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BRIEF COMMUNICATION

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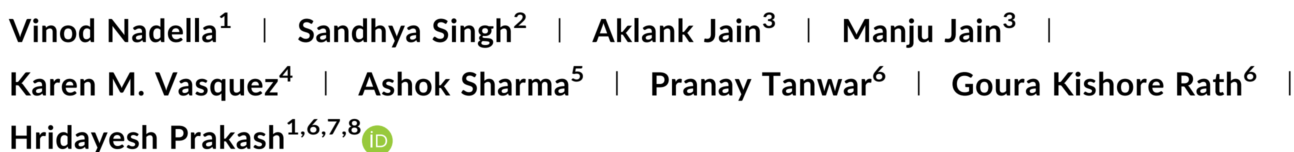
Low dose radiation primed iNOS + M1macrophages modulate angiogenic programming of tumor derived endothelium



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Solid tumors are covered by stroma, which is hypoxic in nature and composed of various non-malignant components such as endothelial cells, fibroblasts, and pericytes that support tumor growth. Tumor stroma represents a mechanical barrier for tumor infiltration of CD8+ effector T cells in particular. In this context, our previous studies have demonstrated the therapeutic impact of Low-Dose Radiation (LDR)-primed and M1-retuned (iNOS+) peritumoral macrophages that produce inducible nitric oxide, have immunological roles on tumor infiltration of effector T cells, cancer-related inflammation, and subsequent tumor immune rejection in a mouse model of pancreatic cancer. These findings suggested a possible modification of tumor endothelium by LDR-primed macrophages. In line with these observations, here we demonstrate the influence of LDR in down-modulating HIF-1 in irradiated tumors in the course of polarization of irradiated tumor-associated macrophages toward an M1 phenotype. Furthermore, we demonstrate that M1 macrophages which are primed by LDR can directly influence angiogenic responses in eNOS+ endothelial cells which produce nitric oxide having both vascular and physiological roles. Furthermore, we demonstrate that naïve macrophages, upon differentiating to an M1 phenotype either by Th1 stimuli or LDR, potentially modify sphingosine-1-phosphate/VEGF-induced angiogenic signaling in tumor-derived endothelial cells with tumorigenic potential, thus indicating the significance of iNOS+ macrophages in modulating signaling in eNOS+ tumor-derived endothelium. Our study suggests that iNOS+ macrophages can activate tumor endothelium which may contribute to cancer-directed immunotherapy in particular.

KEYWORDS

angiogenesis, endothelium, gamma irradiation, immune cells infiltration, M1 macrophages, tumor therapy

1 | INTRODUCTION

Tumor hypoxia and impermissive endothelium are among a variety of factors that contribute to poor prognosis and intrinsic resistance in a variety of tumors against various interventions.[1–4] Solid tumors are often surrounded with stroma rich in eNOS protein, which is important for the maintenance of the tumor microenvironment,[5] endothelium anergy, and intrinsic resistance of tumors to variety of

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tumor-directed therapies.[6] Among various tissue-specific factors that contribute to endothelium anergy, tumor hypoxia and high-grade tumor metabolism are key factors and characteristics of solid tumors, which render them refractory in nature.[7,8] Tumor hypoxia is executed by hypoxia inducible factor 1 (HIF-1), which promotes angiogenesis, in part, by enhancing vascular endothelial growth factor (VEGF) signaling, which interferes with tumor infiltration of CD8+ and retuning of M1 phenotypic macrophages across inert endothelium.[9–11] A large variety of tumors rely on VEGF/HIF-1 and sphingosine-1 phosphate (S1P), which promote tumor angiogenesis. Apart from this, these tissue-derived factors also promote polarization of tumor infiltrating naïve/inflammatory macrophages toward the M2 phenotype like tumor associated macrophages (TAM).[12,13] We have previously demonstrated the indispensable role of low-dose irradiation-programed iNOS+ (defined as M1) peritumoral macrophages in immunotherapy against non-resettable tumors of the pancreas, skin (eg, melanoma),[14,15] and colon (unpublished data). This was accompanied by changes in cancer-related inflammation, endothelium anergy, and angiogenesis, which are limiting factors for tumor infiltration of T cells. Interestingly, our macrophage depletion experiments in Rip1Tag5 mice furthermore demonstrated that selective depletion of M1 macrophages promoted glucose metabolism,[15] indicating a neuroendocrine impact of low-dose radiation (LDR) primed M1 macrophages in these mice.

