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## Blockade of TNF $\alpha$ signaling in tumor-associated macrophages: a new radiosensitizing strategy

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### Abstract

Most cancer patients receive radiotherapy during the course of their disease. Improvements in the therapeutic index have been based on physical improvements in delivery. Radiosensitizer development targeting tumor cells has not yielded effective agents. Recent investigations have focused on the tumor stroma as a potential target for radiosensitization. We report that depletion of tumor associated macrophages by systemic or local injection of macrophage depleting liposomal clodronate prior to radiotherapy increased the anti-tumor effects of ionizing radiation (IR) as a large single dose (20 Gy) or fractionated dose (2 Gy  $\times$  10). Co-implantation of tumor cells with BM-derived macrophages (BMDM $\phi$ ) increased tumor radioresistance. Studies using mice with germ line deletions of TNF receptors 1 and 2 (TNFR1,2 $^{-/-}$ ) or TNF $\alpha$  (TNF $^{-/-}$ ) and treatment with a soluble TNF receptor fusion protein (Enbrel<sup>R</sup>) showed that radioresistance mediated by BMDM $\phi$  required intact TNF $\alpha$  signaling. Radiation exposure led to the upregulation of VEGF by macrophages, and neutralizing antibodies to VEGF also enhanced the anti-tumor response to IR demonstrating that the radioprotective effect of TNF $\alpha$  was mediated by VEGF production in tumor associated macrophages (TAM $\phi$ ). These data provide a mechanistic basis for targeting macrophage populations generally, and TNF $\alpha$  induced macrophage VEGF specifically, to improve radiotherapy.

### Keywords

BMDM $\phi$ ; radiation; TNF $\alpha$  signaling; VEGF

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## INTRODUCTION

Radiation therapy is a common cancer treatment usually delivered in a fractionated schedule (daily 1.8–3Gy) for 6–8 weeks. In some selected lung cancers, or limited forms of metastasis, very large doses (1–5 treatments of 10–20 Gy) may be employed (1). Despite research into the radiobiology and physics of radiotherapy, many patients fail within the irradiated target volume. Currently, the most effective radiosensitizers represent the commonly used chemotherapeutic agents (2). These drugs are not designed as selective radiosensitizers and clinical improvement reflected by increased local control is achieved at the expense of significant normal tissue toxicity. More selective radiosensitizing agents and new treatment strategies are required to improve the therapeutic index in radiotherapy.

Research on the mechanisms of tumor radioresistance and the development of radiosensitizers focuses on tumor cells or exploiting differences in the oxygenation status of tumors and normal tissue (3). Recently the tumor-associated stroma has gained attention as an important component of the response to radiotherapy (4, 5) and investigations have centered on the role of the tumor associated vasculature (6, 7). Several clinical trials are underway to investigate the role of angiogenesis inhibitors in combination with radiotherapy (8–11). The results are preliminary and though promising, show mixed results in terms of improving radiotherapy outcomes and unexpected toxicities have been reported (11–13). Conversely, little is known about other components of the tumor associated stroma and the effect of these cells on the response of tumors to radiotherapy.

Macrophages are multifunctional cells of the myeloid lineage, phenotyped as CD14<sup>+</sup>. Macrophages engulf microbes and cell debris, are antigen presenting cells and secrete cytokines and chemokines. Macrophage function is described within the context of normal physiological or pathological microenvironments. Macrophages are designated as M1 classically activated macrophages or M2 alternatively activated macrophages. Pro-inflammatory M1 macrophages are activated by LPS and IFN $\gamma$ , secrete TNF $\alpha$  and IL-12 and support T cell function. M2 macrophages are considered to be anti-inflammatory and immunosuppressive, expressing F4/80, CD11b and CD206, secrete IL-10, IL-4 and TGF $\beta$  and down regulate T cell function (14, 15) Tumor associated macrophages (TAM $\phi$ ) are usually characterized as M2. However, functional overlap exists between characteristics of M1 and M2 macrophages. Additional/expanded definitions of TAM $\phi$  populations have been investigated as determinants of tumor progression and metastasis. These populations include CD11b<sup>+</sup>/Gr1<sup>+</sup> myeloid suppressor cells as well as Tie2<sup>+</sup> expressing macrophages (15, 16). Emerging data suggest key roles for these various monocyte/macrophage cells in generating pro-angiogenic signals during tumor growth.

Radiation was originally reported to prime the anti-tumor effects of macrophages through production of TNF $\alpha$  and nitric oxide (NO) (17–20). However, Milas, et al., investigated the association between TAM $\phi$  and the response of tumors to radiotherapy and noted a trend toward decreasing radiocurability with increasing TAM $\phi$  content. These investigators recognized the contradiction between potential beneficial (phagocytic function) and deleterious functions (pro-angiogenic phenotypes) of TAM $\phi$  (21–23). Recently, Tsai, et al., reported that irradiated macrophages contributed to tumor growth through increased

secretion of COX-2, iNOS and Arg-1 although no direct mechanisms for promotion of tumor growth were described (24). Brown, et al., reported that cells of the myeloid/macrophage lineage contributed to tumor regrowth following radiation by increasing tumor vasculogenesis when tumor angiogenesis was suppressed by prior irradiation of the tumor bed (25). Collectively these studies raise the possibility that macrophage populations contribute to tumor radioprotection. Moreover, radiation of the tumor microenvironment might upregulate macrophage derived cytokines that promote tumor growth, survival, angiogenesis and vasculogenesis.

TNF $\alpha$ , which has anti-tumor effects at high concentrations, also promotes tumor angiogenesis, tumor cell survival and metastases at lower levels (26). A recent report suggested that TNF $\alpha$  mediates the differentiation of monocytes into angiogenic cells that support tumor angiogenesis (27–29). Radiotherapy, while directly inducing tumor cell death, may upregulate pro-angiogenic and pro-survival factors within the tumor microenvironment. We and others have found that radiation upregulates TNF $\alpha$  production by tumor cells and cells of the myeloid lineage (30, 31). Here we report that depletion of macrophages by liposomal chlodronate prior to irradiation increases the anti-tumor effects of IR, whereas co-implantation of tumor cells with BM-derived macrophages (BMDM $\phi$ ) results in increased tumor radioresistance. The radioprotective effect of BMDM $\phi$  requires functional TNF $\alpha$ /TNFR signaling and induction of macrophage secreted VEGF. We report that blockade of macrophage VEGF induction by Enbrel<sup>R</sup> (soluble TNF receptor dimeric fusion protein) used in several murine studies, increased tumor radiosensitivity (32, 33). We also report that blocking VEGF with neutralizing antibodies improves the anti-tumor effects of IR. These data provide a rationale for targeting macrophage populations generally and TNF $\alpha$  induced VEGF signaling specifically when designing radiotherapeutic strategies.

