU labeling solution and antibody are components of the 5-Bromo-2'-deoxy-Uridine Labeling and Detection Kit III (Ref 1144611001, Roche). BrdU incorporation was detected at 450nm using a spectrophotometer.

Nuclear and Cytoplasmic Extraction
Stem cells were infected with lentiviral shRNA for ablation of either TNF-R1 or p65 gene prior to treatment with 100ng/ml of TNF-α for 48 hours. A non-coding shRNA was also used as a control for the gene knockdown. Nuclear and cytoplasmic fractions from the stem cells were harvested using a kit containing NE-PER® Nuclear and Cytoplasmic Reagents (Thermo Scientific), according to the manufacturers' instructions. A total of 20µg of proteins was applied to SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences), followed by a standard western blotting procedure. Polyclonal antibodies against p65 (Abcam, UK), α-tubulin (Sigma Alrich) and Histone H1 (Santa Cruz Biotechnology, Inc.) were used to detect the respective proteins.

Cell Death (Apoptosis) Detection Assays
For detection of apoptosis in vitro, stem cells were seeded in a 6-well plate at 1x10^5 cells and cultured in the presence or absence of either TNF-α (100ng/ml) or J774.1 macrophages (with a 0.4µm insert). Forty eight hours later, the cells were trypsinized and stained with Annexin-V (eBioscience) according to the manufacturer's instructions. The percentage of apoptotic (annexin-V positive) cells were quantified using flow cytometric analysis. The levels of apoptosis in in vivo matrigel plugs (refer in vivo angiogenesis assay) were identified by dUTP nick-end labeling (TUNEL) using an apoptosis detection kit according to the manufacturer's protocol (ApopTag Red In Situ apoptosis detection kit; Chemicon Inc.). Tissue samples were counterstained with DAPI (1:1,000 in PBS) for 3mins at room temperature and mounted with fluorescent mounting media (Dako) before image acquisition using the Axio Imager.M2 microscope and AxioVision Digital Imaging System (Carl Zeiss Ltd.).

Statistical Analysis
Data was presented as the mean and standard error of the mean (S.E.M.) of at least three independent experiments and analysed using Graphpad Prism V.4. Statistical analysis was performed with analysis of variance (ANOVA) followed by Dunnett’s multiple comparison tests and significance was depicted by asterisks, *: P<0.05, **: P<0.01, ***: P<0.005.

References

