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BREAKING THE “HARMONY” OF TNF- α SIGNALING FOR CANCER TREATMENT

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Abstract

Tumor necrosis factor-alpha (TNF- α) binds to two distinct receptors, TNFR1/p55 and TNFR2/p75. TNF- α is implicated in the processes of tumor growth, survival, differentiation, invasion, metastases, secretion of cytokines and pro-angiogenic factors. We have shown that TNFR2/p75 signaling promotes ischemia-induced angiogenesis via modulation of several angiogenic growth factors. We hypothesized that TNFR2/p75 may promote tumor growth and angiogenesis. Growth of mouse Lewis lung carcinoma (LLC1) and/or mouse melanoma B16 cell was evaluated in wild type (WT), p75 knockout (KO) and double p55KO/p75KO mouse tumor xenograft models. Compared to WT and p55KO/p75KO mice, growth of tumors in p75KO mice was significantly decreased (two-fold) in both LLC and B16 tumors. Tumor growth inhibition was correlated with decreases in VEGF expression and capillary density, as well as bone marrow (BM)-derived endothelial progenitor cells (EPCs) incorporation into the functional capillary network, and an increase in apoptotic cells in LLC xenografts. Gene array analysis of tumor tissues showed a decrease in gene expression in pathways that promote tumor angiogenesis and cell survival. Blocking p75 by shRNA in cultured LLCs led to increases in TNF-mediated apoptosis, as well as decreases in the constitutive and TNF-mediated expression of angiogenic growth factors (VEGF, HGF, PLGF), and SDF-1 α receptor CXCR4. In summary, p75 is essential for tumor angiogenesis and survival in highly vascularized murine lung tumor xenografts. Blocking p75 expression may lead to tumor regression. This may represent new and effective therapy against lung neoplasms and potentially tumors of other origin.

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Keywords

TNF- α ; TNFR2/p75; cancer; angiogenesis; EPC; microenvironment

Introduction

Tumor necrosis factor-alpha (TNF) has pluripotent effects on tumorigenesis and tumor progression and it is produced by tumor and tumor microenvironment (Elbaz & Mahmoud, 1994; Goillot et al., 1992; Komori et al., 1993; Liu et al., 1998; Montesano et al., 2005; Rivas et al., 2008; Suganuma et al., 1999). The effect of TNF on angiogenesis, however, is controversial (Frater-Schroder et al., 1987; Leibovich et al., 1987; Sato et al., 1987). In cell culture, the effect of TNF on angiogenesis depends on the concentration and duration of the treatment (Fajardo et al., 1992; Frater-Schroder et al., 1987; Leibovich et al., 1987; Sato et al., 1987; Slowik et al., 1993). In endothelial cells (ECs), TNF increases the expression of angiogenic factors, such as VEGF, bFGF, IL-8 (Yoshida et al., 1997). In addition, impaired TNF signaling in ECs was associated with enhanced apoptotic response in cutaneous microvasculature in adult tissue (Fajardo et al., 1992).

TNF exerts its function via binding to p55 (TNFR1) and p75 (TNFR2). Both p55 and p75 bind to TNF receptor-associated factor 1 and 2 (TRAF1 and TRAF2) (Rothe et al., 1994; Sethi et al., 2008), thus providing a mechanism for shared activity between the two receptors. On the other hand, the cytoplasmic domains of p55 and p75 are significantly different and these receptors are reported to trigger divergent signaling pathways upon interaction with TNF (Barbara et al., 1996; Jacobsen et al., 1994; Rothe et al., 1994; Sethi et al., 2008). The intracellular signaling involving p55 has been well deciphered. Studies have shown that p55 activates both apoptosis and cell survival signaling pathway and the pro-apoptotic effects of p55 are related to the death domain on its cytoplasmic part of the receptor (Barbara et al., 1996; Bhardwaj & Aggarwal, 2003; Jacobsen et al., 1994). Inversely, p55, via activating NF- κ B, increases the expression of genes that in turn suppress TNF-mediated apoptosis (Barbara et al., 1996; Bhardwaj & Aggarwal, 2003; Jacobsen et al., 1994). Compared to p55, few signaling pathways and functions of p75 have been elucidated, especially in the context of tumor angiogenesis (Frater-Schroder et al., 1987; Slowik et al., 1993). Unlike p55, the cytoplasmic domain of p75 lacks the death domain (Mukhopadhyay et al., 2001). Studies have shown that in ECs, Bmx/Etk (endothelial/epithelial tyrosine kinase) binds to p75 constitutively. TNF stimulation induces reciprocal phosphorylation between p75-bound Bmx/Etk and VEGFR2, which leads to the activation of the Akt pathway, and in turn results in EC migration and angiogenesis (Zhang et al., 2003). Studies from our laboratory have shown that p75 signaling promotes ischemia-induced angiogenesis via modulation of several angiogenic growth factors in mice (Goukassian et al., 2007).

We hypothesize that p75 signaling may be essential for the angiogenic and survival processes in tumor tissue and blocking p75 signaling may affect tumor cell survival. We also investigated p75-mediated effects on p38 (MAPK) activation in bone marrow (BM)-derived endothelial progenitor cells (EPCs). BM-derived EPCs are known to be critical regulators of the angiogenic switch in progression of micro-metastasis to lethal macro-metastasis (Gao et

al., 2008; Rafii & Lyden, 2008) and tumor-derived TNF signaling had been link in vivo to differentiation of myeloid progenitors cells to “byphenotypic” myeloid/endothelial cells (Li et al., 2009). In this study, we identified the role of functional p75 signaling in BM-derived EPCs in vitro and in the context of tumor angiogenesis and tumor growth in vivo.