## MATERIALS AND METHODS

### Mice and Tumor Cell Lines.

C57BL/6 mice WT, C57BL/6–129S-Tnfrsf1a<sup>tm1Imx</sup>Tnfrsf1b<sup>tm1Imx</sup>/J (TNFR1,2<sup>-/-</sup>), B6:–129S-Tnf<sup>tm1Gkl/J</sup> (TNF<sup>-/-</sup>) and C57BL/6 -Tg (UBC-GFP) 30SchaJ (GFP<sup>+/+</sup>) breeding pairs were purchased from Jackson Laboratories (Bar Harbor, ME). TNF<sup>-/-</sup> mice maintained in the 129/SvEv and C57BL/6 backgrounds after 6 generation backcross have been previously used to study the effects of TNF $\alpha$  on tumor promotion and antigen presentation (29, 34). B16F1 melanoma cells were cultured as described (35) B16.SIY melanoma cells expressing model antigen SIY, which can be recognized by CD8<sup>+</sup> T cells in the context of K<sup>b</sup> were cultured as described. The care and treatment of experimental animals was in accordance with institutional guidelines.

### Generation of BM-derived Macrophages (BMDM $\phi$ ).

Femoral BM cells were obtained from mice and cultured in complete RPMI supplemented 10% FCS and 30% pretested conditioned medium from the L929 cell line as a source of M-CSF for the first 5 days, followed by complete RPMI supplemented with 10% FCS and 10ng/ml recombinant M-CSF (R&D Systems, Minneapolis, MN) for additional 5 days.

These BM-derived macrophages were >95% CD14<sup>+</sup> and >90% CD11b<sup>+</sup>F4/80<sup>+</sup> analyzed by FACS.

### **Tumor Induction and Irradiation.**

Tumors cells ( $5 \times 10^5$  B16F1, or B16.SIY) were inoculated subcutaneously (s.c.) into the right hind limb. Tumors were measured with calipers and volume calculated as length  $\times$  width  $\times$  depth/2. At day 10–12 local radiotherapy (single dose 20 Gy, or fractional doses 2 Gy  $\times$  10) was delivered. In some experiments, B16F1/B16.SIY cells were co-injected with BMDM $\phi$  at a 4:1 ratio. Elsewhere macrophages were depleted by i.p. or intratumoral administration of Lip-Clod every 5–7 days (36). Cl<sub>2</sub>MDP (or clodronate) was a gift of Roche Diagnostics GmbH, Mannheim, GR (37). Other reagents include: phosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany), and cholesterol (Sigma, St. Louis, MO). Macrophage depletion was confirmed by FACS and immunohistochemistry of spleen and tumor cryosections with greater than 90% reduction compared with the control. Blockade of VEGF using neutralizing IgG against mouse VEGF-164 (R & D Systems) has been previously described (38). Briefly, goat IgG against mouse VEGF-164 was suspended in PBS and administered via i.p. injection (10  $\mu$ g/mouse, 3 h before IR and 3, 8 days after IR). Control mice received goat IgG (Sigma).

### **Colony Forming Assay.**

200 control or irradiated B16F1 or B16.SIY cells were seeded in 5cm culture dishes in RPMI containing 10% FCS, with or without 30% spent supernatant collected from either WT, TNF<sup>-/-</sup> or TNFR1,2<sup>-/-</sup> BMDM $\phi$  ( $5 \times 10^6$  cells). After 7 days, cells were washed and stained with crystal violet. Colonies with more than 50 cells were counted.

### **Protein Array and Luminex.**

Spent culture supernatants were collected and incubated with membranes coated with 62 anti-mouse cytokine antibodies (RayBiotech, Inc., Norcross, GA) according to the manufacturer's instruction. An antibody labeled with biotin coated on the upper left and lower right corners of the membrane serve as positive control. The film was scanned and spots were quantified by densitometry analysis using UN-SCAN-IT gel automated digitizing system software (Silk Scientific, Orem, UT). 32 cytokines were quantified using Mouse Cytokine/Chemokine Premixed 32 Plex (Milipore, Billerica, MA). Median Fluorescent Intensity (MFI) from each well was acquired and the relative concentration of each cytokine/chemokine was calculated.

### **FACS and Cell Sorting.**

Tumors were excised, sectioned and digested in DMEM supplemented with 2% FCS and 1.5 mg/ml of collagenase D (Sigma) for 30 min in a 37°C shaking incubator to collect single-cell suspension. Cells were stained with anti-CD11b, anti-CD206, anti-F4/80, anti-TNFR1, anti-TNFR2 (Bio Legend, San Diego, CA), washed and analyzed on a LSR flow cytometer. Frequency of CD11b<sup>+</sup> and F4/80<sup>+</sup> TAM $\phi$  were analyzed and sorted on a Mo Flow to collect TAM $\phi$ . Purity reached >95% CD11b<sup>+</sup> F4/80<sup>+</sup> cells.

### Western Blot.

Cells were lysed with cell lysis buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na<sub>2</sub>EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 µg/ml leupeptin and protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). Protein concentrations were determined using the Bio-Rad protein assay kit and 10 µg from each sample was analyzed on 10% SDS–PAGE gels. Protein bands were transferred onto PVDF membranes, probed with rabbit anti-mouse VEGF (Santa Cruz Biotech, Santa Cruz, CA) for 2 h, then HRP-conjugated anti-rabbit for 1 h. Membranes were washed and immunodetected using ECL Kit (Amersham Biosciences, Pittsburg, PA). Anti-β-actin stained membranes were used as loading control.

### Histopathology and Immunohistochemistry.

Tissues were fixed in 10% neutral formalin or 4% paraformaldehyde. Sections were blocked with 5% goat serum and incubated with VEGFR2 antibody (Cell Signaling Technology, MA, 1:200 dilution) for 1 h. After washing with TBS, biotinylated anti-rabbit secondary antibody followed by ABC-AP (Vector Labs, Burlingame, CA) was applied for 30 min each. Vector blue substrate was used for color development. For the following NG2 Chondroitin Sulfate Proteoglycan staining, the slides were washed, blocked with Avidin/Biotin blocking kit, then 10% goat serum for 10 min. Slides were incubated in 1:100 dilution of NG2 antibody (Millipore, Billerica, MA) overnight at 4°C. After applying biotinylated secondary antibody and ABC-AP solution, tissues were covered with Vulcan fast red substrate (Biocare Medical, Concorc, CA) for 15 min. Slides were washed and counter stained with methyl green. Sections were dehydrated in 100% ethanol, cleared by HistoClear (Wards Natural Science, Rochester, NY), then mounted with VectaMount (Vector Labs). Images were photographed at 200X magnification using a Zeiss camera operated by Openlab software.

### Statistical Analysis.

A random effects model for longitudinal data was used to obtain an overall estimate of the intercept and slope of linear growth for each group. One-way ANOVA with Dunnett's post test was performed using GraphPad InStat version 3.05 ( $p < 0.05$ ).