Here, we elaborate on these findings and further demonstrate the potential of LDR in down-modulating tumor hypoxia in tumors, which may contribute to retuning of TAM toward an M1 phenotype. Interestingly, when M1-retuned macrophages were co-cultured with VEGFR2+ pancreatic β-islet SVR (derivative of SVEN) tumorigenic endothelium, angiogenic signaling was inhibited in these cells, which appear to have been induced by various pro-inflammatoary and Th1 factor secreted by primed macrophages. Thus, these results reveal that TAM, once polarized toward an M1 phenotype in situ, have the ability to activate tumorigenic and eNOS+ endothelium to facilitate tumor infiltration of effector molecules, which may contribute to immunotherapeutic approaches to the treatment of cancer.

2 | MATERIALS AND METHODS

2.1 | Antibodies and reagents

RPMI 1640, lipopolysaccharide (LPS), penicillin-streptomycin solution, $NaNO_2$, sphingosine 1 phosphate, and anti-actin antibody were purchased from Sigma-Aldrich (Taufkirchen, Germany). Sulphanilamide and N-(naphthyl) ethylenediamine dihydrochloride were purchased from E. Merck (Darmstadt, Germany). CD11b+ human and mouse MACS MicroBeads and MS Columns were purchased from Miltenyi Biotec (Auburn, CA). Recombinant mouse IFN cytokines; rabbit polyclonal anti-mouse NOS2, eNOS, and pAKT; and caveolin-1 antibodies were purchased from Cell Signaling (Frankfurt, Germany). VEGFR2 and anti-HIF-1 antibodies were purchased from Novus Biological (Wiesbaden, Germany). VEGF peptide was purchased from PeproTech GmBH (Hamburg, Germany). Goat anti-rabbit and mouse HRP-conjugated secondary antibodies were purchased from Sigma-Aldrich and Santa Cruz Biotechnology (Heidelberg, Germany), respectively. Mouse HIF-1-specific siRNA was purchased from Invitrogen (Schwerte, Germany). Control siRNA was purchased from Qiagen (Hilden, Germany). Goat anti-rabbit Alexa Fluor 488, goat anti-rat-Alexa Fluor 569, anti-rat-Cy3, and goat anti-mouse Cy3-conjugated antibodies were purchased from Dinova (Koenigswinter, Germany).

2.2 | Total body irradiation

The well characterized RipTag-5 (RT5) transgenic mouse model of spontaneous insulinoma[16] was used for this study. At ~25 weeks of age, RT5 mice were irradiated systemically with 2 Gy irradiation at weeks 25 and 26 by using a Gammatron Cobalt 60 units (Siemens, München, Germany) at a dose rate of 0.4 Gy/min. Mice were housed under pathogen-free conditions at the German Cancer Research Center. All animal experiments were authorized by the local government.

2.3 | Cell culture experiments

The RAW264.7A murine macrophage line (ATCC-TIB-71™) and the VEGFR2+ mouse pancreatic β endothelium,[17,18] also known as SVR-1 (CRL-2280), were purchased from the American Type Culture Collection (ATCC, Manassas, VA). HUVEC cells were received from Helmut Augustin (Heidelberg, Germany). These cells were co-cultured both directly and/or indirectly using a cell culture insert (Millipore, Darmstadt, Germany). For direct co-culture experiments, RAW 264.7A murine macrophage cells were seeded 1 day in advance of experimental use to ensure adherence. The next day, cells were washed once with serum-free media and SVR-1 cells were seeded over the monolayer of RAW macrophages and cultured for an additional 12 h for the adherence of the tumor cells over macrophages. These cells were then treated with different stimuli for the time intervals as mentioned in the figures. For indirect cell culture, cells were cultured using a cell culture insert whereby RAW cells were cultured over the membrane and SVR cells were laid down at bottom. The RAW macrophages in the upper chamber or RAW and SVR-1 direct co-cultures were treated with various innate stimuli or irradiated by using a Gammatron Cobalt 60 unit (Siemens) at a dose rate of 0.4 Gy/min. For evaluating the activation potential of irradiation and/or various stimulations, nitric oxide production was quantified in the culture supernatant by using a standard Griess reagent method.[19] For studies with CD11b+ peritoneal macrophages, C57BL/6j mice were injected with 1 mL of 4% brewer thioglycolate medium intraperitoneally and peritoneal lavage was harvested on the third day post-injection. Peritoneal lavage was centrifuged at 1500 rpm for 8 min and the cell pellets were resuspended in fresh serum-free RPMI. CD11b+ macrophages were purified by using a MACS-based separation method (Miltenyi Biotec) and were cultured in serum-containing medium overnight.