Results

TNFR2/p75 knockout microenvironment does not support LLC and B16 melanoma tumor growth

Twenty-one days after inoculation, tumor (LLC) volumes were larger in WT and Double-KO (Dbl-KO) mice compared to p75KO mice (Supplementary Fig. S1). A similar growth was observed in all three genotypes between day 0–7 (Fig. 1A). Compared to day 7, tumors in WT and Dbl-KO mice became ~370% ($p<0.02$) and ~1030% ($p<0.008$) larger on days 14 and 21, respectively, whereas in p75KO mice, tumors became only ~180% ($p<0.05$) and ~420% ($p<0.01$) larger on days 14 and 21, respectively. Kinetics of tumor growth and tumor volumes in Dbl-KO mice were comparable to WT mice with no statistical difference at any time point. Compared to WT and Dbl-KO mice, tumor growth in p75KO mice was reduced by ~30% ($p<0.05$) on day 14 and ~50% ($p<0.001$) on day 21 (Fig. 1A). These results suggest that the absence of signaling through p75, but not both TNF receptors (i.e., Dbl-KO) in tumor tissue microenvironment inhibits tumor growth.

B16 mouse metastatic melanoma tumor growth was evaluated in WT and TNFR2/p75 mice, as well (Supplementary Fig. S2). Tumor growth kinetics revealed that between days 0–7 tumors grew similarly in WT vs p75 mice. Compared to day 7, melanoma tumors in WT mice became ~1195% larger (12-fold) on day 14, whereas at the same time period tumor growth was inhibited in p75KO mice by more the two times ($p<0.02$) on day 14 (only 474% increase between day 7 vs day 14). These results indicate that in the absence of the signaling via TNFR2/p75, at least in the tumor tissue microenvironment, melanoma growth can be inhibited ~2.5-fold. Please note that in these experiments B16 cells were unaltered (so-called “wild type”) and the tumor growth inhibition was predominantly dependent on unsupportive host tissue in p75KO mice. LLC (Fig. 1A) and B16 melanoma xenograft studies (Supplementary Fig. S2) taken together suggest that this may be a common response of vascularized tumors of different origin, at least in part, due to decreased tumor angiogenesis.

TNF expression is similar in the tumor tissue from WT and p75KO mice

Immunostaining showed that the intensity of TNF staining in tumor or normal tissue (2 cm distant from tumor) was not different between WT and p75KO mice (Fig. 1B and Supplementary Fig. S3A, B). However, compared to normal skin (Supplementary Fig. S3B), TNF expression was ~10- and 9-fold ($p<0.001$) higher in tumor tissue from WT and p75KO mice, respectively. ELISA of tumor homogenates confirmed that TNF levels were similar in tumor tissue from WT vs. p75KO mice (1010 ± 166 vs. 976 ± 262 pg/ml, $p=NS$) (Fig. 1C).

VEGF expression and capillary density are decreased and apoptosis is increased in tumors from p75KO mice

Compared to WT tumors, the intensity of VEGF immunostaining in tumor tissue from p75KO mice was decreased more than 50% ($p < 0.001$) (Fig. 1D and Supplementary Fig. S4A). Accordingly, ELISA of tumor homogenates showed ~40% ($p < 0.002$) decrease in VEGF levels in the tumor tissue from p75KO mice (Fig. 1E). CD31 immunostaining was significantly decreased in tumors from p75KO mice (80% decrease, $p < 0.001$, Fig. 1F and Supplementary Fig. S4B). FACS analysis revealed a 41% ($p < 0.045$) decrease in CD31 (+) cell in the whole tumor tissue from p75KO vs WT mice (Fig. 1G). TUNEL staining was increased ~40% ($p < 0.05$) in tumor tissue from p75KO mice (Fig. 1H and Supplementary Fig. S4C).

Decreased tumor angiogenesis in the periphery of the tumor in p75KO mice

As shown in Fig. 2A and 2B, apoptosis (as measured by TUNEL) in tumors from p75KO mice were increased (Fig. 2B, left image and triple overlay). This was associated with a significant decrease in capillary network in tumor but not peri-tumoral tissue (Fig. 2B, middle image and triple overlay), indicating substantial deficiency in tumor angiogenesis in the tumor-microenvironment interface in p75KO mice. These results suggest that WT host tissue supports tumor angiogenesis and survival, whereas both biological processes are compromised in p75KO mice.

Incorporation of BM-derived EPCs into tumor vasculature is decreased in tumors from p75KO mice

The recruitment of BM-derived cells was not different in chimeric (syngeneic GFP bone marrow transplanted) WT vs. p75KO mice (Fig. 2C and D, green fluorescence and arrows). BM-derived EPCs (Fig. 2E and F, yellow fluorescence and arrows) were incorporated into functional tumor vessels (GFP + BM-derived cells also positive for BS1-lectin-rhodamine perfusion - red) in tumors of WT mice, whereas this was significantly decreased (~45%, $p < 0.05$) in tumors from p75KO mice (Fig. 2E and F, yellow/arrows vs. green/arrowheads). This finding suggests that incorporation of BM-derived EPC into functional vascular network within the tumor tissue requires, at least in part, the presence of TNF receptor p75 in tumor stroma and/or on EPC themselves.