## RESULTS

### Depletion of Macrophages Increases the Anti-tumor Effects of Ionizing Radiation.

Macrophages were depleted by i.p. injection of liposomal clodronate (Lip-Clod) (37) one day before injection of  $2 \times 10^6$  B16.SIY cells and every 5 days thereafter in WT mice. 90% of the macrophages were specifically depleted in the spleen and tumor microenvironment as analyzed by FACS (36, 39, 40) (Supplemental Figure 1). 20 Gy was administered when tumors reached 150–200 mm<sup>3</sup>. The combination of Lip-Clod and 20 Gy significantly delayed tumor regrowth compared to 20 Gy alone ( $335 \pm 207 \text{ mm}^3$  vs  $3215 \pm 1849 \text{ mm}^3$ , day 22,  $p = 0.002$ ), or Lip-Clod alone ( $273 \pm 198 \text{ mm}^3$  vs  $4987 \pm 2556 \text{ mm}^3$ , day 18,  $p = 0.041$ , Figure 1a). Lip-Clod also enhanced the anti-tumor effects of IR after tumors were established compared to 20 Gy alone ( $2678 \pm 1243 \text{ mm}^3$  vs  $4599 \pm 889 \text{ mm}^3$ , day 22,  $p = 0.071$ , Figure 1b). We also injected Lip-Clod intratumorally and observed that tumor regrowth was

significantly delayed compared to IR alone ( $1669\pm 749\text{mm}^3$  vs  $5317\pm 1322\text{mm}^3$ , day 26,  $p=0.037$ , Figure 1c).

### **TNF $\alpha$ Signaling in BMDM $\phi$ Mediates Tumor Radioresistance.**

TNF $\alpha$  is reported to both enhance tumor growth and mediate anti-tumor effects. To study the role of TNF $\alpha$  produced by macrophages, we cultured BMDM $\phi$  from WT and TNF $^{-/-}$  mice and co-injected these BMDM $\phi$  with  $5\times 10^5$  B16.SIY cells into WT mice. TNF $^{-/-}$  and WT BMDM $\phi$  exhibit similar levels of cell surface markers with >95% CD14 and 90% CD11b detected by FACS. They also expressed similar levels of iNOS but TNF $^{-/-}$  BMDM $\phi$  expressed significantly lower levels of Arg1 (data not shown). The percentages of F4/80 $^+$  TAM $\phi$  was similar between the WT and TNF $^{-/-}$  groups, although TNF $^{-/-}$  cells had diminished levels of surface CD206 staining (Supplemental Figure 2). Co-injection of WT BMDM $\phi$  with B16.SIY cells significantly accelerated tumor regrowth after 20 Gy compared to 20 Gy alone ( $1384\pm 553\text{mm}^3$  vs  $125\pm 36\text{mm}^3$ , day 22,  $p=0.030$ , Figure 2a, b). The regrowth of irradiated tumors in which B16.SIY cells were co-injected with WT BMDM $\phi$  occurred significantly earlier compared to irradiated tumors in which B16.SIY cells were co-injected with TNF $^{-/-}$  BMDM $\phi$  ( $1384\pm 553\text{mm}^3$  vs  $337\pm 261\text{mm}^3$ , day 22,  $P=0.037$ , Figure 2 b, c). Our data demonstrate that depletion of macrophages enhances the anti-tumor effects of IR and co-implantation of macrophages reverses this effect. These results also implicate macrophage secreted TNF $\alpha$  in the resistance of tumors to IR and suggest that macrophage derived TNF $\alpha$  and/or TNF $\alpha$  signaling in TAM $\phi$  contributes in part to B16.SIY tumor radioresistance.

### **TNF $\alpha$ Signaling in BMDM $\phi$ Promotes Tumor Growth.**

To investigate the role of TNF $\alpha$  produced specifically by BMDM $\phi$ , we injected B16.SIY cells into TNF $^{-/-}$  mice transplanted with WT BM or TNF $^{-/-}$  BM. FACS analysis demonstrated that the BM was reconstituted with >70% of donor cells (Supplemental Figure 3). Animals underwent macrophage depletion with Lip-Clod and tumor growth was compared to controls. Tumor volume in WT BMT mice was significantly reduced by macrophage depletion ( $3067\pm 615\text{mm}^3$  vs  $825\pm 174\text{mm}^3$ , day 60,  $p=0.017$ , Supplemental Figure 4a). No tumor growth was observed in TNF $^{-/-}$  BMT mice, demonstrating an essential role for TNF $\alpha$  produced by BMDM $\phi$ . We next injected B16.SIY cells into TNFR1,2 $^{-/-}$  mice after WT or TNFR1,2 $^{-/-}$  BMT, with or without macrophage depletion. WT BMT in TNFR1,2 $^{-/-}$  mice promoted tumor growth 30 days earlier than in TNFR1,2 $^{-/-}$  BMT mice, which was significantly delayed when macrophages were depleted ( $867\pm 64\text{mm}^3$  vs  $4353\pm 888\text{mm}^3$ , day 45,  $p=0.013$ , Supplemental Figure 4b). No significant difference was observed in TNFR1,2 $^{-/-}$  mice with TNFR1,2 $^{-/-}$  BMT when macrophages were depleted ( $65\pm 15\text{mm}^3$  vs  $73\pm 18\text{mm}^3$ , day 45,  $p=0.549$ ). In summary, tumors grew more slowly in TNFR1,2 $^{-/-}$  BMT mice while WT BMT permitted tumor growth. However, tumor growth was suppressed when macrophages were depleted. These results suggest that autocrine/paracrine TNF $\alpha$  signaling in BMDM $\phi$  is essential in promoting tumor growth.

### **Autocrine/paracrine TNF $\alpha$ Signaling in BMDM $\phi$ Mediates Radioresistance.**

We co-injected BMDM $\phi$  from WT or TNFR1,2 $^{-/-}$  with B16.SIY cells into TNFR1,2 $^{-/-}$  mice. Co-injection of WT BMDM $\phi$  significantly accelerated tumor regrowth after 20 Gy



compared to IR alone ( $782 \pm 179 \text{mm}^3$  vs  $78 \pm 14 \text{mm}^3$ , day 22,  $p=0.003$ , Figure 3a, b). The regrowth of irradiated tumors co-injected with WT BMDM $\phi$  was significantly accelerated compared to the response of tumors co-injected with TNFR1,2-/- BMDM $\phi$  ( $782 \pm 179 \text{mm}^3$  vs  $283 \pm 157 \text{mm}^3$ , day 22,  $p=0.041$ , Figure 3b, c). These results indicate that intact TNF/TNFR signaling in macrophages is required for accelerated tumor regrowth after IR. We repeated these experiments using fractionated IR and report that the growth of tumors co-injected with WT BMDM $\phi$  was significantly increased compared to tumors co-injected with TNFR1,2-/- BMDM $\phi$  ( $384 \pm 64 \text{mm}^3$  vs  $38 \pm 7 \text{mm}^3$ , day 22,  $p=0.010$ , Supplemental Figure 5). These data further suggest that TNF $\alpha$ /TNFR signaling in BMDM $\phi$  mediates radioresistance.