Macrophage monolayers were washed on the following day once with serum-free medium to remove any unbound cells. Cells were irradiated with different doses using a Gammatron Cobalt 60 unit (Siemens) at a dose rate of 0.4 Gy/min. Cells were cultured further for 48 h and the supernatants were collected and centrifuged at 5000 rpm to remove cellular debris. Next, cell-free conditioned media were collected and used for experiments as mentioned in the results.

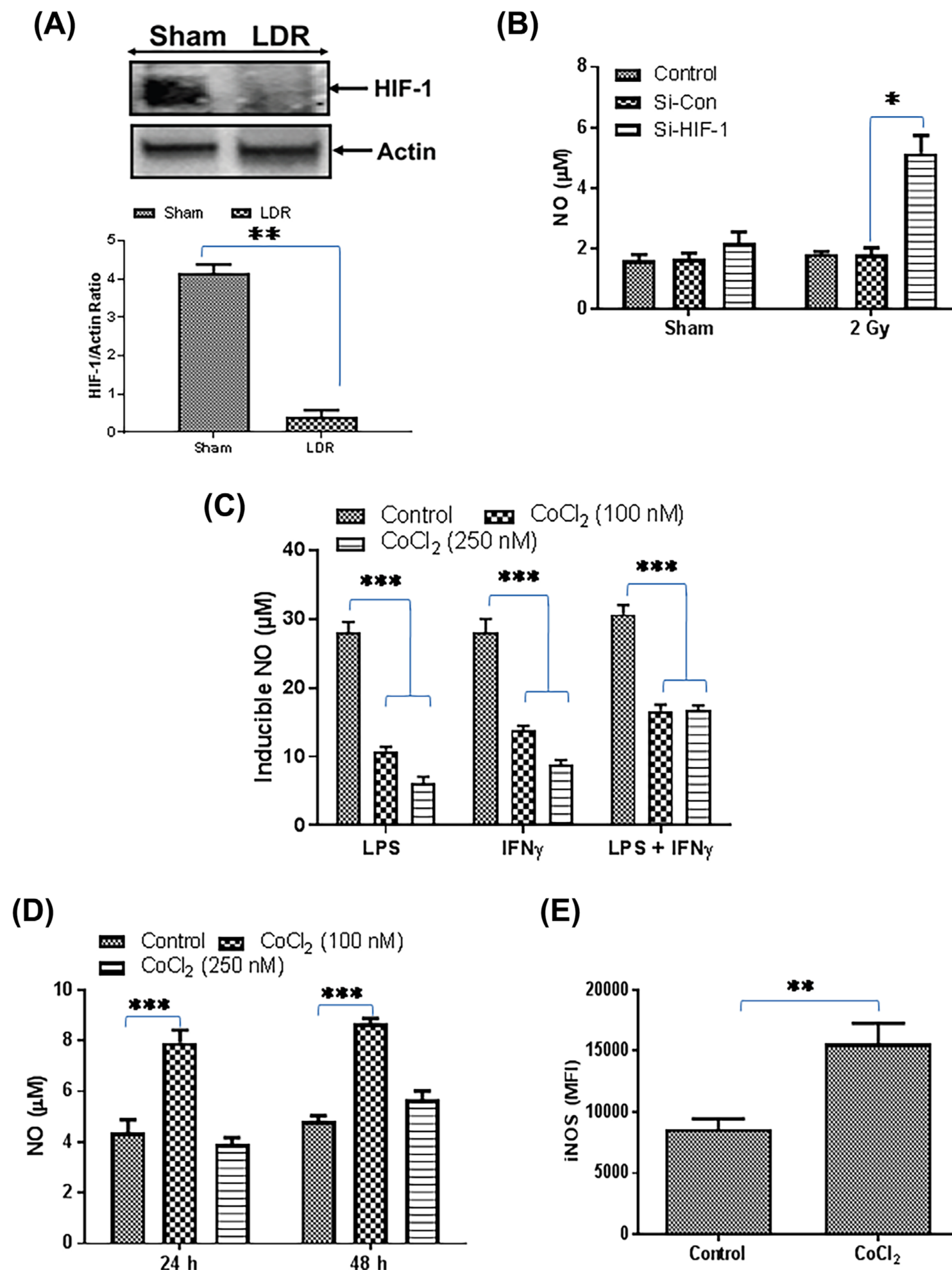

FIGURE 1 Dual role of HIF-1 in macrophage retuning. A, RT5 mice (25 weeks of age) were irradiated twice with 2 Gy irradiation and were analyzed for the expression of HIF-1 as an indicator of tumor hypoxia. A representative blot from several tumor lysate repeats with similar outcomes is shown. β-actin was used a loading control. Densitometric analysis of all the Western blots were quantified by ImageJ software and the mean densitometry values were plotted in terms of relative protein expression. B, HIF-1 proteins were depleted in RAW macrophages by siRNA-based methods and the macrophages were then irradiated with a dose of 2 Gy and the impact of this treatment was analyzed on the level of NO in the cell culture supernatants. C, RAW macrophages treated with various doses of $CoCl_2$ (to stabilize the expression of HIF-1) were stimulated with Th1 effectors (ie, LPS and IFNγ) and NO levels were quantified 24 h posttreatment by using a Giess reagent method. D, Naïve RAW macrophages were treated with $CoCl_2$ and the impact of treatment on the levels of NO was analyzed. Shown is the mean level (μM) of NO ± SEM from three independent experiments. E, Expression of iNOS in CD11b+/Gr-1(−) RT5 tumor macrophages under the influence of $CoCl_2$ treatment was analyzed. Statistical analyses were conducted using either Student's *t* tests, one-way or two-way ANOVA, followed by Bonferroni post-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). All the statistical analyses was carried out with GraphPad Prism Version 7.0 software