Angiogenic, pro-survival and anti-apoptotic gene expression is decreased in tumors from p75KO mice

Combined data from angiogenic, apoptotic and cancer pathways microarrays (total of 360 genes tested) revealed that 29 genes were significantly regulated in tumors from p75KO vs. WT mice (28 genes were down-regulated and only one, Bcl2l1, was up-regulated). Strikingly, 17 out of 28 down-regulated genes are involved in regulation of angiogenesis (i.e., VEGF, HGF, Pgf, IL1b, p38 Mapk14, Cxcl2, etc). Compared to tumors from WT mice, tumors from p75KO mice showed ~2-5-fold decreases in the expression of angiogenic growth factors, cytokines and chemokines (Fig. 3A–C). The expression of anti-apoptotic (Bag 1,3,4, Bnip3, Dad1 and Faim) and Bcl2 family (Bnip3l and Bok) genes were decreased ~2-11-fold in tumors from p75KO mice compared to WT mice (Fig. 4A–C). Furthermore,

absence of p75 in the host tissue had little effects on the expression of genes in cancer pathways (Supplementary Fig. S5A–C). In agreement with the gene arrays, qRT-PCR results showed that as in gene arrays the expression of Ang-1, Ang-2, Hif-1 α and MMP2 was not modified in the LLC tumors from WT vs. p75KO mice; whereas Cxcl2 expression was decreased in tumors from p75KO vs. WT mice (Supplementary Fig. S6A–E). In summary, microarray results suggest that signaling through TNFR/p75 in microenvironment plays an important role in supporting angiogenesis and cell survival in LLC tumors. These results also suggest that inhibition of tumor growth in p75KO mice is, predominantly, a consequence of unsupportive tumor microenvironment. (For the results of B16 mouse melanoma angiogenic gene array analysis, please see Supplementary Figure7).

TNFR2/p75 is required for the activation of p38 MAPK in BM-derived EPCs

In cultured BM-derived EPCs obtained from WT mice and in cultured LLCs, TNF treatment activated p38 MAPK in a time-dependent manner (Fig. 5A,B and 5E,F), whereas in EPCs obtained from p75KO mice, TNF-mediated p38 activation was significantly weaker (~0.4-fold, p =NS, Fig. 5C–D). These results suggest that (a) signaling via TNF receptor p75 is necessary for proper p38 activation in BM-derived EPCs and (b) that p38 is constitutively activate in LLCs and there is significant TNF-mediated p38 activation in LLCs.

TNF-mediated apoptosis is increased in TNFR2/p75 knockdown LLCs

To further substantiate the clinical implications of our findings in p75KO mice implanted with unaltered tumor cells, and due to the fact that we can not create p75 knockout host humans, but with current drugs and technologies we can inhibit/block efficiently p75 receptor in the whole tumor tissue (tumor cells, EC, fibroblasts, inflammatory cells, BM-derived cells) we performed a series of experiments using p75 receptor knockdown (KD) cancer cells. We generated p75 receptor knockdown LLCs (p75KD/LLCs) using shRNA technology (Supplementary Materials and Methods). qRT-PCR data of percent inhibition of p75 receptor in stably transfected p75KD/LLCs is presented in Supplementary Fig. S8. p75KD/LLCs (plasmid combinations 1+2 and 1–4) showed ~74% or ~90% inhibition of p75 receptor expression, respectively (Supplementary Fig. S8). Compared to control, scrambled plasmid transfected LLCs, TNF treatment induced ~56% and ~90% apoptotic cells (cells in sub-G0/G1 population) (Riccardi & Nicoletti, 2006) in p75KD/LLCs (1+2) and p75KD/LLCs (1–4), respectively (Fig. 6A). Increase in apoptotic cells was in direct correlation with percent inhibition of p75 receptor (Fig. 6A and Supplementary Fig. S8).

Compared to control, p75KD/LLCs (1+2 and 1–4) showed significant TNF-mediated decreases in the expression of Vegfa (0.75-3.5-fold), Hgf (2.5-9-fold), Plgf (3-15-fold), Cxcr4 (5-20-fold) (Fig. 6B–E, grey bars). The constitutive levels of these genes were also decreased in non- treated p75KD/LLC cells (Fig. 6B–E, black bars). Thus, the highest degree of apoptosis (Fig. 6A) and decrease in the expression of angiogenic factors and Sdf-1 α receptor Cxcr4 (Fig. 6B–E) were observed in cells with the lowest p75 receptor expression level (Fig. 6A and Supplementary Fig. S8), which strongly corroborates our findings in mouse tumor tissue (Fig. 1D–H, and Fig. 2A,B).

Discussion

It is well-known that TNF is continuously produced in tumors and tumor microenvironment and this endogenous tumor-produced TNF induces tumor angiogenesis and promotes tumor development and spread (Elbaz & Mahmoud, 1994; Goillot et al., 1992; Komori et al., 1993; Liu et al., 1998; Montesano et al., 2005; Rivas et al., 2008; Suganuma et al., 1999).

Conversely, local administration of high doses of TNF into tumor tissue is anti-angiogenic and has potent anti-tumor effects (Hohenberger et al., 2003). TNFR1/p55 and TNF2/p75, receptors of TNF, have opposing TNF-mediated effects. TNFR1/p55 is known to mediate cytotoxic effects of TNF and cell death (Wong & Goeddel, 1994). p75, however, is implied in cell protective effects of TNF (Barbara et al., 1996; Jacobsen et al., 1994; Slowik et al., 1993; Vandenabeele et al., 1995) in endothelial and hematopoietic lineage cells (Hohenberger et al., 2003; Jacobsen et al., 1994). The role of TNFR2/p75 in mediating tumor growth and angiogenesis is unclear. In order to clarify the role of TNFR2/p75 in mediating TNF-induced angiogenic, survival and apoptosis processes in tumor tissue, we utilized TNFR2/p75 knockout (KO) mice to remove signaling through p75 receptor in tumor-microenvironment and shRNA to knockdown (KD) p75 expression in tumor cells.