### **BMDM $\phi$ Supernatant Does Not Affect Tumor Cell growth or Radiosensitivity.**

We explored the direct effects of BMDM $\phi$  on B16.SIY tumor cell radiosensitivity and/or growth *in vitro*. Supernatant from WT BMDM $\phi$  suppressed B16.SIY colony formation ( $p=0.015$ ), while supernatant from TNF-/- or TNFR1,2-/- BMDM $\phi$  had no effect ( $p=0.259$ ,  $p=0.338$ , Supplemental Figure 6). Unexpectedly, supernatants from TNF-/- and TNFR1,2-/- BMDM $\phi$  cultures increased colony formation in irradiated cells ( $p=0.065$ ,  $p=0.055$ ). Interestingly, the radioprotective effect of TNF-/- or TNFR1,2-/- supernatants *in vitro* differs from the *in vivo* findings with TNF-/- or TNFR1,2-/- macrophages. Supernatant from WT BMDM $\phi$  had no effect on irradiated B16.SIY colony formation ( $p=0.890$ ). Supernatant collected from irradiated WT, TNF-/- or TNFR1,2-/- BMDM $\phi$  had no significant effect on either control or irradiated B16.SIY growth. These results suggest that the radioprotective effects of TNF $\alpha$  signaling in BMDM $\phi$  are not exerted directly on tumor cells but likely on non-tumor cell constituents of the tumor microenvironment.

### **Induction of VEGF Through TNF $\alpha$ /TNFR Signaling in TAM $\phi$ Mediates Rapid Tumor Regrowth Following Irradiation.**

In addition to TAM $\phi$ , tumor stroma is also comprised of matrix proteins and various cell types including blood/lymphatic vessels (41). Recent data suggest that TAM $\phi$  support tumor growth by contributing to angiogenesis and/or vasculogenesis (41–44) in part mediated by TNF $\alpha$ . We employed a protein array and examined 62 cytokines and chemokines in unirradiated BMDM $\phi$  and BMDM $\phi$  treated with 5 Gy. Unirradiated WT and TNFR1,2-/- BMDM $\phi$  produced similar cytokine/chemokine levels including M-CSF, G-CSF, GM-CSF, CCL2, CCL9, IL-6, CXCL2, IL-10, TNF $\alpha$ , IL-12 and low levels of VEGF. Following 5 Gy there was a significant increase of VEGF in WT BMDM $\phi$  but not TNFR1,2-/- BMDM $\phi$ , while TNF $\alpha$  was induced in both WT and TNFR1,2-/- BMDM $\phi$  (Figure 4a). These results were confirmed by Luminex (Figure 4b) and suggest that the induction of VEGF by IR is dependent on TNF $\alpha$ /TNFR autocrine/paracrine signaling in BMDM $\phi$ . Our findings support the hypothesis that VEGF production in TAM $\phi$  through TNF $\alpha$  signaling activated by IR might play an important role in tumor vessel repair and tumor regrowth.

We examined if irradiation leads to TNF/TNFR mediated upregulation of VEGF in tumor macrophages. We injected B16.SIY cells into WT and TNFR1,2-/- mice and 20 Gy was delivered when tumors reached 150–200  $\text{mm}^3$ . Tumors were excised and digested into single cell suspensions. CD11b<sup>+</sup> TAM $\phi$  were sorted and VEGF expression was assayed by western

blot and Luminex. Significantly higher levels of VEGF were detected in CD11b<sup>+</sup> TAM $\phi$  isolated from tumors grown in WT mice compared to TNFR1,2<sup>-/-</sup> mice (Figure 5a, b, p=0.015). Increased TAM $\phi$  VEGF from tumors in WT mice was mirrored by increased tumor neovasculature/angiogenesis post IR visualized in H&E and VEGFR2 stained tissue sections (Figure 5c). We quantified functional vascular structures demonstrating intact blood perfusion by the presence of red blood cells in VEGFR2<sup>+</sup> vessels. In tumors grown in TNFR1,2<sup>-/-</sup> mice, there were significantly decreased functional vessels after IR, compared to either untreated control or tumors grown in WT post IR (p<0.0001 and p=0.002, respectively, Figure 5d). Also, more surviving tumor cells exhibiting intact morphology were present in irradiated tumors in WT compared to tumors in TNFR1,2<sup>-/-</sup> which demonstrated greater radiosensitivity.

### Depleting TNF $\alpha$ with Enbrel® Enhances Tumor Radiosensitivity.

To test this hypothesis that TNF $\alpha$  blockade might be clinically relevant, we injected B16.SIY cells into WT mice and treated them with Enbrel® + 20 Gy (32, 33). Enbrel® treated animals had slightly larger tumors than untreated control animals. Mice treated with Enbrel® + 20 Gy exhibited a reduction in tumor regrowth compared to 20 Gy alone (153±34mm<sup>3</sup> vs 440±97mm<sup>3</sup>, day 28, p=0.022, Figure 6a).

The observation that IR induced VEGF production by macrophages via TNF $\alpha$ /TNFR signaling pathways led us to examine the impact of pharmacologic TNF $\alpha$  inhibition and radiation on tumor angiogenesis. As with genetic deletion of TNFR1,2, Enbrel® treatment prevented upregulation of VEGF by tumor macrophages *in vivo* (Figure 6b, c). We next assessed the effect of IR + Enbrel® on neovascularization. Radiation alone led to a significant reduction in microvessel density as measured by VEGFR2<sup>+</sup> (PBS 24±1.8 versus PBS + IR 4.1±1.4, p<0.0001). Enbrel® alone led to a modest decrease in microvessel density (PBS 24±1.8 versus Enbrel® 13±1, p<0.01), while Enbrel® + IR significantly inhibited angiogenesis when compared to all groups (2.75±0.35 versus Enbrel® alone 13±1, p<0.0001; versus PBS + IR 4.1±1.4, p<0.01, Figure 6d). NG2<sup>+</sup> pericyte coverage was reduced in tumors treated with Enbrel® compared to PBS (98 ±0.2% versus 84±2.3%, p<0.001) and further decreased with Enbrel® + IR (57±22.4%, p=0.03 compared to Enbrel® alone). Vascular size and morphology were also substantially affected by treatment. For example, Enbrel® + IR treated tumors demonstrated narrow lumens suggestive of vascular collapse. Finally, while radiation exposure led to vascular hemorrhage, the addition of Enbrel® intensified hemorrhagic necrosis and regression. These data demonstrate a profound effect of TNF $\alpha$  depletion + IR on angiogenesis and vascular function. Given that irradiated macrophages upregulated VEGF expression, we next hypothesized that blockade of VEGF would likewise enhance radiosensitivity. Treatment of mice with neutralizing antibodies to VEGF led to increased radiosensitivity in B16F1 (806.1±329.1 versus IR alone 2101.8±525.4, p=0.05) and B16.SIY (0.5±0.1 versus IR alone 408.5±65.1, p=0.005) tumors (Supplemental Figure 7) (38).