2.4 | RNA interference

HIF-1 in RAW macrophages was depleted by using a siRNA-based method. 1×10^4 RAW macrophages were cultured in antibiotic-free medium for 24 h and were subsequently transfected with HIF-1 siRNA (Santa Cruz Biotechnology, GmBH) or a scrambled siRNA (Invitrogen) in antibiotic- and serum-free medium with varying concentrations using an oligofectamine transfection reagent from Invitrogen (Darmstadt, Germany). The cells were incubated with the siRNA mixture for 6 h in serum-free medium. The medium thereafter was replaced and cells were incubated for 24-h and 48-h time periods, with efficiency of knockdown assessed by Western analysis.

2.5 | Western analysis

Cells were washed with ice-cold PBS (pH 7.4) and lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, and a protease inhibitor mixture) and sonicated. The lysate was centrifuged at 14 000 rpm for 20 min at 4°C to separate the particulate fraction. Protein concentration was determined by using a BCA kit (Darmstadt, Germany). Twenty micrograms protein per sample was separated on a Nu-PAGE Bis-Tris Mini Gel system and blotted onto a PVDF membrane. Blots were blocked at room temperature for 30 min with 5% nonfat dry milk in TBS-T (20 mM Tris base, 137 mM NaCl, and 0.05% Tween 20, pH 7.5) and then incubated overnight at 4°C with primary antibodies, and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies. Blots were visualized by using an ECL reagent (GE Healthcare, Dornstadt, Germany). β-actin was used an internal loading control.