In view of the fact that the divergent TNF effects has been attributed to TNF concentration, duration of exposure (Fajardo et al., 1992) and also type of targeted cells (Fiers et al., 1995), we analyzed TNF levels in tumor tissues from WT vs. p75KO mice. There was a 9–10 fold increase in TNF levels in LLC tumor implants vs. normal skin in both WT and p75KO mice and the TNF level in tumors from WT and p75KO mice were not different. These results indicate that decreased capillary density in tumors from p75KO mice were not caused by lack or different concentration of TNF in tumors due to mice genotype; rather it may be due to increased cytotoxic TNF signaling through unopposed p55 receptor in tumor tissue microenvironment (Wong & Goeddel, 1994). Moreover, augmented levels of apoptosis in tumor tissues from p75KO mice underscored our hypothesis that absence of signaling via p75 receptor in the tumor microenvironment affects survival processes within the tumor tissue, which creates a hostile environment for tumor and EC cells.

Tumor growth is angiogenesis dependent (Folkman, 1971; Gimbrone et al., 1972; Hanahan & Folkman, 1996). VEGF, a critical growth factor in angiogenesis, is known to be regulated by TNF (Eisenthal et al., 2003; Yoshida et al., 1997). Similar to earlier studies in models of post-natal neovascularization (Goukassian et al., 2007; Luo et al., 2006), we observed a significant reduction in VEGF expression in tumor tissue from p75KO vs. WT mice. This suggests that intact p75 signaling in tumor microenvironment may play an important role in tumor angiogenesis through positive regulation of VEGF expression (Goukassian et al., 2007; Meng et al., 2010). Indeed, VEGF decreases were associated with a significant reduction in capillary network in tumors from p75KO vs. WT mice. Our findings suggest that inhibition of tumor angiogenesis due to decreased expression of VEGF and other angiogenic growth factors and cytokines (Plgf, Cxcl2, IL1b, etc.) may be the primary mechanism of tumor growth inhibition in p75KO mice.

TNF, via p75, elicits potent EC-activating cytokine and growth factor signaling (Ryuto et al., 1996; Yoshida et al., 1997) that induces EC/EPC survival, migration, recruitment and

incorporation into new tumor microvasculature and angiogenesis (Lyden et al., 2001; Peters et al., 2005; Santarelli et al., 2006); whereas activation of p55 signaling by TNF leads to inhibition of EC migration and increase in EC apoptosis (Madge & Pober, 2001). VEGF has been shown to mobilize BM-derived EPCs in murine models and in humans (Asahara et al., 1999; Kalka et al., 2000). In p75KO mice, decreased TNF-induced putative angiogenic signaling (TNF→NF-κB→VEGF), may lead to subsequent decreases in either mobilization, recruitment, homing or survival of BM-derived EPCs in the p75KO tumor tissue (Lyden et al., 2001; Peters et al., 2005; Santarelli et al., 2006). In agreement with these previous reports, we showed that there was a significant decrease in the incorporation of recruited EPCs into functional capillary network in tumors from p75KO mice, which suggests an additional mechanism of impaired angiogenesis in p75KO tumor tissue.

This study further showed that a significant number of angiogenesis genes (p38 Mapk 14, Vegfa and Vegfb amongst others) as well as pro-angiogenic growth and transcription factors, and cytokines were decreased in LLC and B16 tumors from p75KO mice. These factors are directly involved in the regulation of Akt, Erk1/2 Mapk (p42/44 Mapk), p38 Mapk and NF-κB pathways that are known to promote tumor survival, proliferation, migration and angiogenesis processes (Gupta et al., 2003; Mukhopadhyay et al., 2001). It is conceivable that decreases in p38 Mapk and two of Vegf isoforms (a and b) are directly downstream of p75 signaling and are not affected by organ-specific tumor origin, at least in two murine tumor models of lung adenocarcinoma (LLC) and malignant melanoma (B16). The rest of angiogenesis genes that were regulated differently in LLC and B16 tumors were either specific for the type of the tumor origin (lung vs. melanoma) and/or are further downstream of TNF-mediated signaling, such as NF-κB activation (Cxcl2, IL18, IL1b and IL12a) (De Plaen et al., 2006; Park et al., 2001; Kohno & Kurimoto, 1998), which (NFκB) we reported to be decreased in p75KO EPCs in vitro (Goukassian et al., 2007). These findings strongly suggest that blocking p75 receptor signaling in tumor microenvironment and/or tumor cells may negatively affect tumor growth through inhibition of additional pathways beyond VEGF signaling. This phenomenon may be common also for tumors of other origin that require substantial vascularization but this, however, remains to be tested.

Absence of p75 receptor in the host tissue minimally affected expression of genes in cancer pathways microarray, suggesting that different microenvironment does not significantly affect cancer pathways gene expression in the whole tumor tissue. Further experimentation that rigorously determine gene expression changes in cancer cells as well as host microenvironment cells (i.e., stromal cells, endothelial cells, immune cells from peripheral blood, BM-derived progenitor cells, etc.) will be necessary to confer causality of gene expression changes to microenvironmental/host tissue cues and the *vice versa*.

Taken together our findings suggest that blocking p75 TNF receptor in tumor cells affects their survival and tumor promoting properties of the tumor cells themselves (e.g., tumor angiogenesis). This underscores a feasibility that one drug may have a dual anti-tumor effect aimed towards tumor microenvironment, as well as tumor cells. Therefore, we would like to suggest that selective inhibition of TNFR2/p75 signaling in tumor tissue with an antecedent increase in TNF (in tumor tissue) or delivery of small non-toxic exogenous recombinant TNF may present a novel cancer monotherapy or, most likely, part of combination therapy

that will help to sensitize tumor cells and tumor ECs to cytotoxic effects of currently in use anti-tumor (chemotherapy, radiotherapy, anti-angiogenic, etc.) therapies.