## Discussion

We tested the hypothesis that depletion of TAM $\phi$  by Lip-Clod would improve the anti-tumor effects of radiotherapy as determined by a delay of tumor regrowth. The effects of macrophage depletion were most marked when macrophages were depleted before tumors were established. TAM $\phi$  have been reported to secrete a variety of cytokines and other proteins that promote tumor growth. Our results demonstrate that the intact TNF $\alpha$ /TNFR signaling in TAM $\phi$  is important in the autocrine/paracrine secretion of cytokines by BMDM $\phi$ . TNF $\alpha$  is a proinflammatory cytokine which is cytotoxic to some tumor cell lines and tumors *in vivo* in high concentrations (45, 46). In our study, we were unable to demonstrate that radioresistance was mediated by a direct effect on B16.SIY cells using supernatant from WT BMDM $\phi$ . This raised the possibility that TNF $\alpha$  stimulated TAM $\phi$  promoted radioresistance by effects on the tumor microenvironment. TNF $\alpha$  at physiological concentrations has recently been implicated in cancer induction and support of tumor angiogenesis, in part, through the attraction of BM derived myeloid precursor cells that contribute to tumor blood vessel formation and stabilization (42, 47). We investigated if TNF/TNFR signaling in macrophages regulates the production of angiogenic cytokines by irradiated macrophages. Our data demonstrate that inhibition of TNFR signaling by both genetic and pharmacologic means prevents increased production of VEGF by irradiated macrophages. Immunohistochemical analyses demonstrate that the combination of TNF $\alpha$  inhibition plus + IR led to both significant decreases in neovascularization as well as vascular function as evidenced by reduced pericyte coverage and increased hemorrhage.

A recent report noted that macrophage secreted VEGF paradoxically slowed tumor growth in part by inducing tumor vessel abnormalities such as tortuosity and leakiness resulting in tumor hypoxia while tumor secreted VEGF “normalized” tumor vasculature (48). Interestingly, when macrophage VEGF was deleted tumors grew more rapidly due to a more “normalized” vasculature but tumors were also more sensitive to cyclophosphamide. Our results are consistent with this report demonstrating the Enbrel® treated tumors were actually more sensitive to IR. In contrast to the report of Stockmann et al (48), TNF $\alpha$  blockade did not increase vessel pericyte coverage, suggesting that TNFR signaling in tumor macrophages likely affects several angiogenic pathways in addition to VEGF. Nonetheless, blockade of VEGF upregulation by inhibition of TNF $\alpha$  signaling represents an alternative clinically relevant method to enhance radiosensitivity via targeting of TAM $\phi$ . Paradoxically supernatant from macrophages defective in TNF signaling through germ line deletions in TNF or TNFR1,2 conferred modest radioprotection on B16.SIY cells *in vitro* through an unknown mechanism(s). Together, these data suggest that macrophage blockade mediates *in vivo* radiosensitivity predominantly through effects on the microenvironment. Active TNF $\alpha$  signaling in irradiated macrophages appears necessary for upregulation of macrophage derived VEGF resulting in enhanced preservation of the irradiated vasculature.

Tumor angiogenesis is distinguished from post-natal vasculogenesis in that tumor angiogenesis is proposed to occur through endothelial migration and sprouting from preexisting blood vessels, whereas vasculogenesis is proposed to occur by the recruitment of BM cells to the site of tumor angiogenesis or local inflammatory damage. While vasculogenesis was previously described as direct incorporation of progenitor cells into

newly emerging vasculature, it also involves the recruitment of bone marrow-derived angiogenic populations that enhance angiogenesis through paracrine mechanisms. The extent to which tumor neovascularization depends on local endothelial cells or infiltrating angiogenic cells is controversial. Recently, tumor regrowth by local myelomonocytic CD11b<sup>+</sup> cells were reported to induce tumor vasculogenesis following irradiation through secretion of MMP-9 in a model where tumor angiogenesis was suppressed by prior irradiation of the tumor bed (25). Additionally TAM $\phi$  secrete a variety of proangiogenic proteins including IL-8, TNF $\alpha$  and VEGF. Therefore, both angiogenesis and vasculogenesis may be supported by TAM $\phi$  derived factors. While our data using Enbrel<sup>R</sup> suggest that pharmacologic inhibition of macrophage TNFR signaling enhances radiosensitivity, our Lip-Clod data demonstrate that reducing macrophage populations in tumors represents another potential anti-angiogenic and anti-vasculogenic strategy. Systemically delivering liposomal clodronate to patients may not be feasible. However, CSF-1 receptor kinase inhibitors may block macrophage differentiation and function, providing an alternative biological tool to inhibit pro-inflammatory cytokine production from macrophages (49). The availability of these small molecules and monoclonal antibodies targeting either CSF-1R or CSF-1 to deplete TAM $\phi$  number and function may allow us to translate these strategies to promote radiationsensitivity in future studies.

We also report that blocking VEGF enhances the effect of IR (38, 50). Though anti-VEGF therapy could block VEGF derived from either vasculogenic myeloid populations or tumor cells, our results confirm that direct VEGF blockade is another therapeutic strategy to increase radiosensitivity. Despite the clear correlation between tumor vascularization and VEGF expression, TNF can also modulate the expression of other antiangiogenic factors, including thrombospondin and angiostatic chemokines like CXCL10, CXCL9. Therefore, targeting the TNF $\alpha$ /TNFR signaling in TAM $\phi$  may enhance radiosensitivity through additional pathways beyond VEGF signaling.