2.6 | Fluorimetric analysis

Various intracellular signaling proteins were measured by using a Fluorimetric method.[14,20] Briefly, cells were cultured in flat bottom black 96-well plates and subsequently irradiated, stimulated, and incubated for various time intervals, as mentioned. After incubation, the cell monolayers were washed once with PBS and fixed with 4% PFA for 10-15 min at room temperature and permeabilized with 0.1% Triton-X for 5 min. The cell layers were then blocked in PBS containing 1% BSA and 1% serum from the host of secondary antibodies. Next, the cell layers were incubated with primary antibody at 4°C overnight in the dark. The next day, the cells were washed twice with 1X PBS. The cells were then incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature in the dark. The cell layers were washed again twice with PBS. The plates were read at 488 nm for Alexa-Fluor 488-labeled antibodies or at 594 nm for Alexa-Fluor 594-labeled antibodies, respectively, with a reference wavelength of 630 nm using a 96-well fluorimeter (TECAN Infinite 200 PRO Systems, Mainz, Germany). Each sample/well was read at multiple fields for uniformity and the mean fluorescence intensities of stained cells from each well were calculated.

2.7 | Statistical analysis

All results were expressed as the mean ± SEM of three independent experiments performed in triplicates. Statistical analyses were conducted using either Student's *t* tests, one-way or two-way ANOVA followed by Bonferroni post-test (**P* < 0.05; ***P* < 0.01; ****P* < 0.001). All the statistical analyses were carried analyzed using GraphPad Prism Version 7.0 software.

3 | RESULTS AND DISCUSSION

3.1 | Dual role of HIF-1 in macrophage polarization

Whole body irradiation of insulinoma-bearing RipTag5 mice led to the complete degradation of HIF-1 in the tumors of treated mice (Figure 1A), which correlated with the reduced levels of key angiogenic proteins in these tumors and M1 retuning of TAM in these mice.[14,15] Degradation of HIF-1 and concurrent upregulation of iNOS in the irradiated tumors and purified CD11b+ TAM from insulinoma-bearing mice[14,15] indicated that depletion of HIF-1 following irradiation may

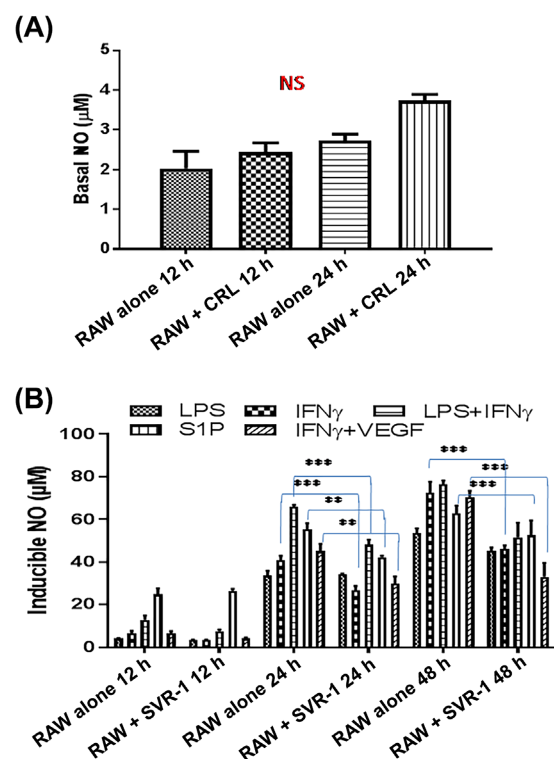

FIGURE 2 VEGFR2+ tumor-derived endothelium inhibits M1 programing of naïve macrophages. A, Naïve RAW macrophages were cultured with or without SVR endothelium and NO was quantified in the culture supernatant at the indicated time intervals. B, RAW macrophages were cultured with SVR cells and were stimulated as indicated and NO levels were quantified in the culture supernatant at the indicated time intervals. Shown are the mean levels of NO (μM) ± SEM from three independent experiments. Statistical analysis was conducted using a two-tailed paired *t*-test with 95% confidence intervals (**P* < 0.05; ***P* < 0.01)

contribute in the upregulation of iNOS in macrophages in these mice.[14] On the basis of these results, we anticipated that HIF-1 depletion would enhance NO levels in macrophages. To test this, we depleted HIF-1 in RAW macrophages using an siRNA-based approach and NO levels were measured in the culture supernatants. Consistent with our hypothesis, depletion of HIF-1 in these macrophages enhanced NO levels, particularly in the irradiated macrophages (Figure 1B), which is in line with recent reports[21,22] and our published data.[14]