Materials/Subjects and Methods

Mouse Tumor Models, Cell Culture and Tumor Inoculation

Six to eight weeks old WT {C57BL/6J – control of the mixed C57BL/6 and 129 strains background defined by the vendor as N10F34, meaning that these two strains were backcrossed 10 times (N10 is number of backcross generations) and inbred 34 times (F34 is number of filial or inbreeding generations)}, p75KO (B6.129S2-Tnfrsf1b^{tm1Mwm/J}) and p55KO/p75KO (B6.129S-Tnfrsf1a^{tm1Imx}Tnfrsf1b^{tm1Imx/J}) (Dbl KO) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were handled in accordance with the guidelines set by the IACUC at St Elizabeth's Medical Center (Boston, MA). LLC cells were obtained from ATCC (Manassas, VA). After trypan blue exclusion (95% viability), 5×10^5 LLC/mouse were re-suspended in 100 μ l 1 \times PBS, mixed with 100 μ l of growth factor reduced and phenol free Matrigel (BD Biosciences, CA) and injected subcutaneously into right flanks. Tumor growth was monitored using electronic calipers on days 7, 14 and 21. Tumor volumes (mm^3) were calculated using $V = 0.52 \times L \times W^2$ formula. (For B16 tumor inoculation model, please see Supplementary Materials and Methods).

Immunostaining, Imaging and Analyses

For immunostainings, tumors were bisected in half, embedded in OCT compound (Tissue-Tek, Torrance, CA, USA) and snap-frozen in liquid nitrogen. Cryosections (6–8 μ m) of WT and p75KO tumors were fixed in acetone (4°C) for 10 minutes (Goukassian et al., 2007) and processed for various immunostaining. Topro-3 was used to visualize nuclei (Invitrogen, Carlsbad, CA, USA). Primary anti-TNF- α rabbit polyclonal (Abcam, Cambridge, MA, USA) and rhodamine-conjugated goat anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were used for detection of TNF expression in tumors and normal skin (2–3 mm away from tumors). Primary anti-VEGFA rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and TRITC-conjugated goat anti-rabbit secondary antibodies were used for detection of VEGF expression. Anti-CD31 primary (BD Pharmingen, San Jose, CA, USA) and Alexa Fluor 555 goat anti-rat secondary antibodies (Invitrogen) were used to determine vascular network in tumors. To analyze apoptotic processes, tumor tissues were stained with ApopTag Fluorescein in Situ Apoptosis (TUNEL) Kit (Chemicon International, Temecula, CA, USA). All immunostainings were imaged using laser scanning confocal microscope (Meta510, ZEISS, Thornwood, NY, USA). Graphs represent pooled data of mean pixel intensity in 7–8 fields of 176,400 μm^2 /mouse from at least 5 animals/genotype using NIH ImageJ program (v1.40, NIH, Bethesda, MD, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of VEGF and TNF in the whole tumor tissue were assessed by ELISA (RayBiotech, Norcross, GA, USA). The plates were read using the Tecan Spectra model 96 Well Microplate Reader (MTX Lab Systems, Vienna, VA, USA).

FACS Analysis of tumor-associated CD31 positive cells

Whole tumors from WT and p75KO mice were collected on day 14 post-inoculation, minced into small pieces and digested in enzymatic medium containing Collagenase A (Roche, IN). Cell suspension was stained with rat anti-mouse CD31 antibody and 7AAD viability staining solution (both, eBiosciences). Percent of tumor-associated CD31 (+) cells were analyzed by FACSCanto II (BD, Franklin Lakes, NJ). The data was analyzed using Summit v4.0 software (DakoCytomation, CA). (Supplementary Materials and Methods).

Bone Marrow Transplant (BMT) Mouse Model

To track BM-derived cells into tumor tissues, 3×10^6 donor BM mononuclear cells (MNCs) from green fluorescent protein (GFP) positive WT and p75KO mice were injected through tail vein into lethally irradiated syngeneic WT or p75KO mice as described by us previously (Goukassian et al., 2007). On day 28 post-BMT, WT/WT-GFP and p75KO/p75KO-GFP BMT mice were inoculated with LLCs into the right flank and tumor growth was monitored. Mice were euthanized and tumors were harvested 21 days after the inoculation. To evaluate patent/functional vessels, mice were injected with biotinylated BS1/lectin (Invitrogen) 30 minutes prior to sacrifice as described (Coleman et al., 2010). Tumors were harvested and processed for immunostaining using Cy5-labeled streptavidin (Invitrogen) to visualize BS1/lectin-stained, thus, perfused vessels.

Gene Array Analysis and qRT-PCR

A pi-shaped piece of tumor tissue was bisected from WT and p75KO mice and homogenized. Total RNA was isolated from tumors (RNeasy Mini Kit, QIAGEN, Valencia, CA, USA) and converted to cRNA using the SuperArray TrueLabeling-AMP 2.0 (SuperArray, Frederick, MD, USA). The gene array membranes were processed according to the manufacturer's (Oligo GEArray Kit, SuperArray). Gene array membranes were exposed to Amersham Hyperfilms and films were analyzed using Bio-Rad densitometry machine and software. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on five genes (Ang-1, Ang-2, Mmp2, Hif1- α , Cxcl2) to confirm gene array analysis results. Supplementary Table S1 contains specific forward and reverse primer sequences. The samples were analyzed using Applied Biosystems 7300 Real Time PCR machine and software.