In summary, we utilized both genetic and pharmacological inhibition of TNF $\alpha$  signaling to study the role of tumor macrophages in promoting tumor radioresistance. Rather than directly affecting tumor cells, active TNF $\alpha$  signaling in irradiated macrophages results in the production of angiogenic cytokines such as VEGF. We have used co-implantation models to demonstrate that macrophages are sufficient for this effect, and Lip-Clod to demonstrate that macrophages are required. While Lip-Clod might have off-target cellular affects, previous studies suggest that Lip-Clod selectively depletes macrophages as opposed to other hematopoietic cells. Since the effects of Lip-Clod on other stromal components remains unclear, we also utilized bone marrow transplantation studies together with Lip-Clod to study the effects of depletion of bone marrow-derived cells. It nonetheless remains possible that active TNF $\alpha$  signaling in cellular components of the microenvironment might mediate radioresistance as well. Other limitations of our study include 1) the lack of a tissue specific promoter such as LysM to delete TNF $\alpha$  specifically in myeloid cells, 2) identification of which BM derived macrophage populations (M1 versus M2) mediate tumor vascular formation/stabilization following IR, and 3) whether VEGF secreted by macrophages attracts additional local angiogenic cells which contribute to tumor vascularization and regrowth following radiotherapy. In spite of these caveats, our results suggest that blockade

of TNF/TNFR signaling in TAM $\phi$  is an attractive target to improve the efficacy of radiotherapy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

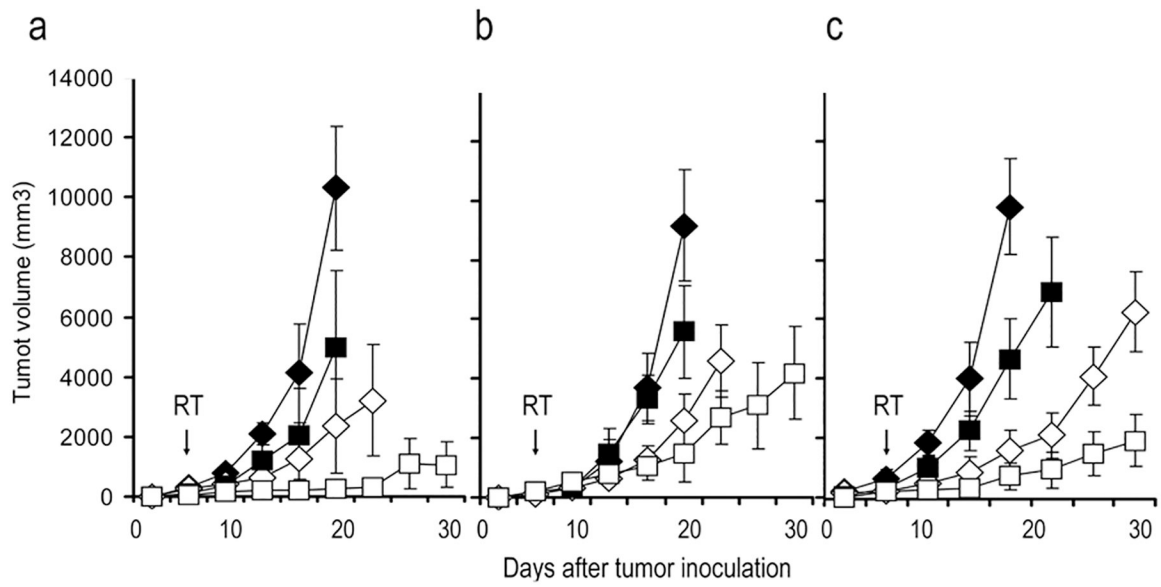
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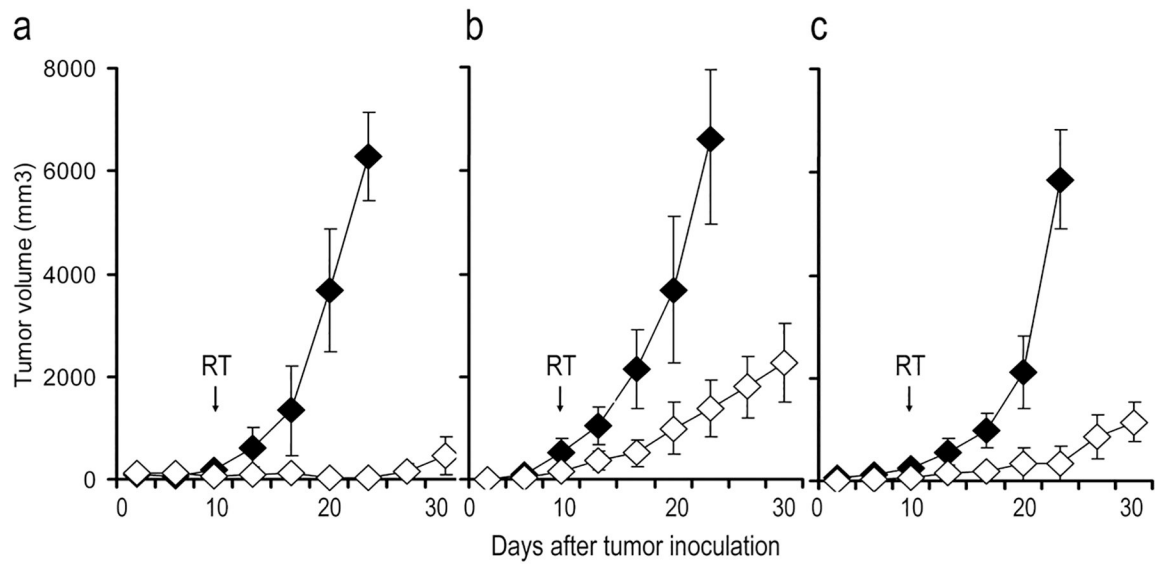
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**Figure 1. Depletion of macrophages enhances radiation.**

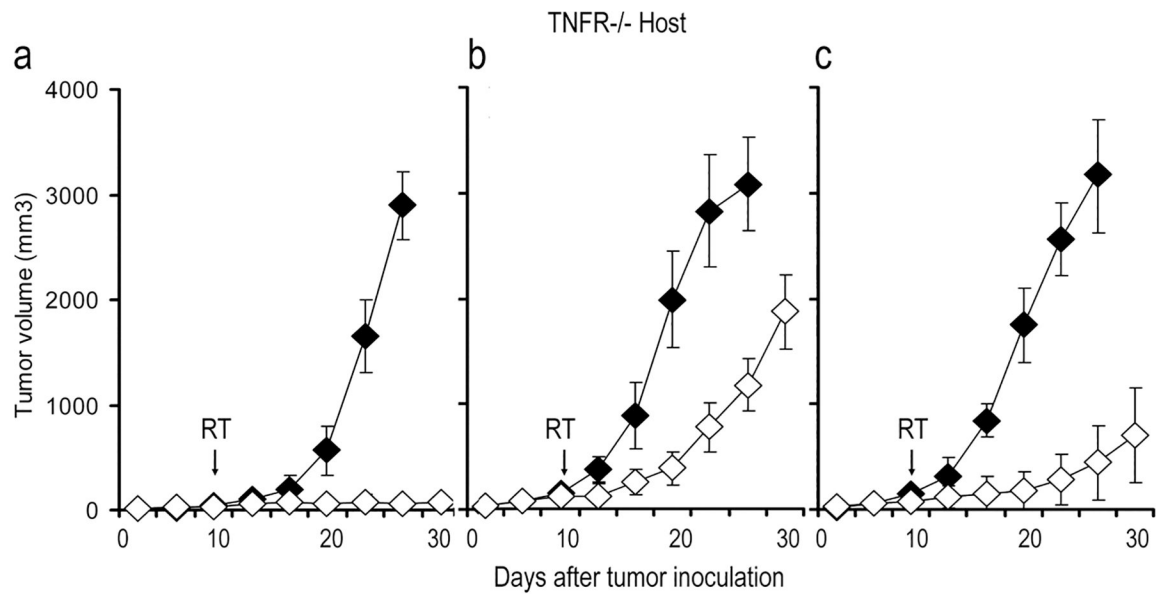
(a). Systemic macrophage depletion with Lip-Clod (200 $\mu$ l i.p.) prior to inoculation of B16.SIY cells significantly ( $p=0.002$ ) enhanced IR (20 Gy) compared with IR alone in WT ( $n=11-16$ /group). (b). Systemic depletion of macrophages after tumors were established enhanced IR ( $p=0.071$ ) compared to IR alone ( $n=11-16$ /group). (c). Intratumoral macrophage depletion significantly ( $p=0.037$ ) delayed tumor regrowth compared to IR alone ( $n=10-12$ /group).  $\blacklozenge$  Lip-PBS;  $\blacksquare$  20 Gy + Lip-PBS;  $\blacksquare$  Lip-Clod;  $\square$  20 Gy + Lip-Clod