HIF-1 is a master transcription factor and when stabilized, in addition to executing hypoxia related physiological responses, it also promotes angiogenic signaling in the polarization of tumor infiltrating Th1 primed macrophages.[23,24] To test this, we stabilized HIF-1 in the naïve macrophages by treating them with a hypoxia-mimetic agent, $CoCl_2$,[25,26] and treated the cells with Th1 stimuli. Indeed, the results from these experiments suggested that stabilization of HIF-1 in the macrophages significantly inhibited the generation of NO in the culture supernatant of iNOS+ Th1-primed inflammatory macrophages (Figure 1C). These findings correlated with results in M1 programing of irradiated TAM in RipTag5 mice. Several studies with HIF-1 specific knockout animals have demonstrated the contribution of HIF-1 proteins in Th1 effector responses of iNOS+ and infected macrophages.[22–28] Thus, HIF-1 expression is likely an important contributor to M1 programing of naïve macrophages in tumor-free environments, suggesting a dual fate of HIF-1 on macrophage polarization. To clarify this, naïve RAW macrophages were treated with different concentrations of $CoCl_2$ and NO titers in treated cell culture supernatants were quantified. Supporting our hypothesis, the results indicated that low levels of HIF-1 enhanced NO titers in naïve RAW macrophages (Figure 1D), suggesting that low levels of HIF-1 support the differentiation of naïve macrophages toward an M1 phenotype. Surprisingly, an increase in the expression levels of iNOS proteins after treatment with $CoCl_2$ in purified CD11b+ macrophages from Rip Tag5 mice demonstrated the influence ofHIF-1 stabilization on re-polarization of TAMs in this system (Figure 1E). Tissue/tumor hypoxia is known to control both macrophage activation and their tissue infiltration during inflammatory responses. For example, a recent report suggested that