TNF treatment and FACS Analysis and qRT-PCR of p75KD/LLCs

p75 knockdown LLC (p75KD/LLC) cell lines transfected with 1+2 and 1+2+3+4 p75shRNA plasmids (Supplementary Materials and Methods) were treated with 80 ng/ml mrTNF (BD Biosciences). Twenty four hours post-TNF treatment, cells were stained with 18 μ g propidium iodide (SIGMA, St. Louis, MO, USA) in 1 mL 1 \times PBS (MediaTech, Herndon, VA, USA) with 8 μ g RNase-A (SIGMA) for 30 minutes and analyzed for DNA content using fluorescent activated cell sorter (Becton-Dickinson, CA) (Goukassian et al., 2007). The percentage of apoptotic cells (the sub-G0/G1 population) was determined using CellQuest Pro (BD Biosciences). mRNA expression of angiogenic factors and receptors was determined by qRT-PCR. For specific forward and reverse primers sequences for each gene (VEGF-A, HGF, PLGF, Ang-1, Ang2 and CXCR4) please see Supplementary Table S1.

Western Blot Analysis

WT and p75KO BM-derived EPCs were isolated from tubular bones, selected *ex vivo*, and used between days 6 and 8 after the initial plating as described in our laboratory (Goukassian et al., 2007). LLCs were maintained as separate culture according to ATCC recommendations. At 60–70% confluence medium was removed and fresh media was added with or without 80 ng/ml mrTNF. Cells were harvested 30 and 60 min after TNF and no-TNF (control) treatments and processed for western blot analysis for detection of total p38 (T-p38) and phosphorylated p38 (P-p38) protein using antibodies against phospho- or total-specific p38 MAPK (Cell Signaling Technology, Danvers, MA, USA). Anti- β -actin-staining was used as loading control. ECL kit (Amersham Biosciences; GE, Piscataway, NJ, USA) was used for immunodetection.

Statistical Analysis

All results were expressed as mean \pm SEM. Statistical analyses were performed using Student's t-Test (unpaired) and ANOVA/ANCOVA by Fisher's PLSD (StatView, SAS Institute, Gary, NC, USA). Differences were considered significant at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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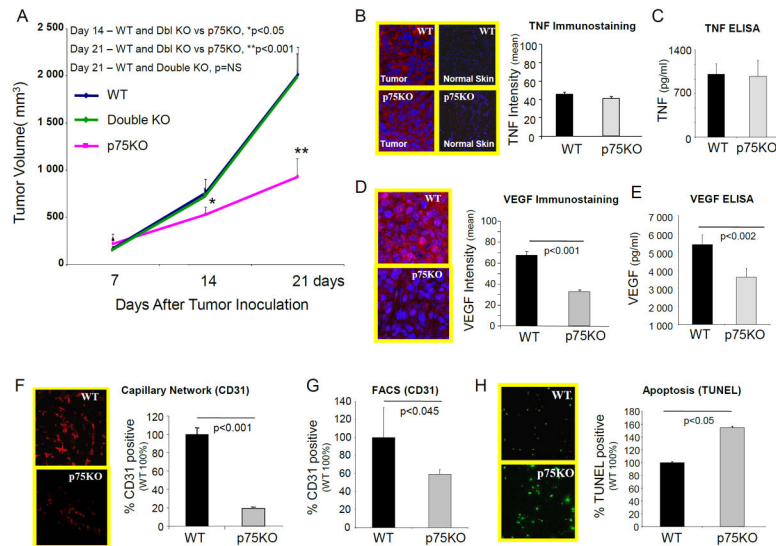


Figure 1. Tumor growth inhibition in p75KO mice is associated with decreased expression of VEGF and capillary density, as well as increased apoptosis, while TNF expression is similar in WT and p75KO tumors

A, Graphic representation of LLC tumor volumes in WT, p75KO and Dbl-KO mice. Graphs represent pooled data from 3 independent experiments (N=18–24/treatment group). B, Quantification of TNF immunostaining (red) in tumors and normal skin of WT and p75KO mice shown as percent of mean pixel intensity using NIH Image J program (here and elsewhere). C, TNF protein release (pg/ml) measured in tumor homogenates from WT and p75KO mice (p=NS). D, Quantification of VEGF immunostaining (red) in tumors from WT and p75KO mice shown as percent of mean pixel intensity (p<0.001, WT vs. p75KO). E, VEGF protein release (pg/ml) measured in tumor homogenates (p<0.002, WT vs. p75KO). F–G, Representative images (right panel) and quantification of – F, CD31 (red) immunofluorescence, and G, Quantification of CD31 (+) cells in the whole tumor tissue from WT vs p75KO mice using FACS analysis, when WT is set as 100%. Please note, compared to FACS analysis, tumor associated CD31 (+) cells were twice as higher in immunofluorescent studies, suggesting a significant heterogeneity in tumor vascular network and a superior quantitative nature of the FACS analysis of tumor associated CD31 (+) cells. H, Representative images (right panel) and quantification of –H, TUNEL (green) immunostaining in WT and p75KO tumors, shown as percent of mean pixel intensity, when WT is set as 100%. Results in all graphs are pooled data (mean+SEM) from 3 independent experiments 7–8 fields of 176,400 μm^2 (image size here and elsewhere) per mouse, N= 5 mice/genotype.