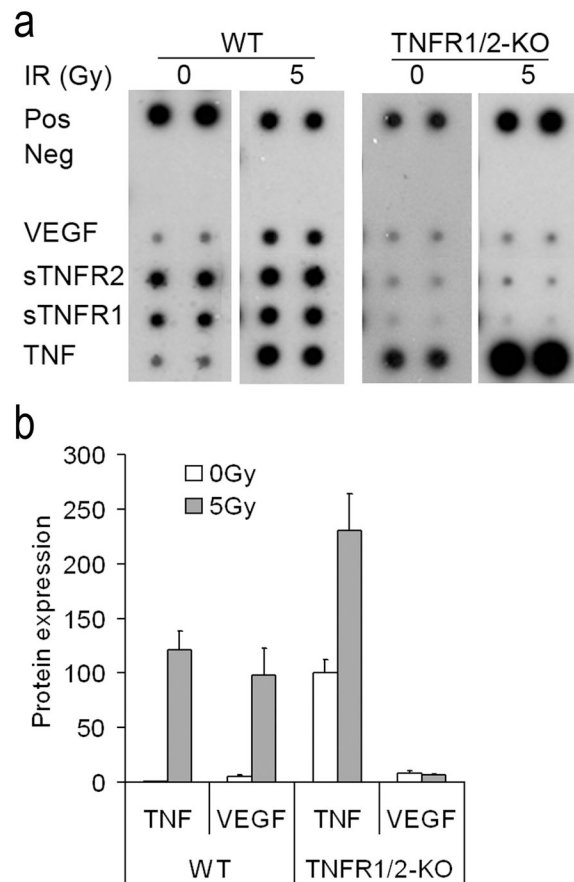
**Figure 2. TNF $\alpha$  signaling in BMDM $\phi$  mediates tumor radioresistance.**

(a). Base line radiation response to 20 Gy of B16.SIY tumors growing in WT (n=12/group).  $\blacklozenge$ B16; IR in B16 (b). Co-injection of WT BMDM $\phi$  with B16.SIY tumor cells significantly accelerates tumor regrowth following 20 Gy compared with baseline (a), (p=0.03, n=12/group).  $\blacklozenge$ WT-Mac + B16; IR in WT-Mac + B16 (c). Co-injection of TNF<sup>-/-</sup> BMDM $\phi$  with B16.SIY cells significantly decreased tumor regrowth following 20 Gy compared to WT (b), (p=0.03, n=12/group).  $\blacklozenge$  TNF<sup>-/-</sup> Mac + B16; IR in TNF<sup>-/-</sup> Mac + B16



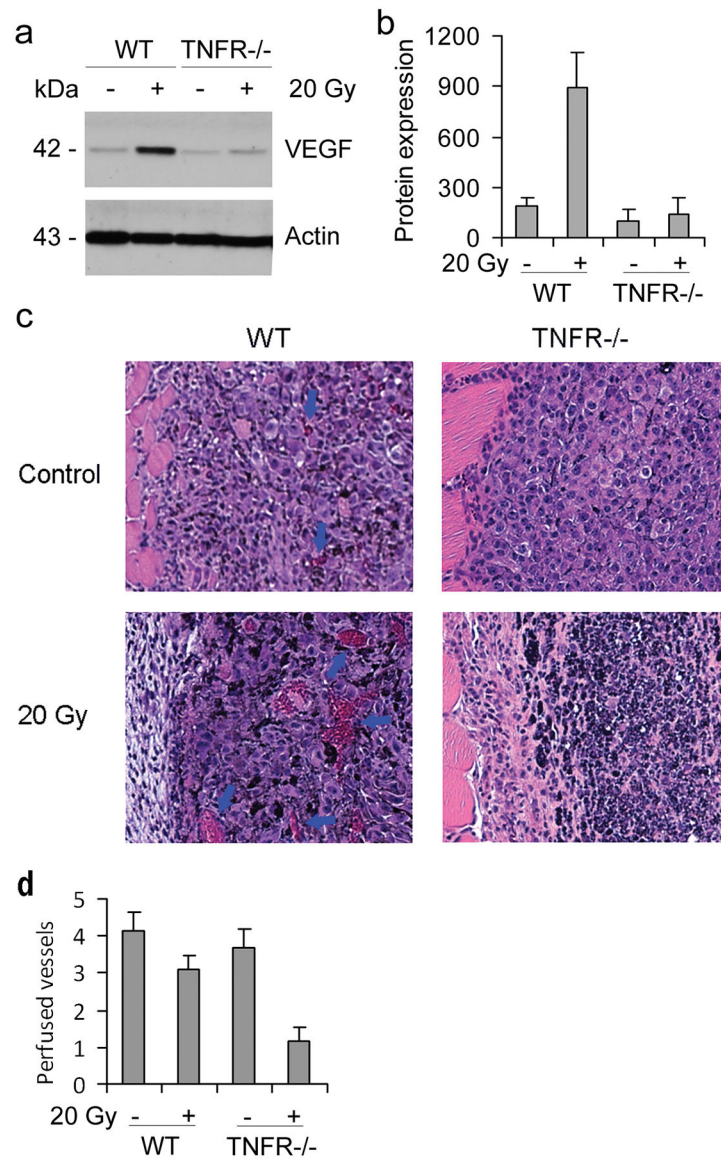
**Figure 3. TNF $\alpha$  signaling in BMDM $\phi$  mediates tumor radioresistance through autocrine/paracrine signaling.**