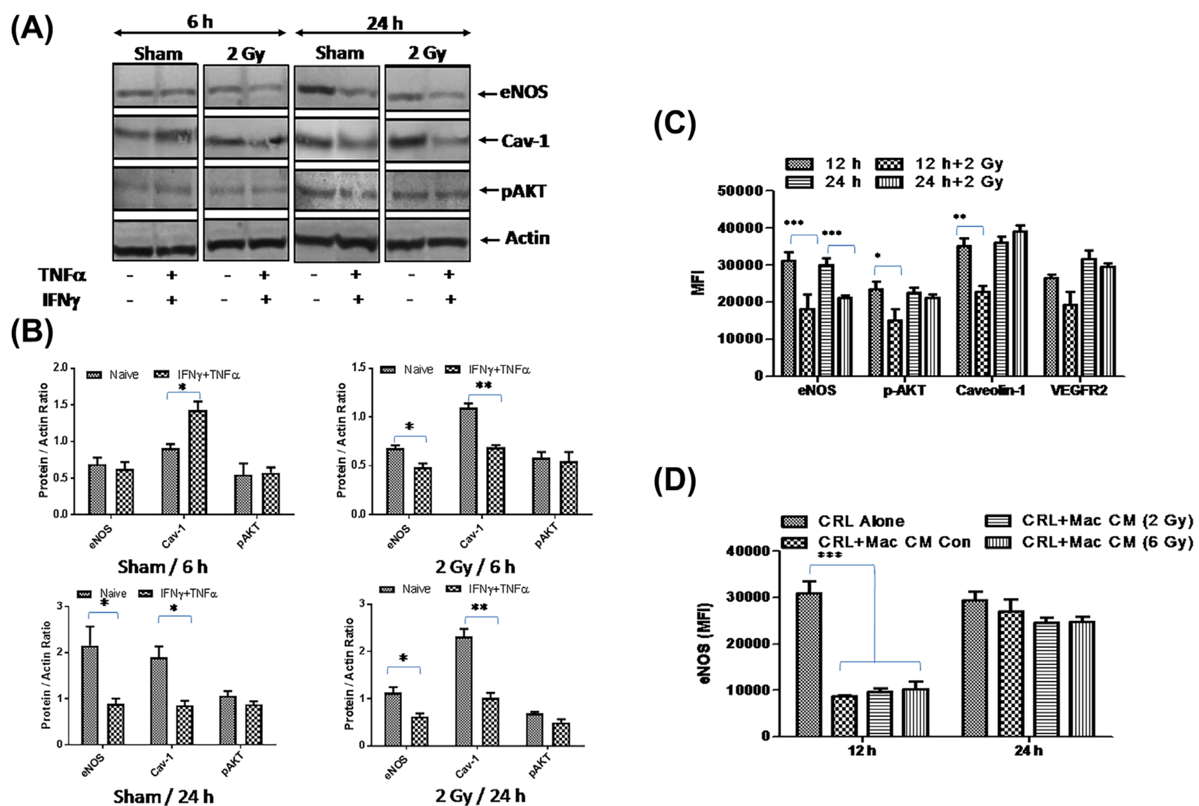

FIGURE 3 Gamma irradiation modifies impermissive VEGFR2+ tumor-derived endothelium. A, VEGFR2+ pancreatic β-islet SVR (derivative of SVEN) endothelial cells were irradiated with gamma irradiation (2 Gy) viz-a-viz stimulation with Th1 effector cytokines and the impact of their stimulation was analyzed on the expression of key proteins involved in eNOS signaling/angiogenesis at 6 and 24 h posttreatment/irradiation. Shown is a representative Western blot from three independent experiments. β-actin was used a loading control. B, Densitometry quantification of representative Western blots shown under (A) were analyzed by ImageJ and the data were plotted as a mean of protein/actin ratio. Statistical analysis was conducted using a two-tailed paired *t*-test with 95% confidence intervals (*$P < 0.05$; **$P < 0.01$). C, SVR cells cultures were irradiated (2 Gy) and eNOS proteins were quantified using a flurometeric-based method at the indicated time intervals. Similarly, (D) SVR cells were cultured in the presence of conditioned media obtained from naïve or irradiated mouse peritoneal macrophages and levels of eNOS proteins were quantified using a flurometeric-based method at the indicated time intervals

HIF-1 promotes the expression of CD68 and CCL2 and enhances macrophage recruitment in tumor tissues.[29–31] Whether HIF-1 promotes M1 or M2 phenotypes in macrophages may depend upon the increased expression of HIF-2 proteins for which the presence of active HIF-1 is essential. Stabilization of HIF-2 in macrophages promoted the degradation of HIF-1 in a protease-dependent manner and in such chronic conditions, HIF-2 could promote M2 polarization in macrophages. Interestingly, low-dose irradiation of macrophages led to the activation of HIF-1 in naïve HIF-2 negative macrophages.[14] These results reinforced that the notion that the presence of HIF-2 and associated signaling pathways, including non-canonical nf-KB and TGF-β in macrophages,[32,33] is important for dictating the fate of HIF-1 for macrophage activation. Thus, these results provide a potential explanation for the dual fate of HIF on NO levels[34,35] and its balance in inflamed macrophages, which may be useful for tumor therapy and open new lines of investigation.

3.2 | Tumor derived endothelium inhibits Th1 programing of naïve macrophages

To determine whether tumorigenic endothelium would influence iNOS+ macrophages or not, we co-cultured VEGFR2+ SVR-1 with RAW macrophages directly or indirectly and measured NO levels (both constitutive and inducible) in the cultured supernatant. The influence of RAW macrophages was analyzed on the expression of various angiogenic proteins in RAW co-cultured SVR-1 cells. We found that co-cultures of SVR-1 cells and naïve macrophages enhanced the constitutive NO levels in culture supernatants, but this remained insignificant over RAW macrophages alone (Figure 2A). However, SVR-1 inhibited the levels of inducible NO in the culture supernatant of Th1 primed iNOS+ macrophages (Figure 2B), suggesting that SVR-1 assisted in driving the polarization of these macrophages to either M2 or TAM phenotypes. This may be due to VEGF-R2-mediated angiogenic programing of RAW macrophages,[36,37]