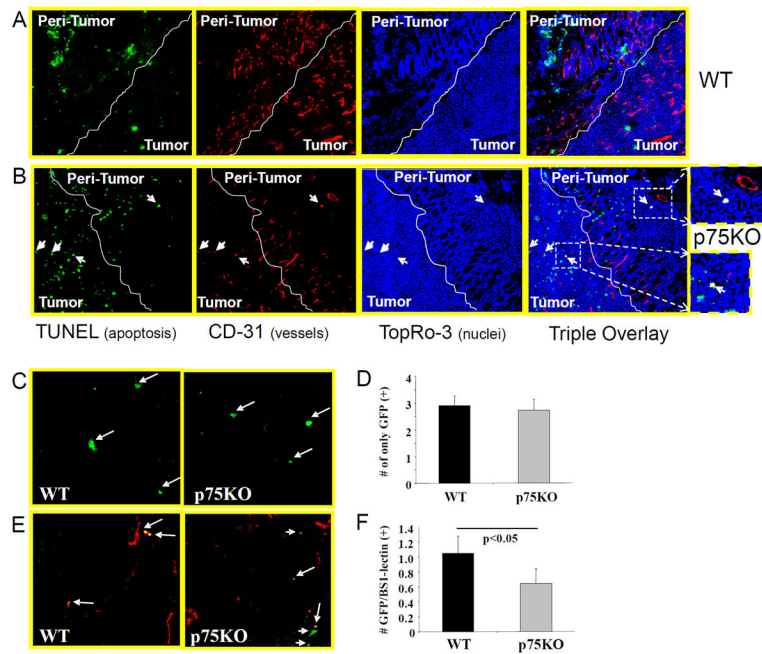


Figure 2. Increased apoptosis in tumors from p75KO mice at the border-zone and reduced incorporation of BM-derived EPCs into functional capillary network in p75KO tumors
Apoptosis and tumor angiogenesis was also evaluated at the interface of tumor/normal tissue by triple staining with Terminal Transferase dUTP Nick End Labeling (TUNEL), CD31 and Topro-3. The peri-tumoral and tumor area was identified by H&E staining of adjacent sections (not shown). Representative images of triple-immunostained tumors (panel on the far right) in the periphery of tumor tissue for TUNEL (green), CD-31 (red) and Topro-3 (blue) in WT (A) and p75KO (B). A–B, TUNEL staining (top panel) in WT tumors. B, Insets in peri-tumoral (top) and tumors (bottom) in p75KOs show double positive (TUNEL/CD31)-yellowish staining, arrowheads) indicating apoptosis of p75KO ECs. C, Representative images of BM-derived (GFP +) cells recruited into WT and p75KO tumor tissue (arrows). D, Quantification of BM-derived GFP (+) cells recruitment into the tumor tissue. E, Representative images of double BM-derived GFP (+)/BS-1/lectin (+) cells, incorporated into functional vessels (yellow staining, arrows). Small arrowheads indicate BM-derived GFP (+) cells that are not incorporated into functional vessels (green) in p75KO tumors. F, Quantification of BM-derived EPCs incorporation into the functional capillary network.

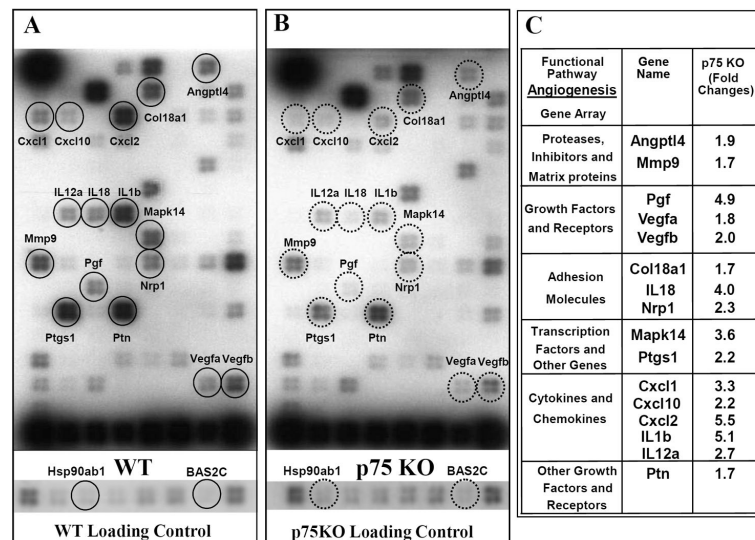


Figure 3. Angiogenic genes expression is decreased in tumor tissue from p75KO mice
 A and B, Representative Angiogenesis pathways microarray of tumors from WT and p75KO mice at 5 minute exposure and the loading controls (bottom, small insets) at 5 second exposure time. Dotted-line circles indicate gene expression decrease in p75KO vs. the same gene in WT (solid-line circles). C, Functional grouping of genes in angiogenesis pathways microarray with the fold changes (dotted-line circles - decreased) in p75KO vs WT tumors. In p75KO tumor tissue decreased gene expression was observed in growth factors, cytokines and chemokines, such as - vascular endothelial growth factor (VEGFA and B), placental growth factor (PGF), chemokine (C-X-C motif) ligands (Cxcl1, Cxcl2, Cxcl10), interleukins (IL1b, IL12a and IL18), pleiotrophin (Ptn, known as, heparin-binding growth factor 8), signal transduction and transcription factors Mapk14 (know as p38), prostaglandin-endoperoxide synthase 1 (Ptgs1), adhesion molecules and proteases such as, collagen, type XVIII, alpha 1 (Col18a1), neuropilin 1 (Nrp1), angiotensin-like 4 (Angptl4) and matrix metalloproteinase 9 – Mmp 9). The densitometry values of Hsp90ab1 and BAS2C genes were used as internal control. An arbitrary cut off was set at 1.6 (up or down). We assigned an arbitrary cut off thresholds for fold changes at 1.6 (here and elsewhere).