(a). Growth of B16.SIY tumors in TNFR1,2<sup>-/-</sup> mice (n=15/group).  $\blacklozenge$  B16;  $\circ$  IR in B16 (b). Co-injection of WT BMDM $\phi$  with B16.SIY cells significantly accelerates tumor regrowth following 20 Gy (p=0.003, n=12/group).  $\blacklozenge$  WT-Mac + B16;  $\circ$  IR in WT-Mac + B16 (c). Co-injection of WT BMDM $\phi$  with B16.SIY cells significantly accelerates tumor regrowth following 20 Gy compared with co-injection of TNFR1,2<sup>-/-</sup> BMDM $\phi$  and B16.SIY cells (p=0.041).  $\blacklozenge$  TNFR<sup>-/-</sup> Mac + B16;  $\circ$  IR in TNFR<sup>-/-</sup> Mac + B16



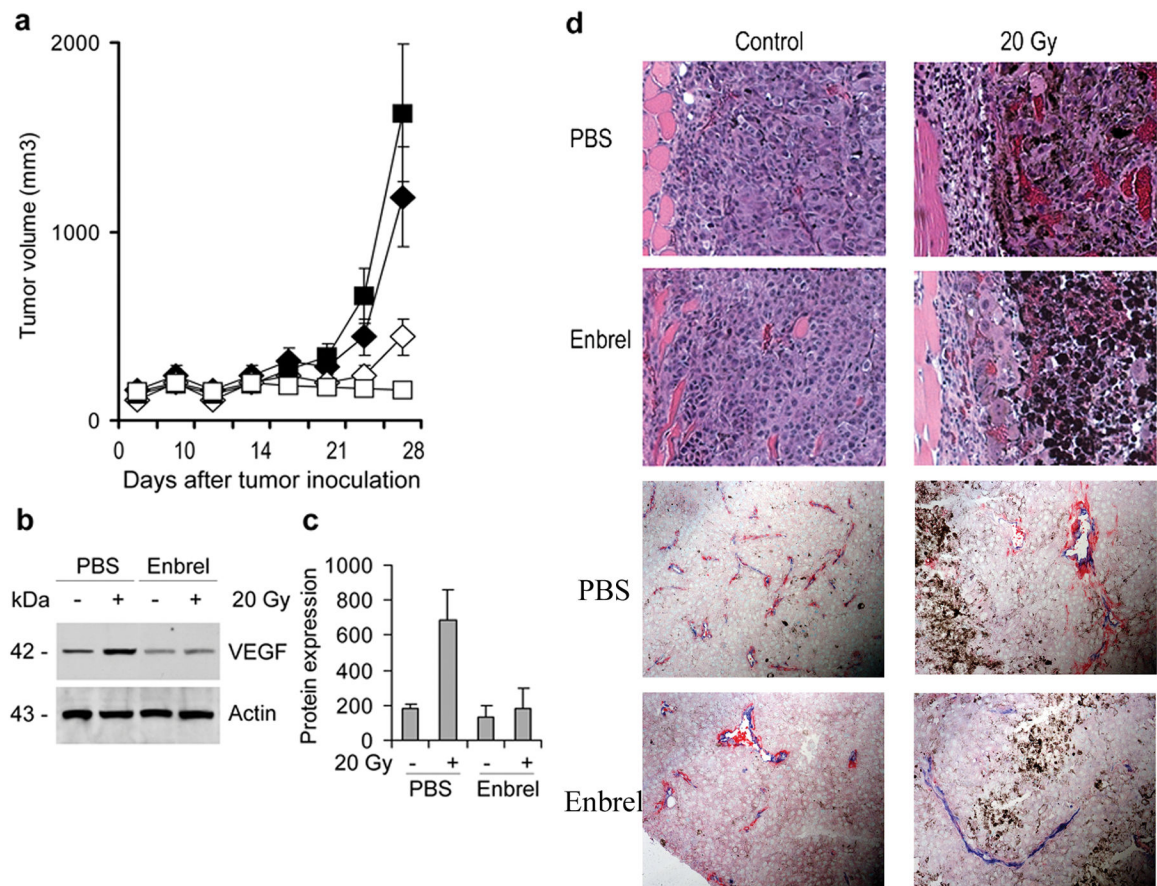
**Figure 4. Radiation induction of VEGF in BMDM $\phi$ .**

(a). A significant increase in VEGF was detected in WT BMDM $\phi$  compared with TNFR1,2 $^{-/-}$  BMDM $\phi$  with 5 Gy (b). Luminex assay confirmation of VEGF induction by IR in WT BMDM $\phi$ . The mean of triplicates from one representative experiment is shown.



**Figure 5. Radiation induction of VEGF in TAM $\phi$ .**

(a). Western blot analysis of VEGF expression of CD11b<sup>+</sup>F4/80<sup>+</sup> TAM $\phi$  isolated from tumors grown in WT and TNFR1,2<sup>-/-</sup>. (b). VEGF levels were significantly elevated in CD11b<sup>+</sup>F4/80<sup>+</sup> TAM $\phi$  from WT compared with TNFR1,2<sup>-/-</sup> (p=0.015, Luminex). (c) Increase neovasculture/angiogenesis in tumors grown in WT compared to TNFR1,2<sup>-/-</sup> post IR. Arrows indicate functional vessels containing red blood cells. (d). VGFR2<sup>+</sup> staining shows a significant decrease in perfused vessels post IR in tumors grown in TNFR1,2<sup>-/-</sup> compared to WT control (p=0.0001) and WT treated with 20 Gy (p=0.002).



**Figure 6. Enbrel® enhances radiosensitivity by blocking the VEGF induction in TAM $\phi$ .** (a). Enbrel® + 20 Gy produced a significant decrease in B16.SIY regrowth compared to 20 Gy alone (p=0.022). ◆ PBS; 20 Gy + PBS; ■ Enbrel; □ 20 Gy + Enbrel (b). Western blot of VEGF expression by CD11b<sup>+</sup>F4/80<sup>+</sup> TAM $\phi$  from WT treated with Enbrel® + 20 Gy. (c). Luminex showing inhibition of VEGF induction in CD11b<sup>+</sup>F4/80<sup>+</sup> TAM $\phi$  isolated from tumors treated with Enbrel® + 20 Gy in WT. The mean of triplicates from one representative experiment is shown. (d). Representative tumor tissue sections showing a high density of intact neovasculature/angiogenesis in WT mice post IR and was reduced with IR + Enbrel® (upper panel); Blue VEGFR2<sup>+</sup> microvessel density and red NG2<sup>+</sup> pericyte coverage were compared between groups (lower panel). Enbrel® + 20 Gy inhibited VEGFR2<sup>+</sup> microvessel repair/neovasculature with less pericyte coverage noted by NG2<sup>+</sup> staining.