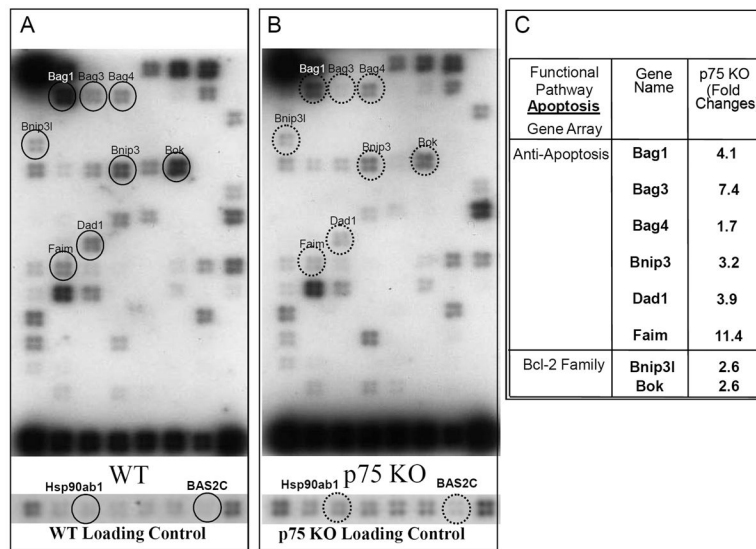


Figure 4. Anti-apoptotic gene expression is decreased in p75KO tumor tissue

A and B, Representative apoptosis pathways microarray of tumors from WT and p75KO mice at 5 minute exposure and the loading controls (bottom, small insets) at 5 second exposure time. Functional grouping of genes in apoptosis microarray with the fold changes of the same gene (solid-line vs. dotted-line circles – decreased) in WT vs p75KO.

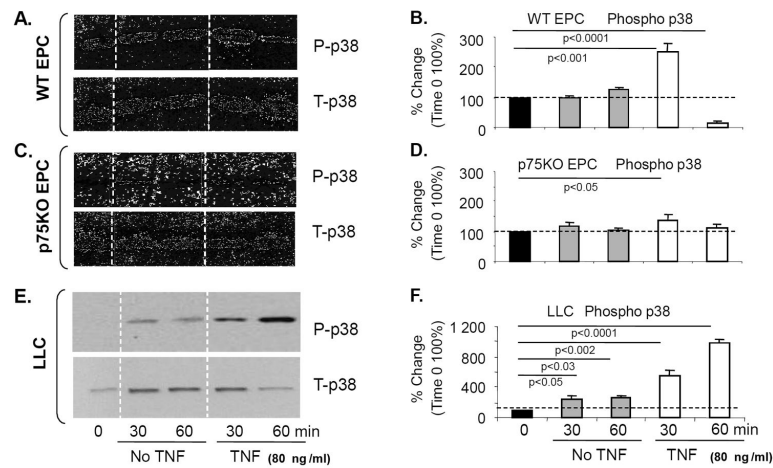


Figure 5. Signaling through TNFR2/p75 is required for proper activation of p38 MAPK in EPCs
A, C, E, Representative images of Phospho-p38 (P-p38) and Total-p38 (T-p38) western blot analysis in WT and p75KO EPCs and unaltered LLCs. B, D, F, Quantification p38 protein level and phosphorylation using densitometric analysis of P-p38 band intensity after adjusting for actin (not shown) and T-p38 band intensity. Band intensity at time 0 for each cell type was set at 100% and percent change over 60 minutes was calculated. Results represent data from three independent experiments. Statistical significance was assigned when $p < 0.05$.

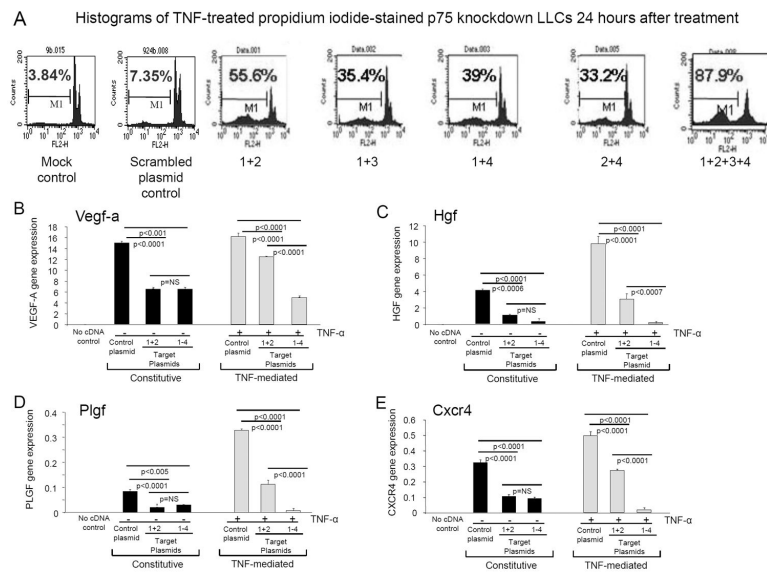


Figure 6. Percent inhibition of p75 receptor expression correlates directly with increase in TNF-mediated apoptosis and decrease in angiogenic gene expression in p75KD/LLCs
 Representative histograms of FACS analysis of propidium iodide (PI)-stained two p75KD/LLC (transfected with p75 shRNA plasmid combinations 1+2 and 1-4), where M1 gates represent sub-G0/G1 population of the cells with less than 2n DNA, presumably, apoptotic cells. B–E, Constitutive and TNF-mediated (80 ng/ml) expression (qRT-PCR analysis) for various angiogenic genes – B, VEGFA, C, HGF, C, PLGF and E, CXCR4 for the same two p75KD/LLCs 24 hrs post-TNF treatment.