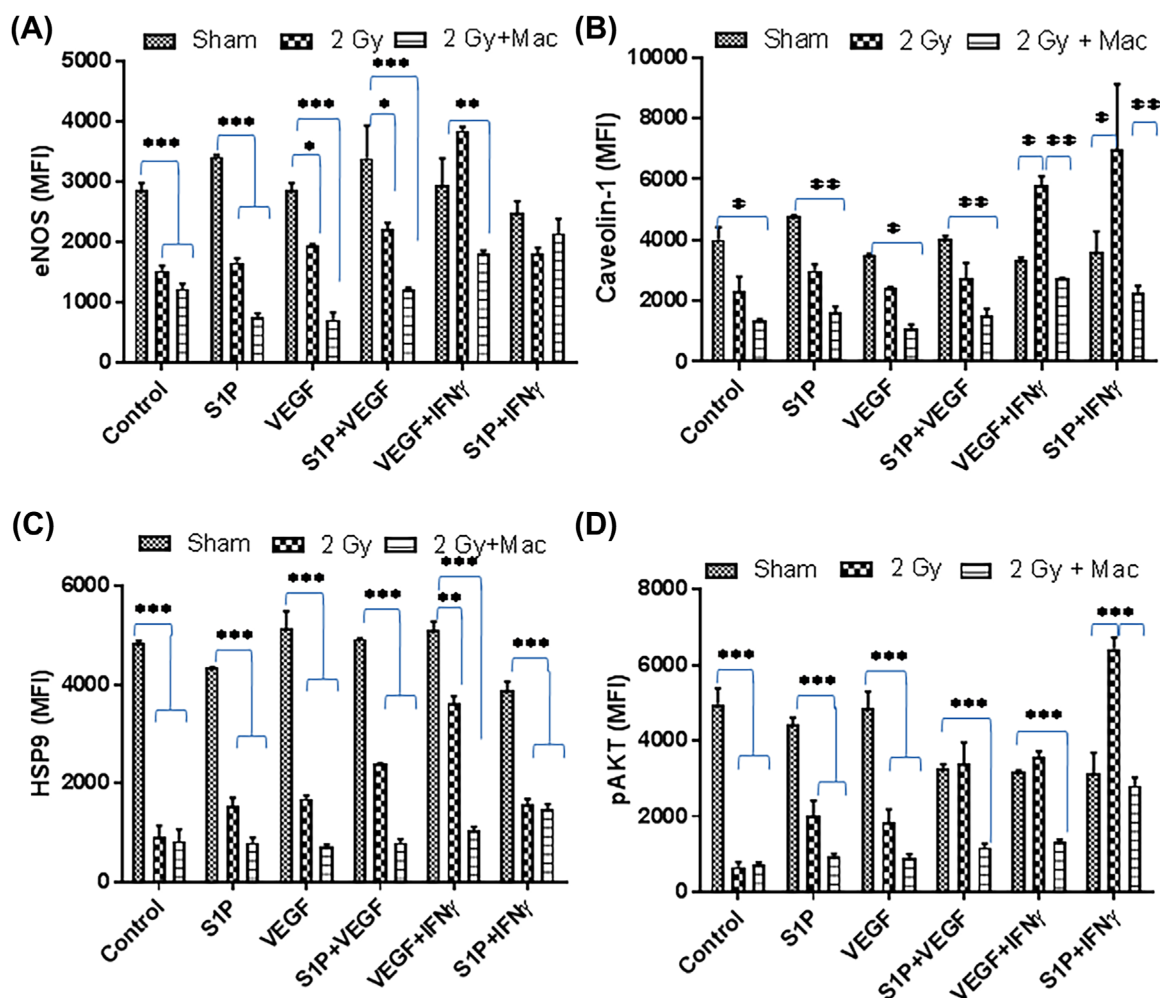

FIGURE 4 Low dose Gamma radiation inhibits angiogenic responses in primary endothelium. Freshly isolated HUVEC endothelial cells were irradiated upon treatment with or without conditioned media obtained from M1-differentiated macrophages and the impact of these treatments was analyzed on various proteins involved in angiogenic signaling. Shown are the MFI of proteins ± SEM from three independent experiments. Statistical analyses were conducted using either Student's t tests or two-way ANOVA followed by Bonferroni post-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$)

which was evident with the drop of inducible NO in the co-cultures (Figure 2B) treated with key angiogenic factors in tumors such as S1P and VEGF.

3.3 | LDR-primed M1 macrophages modulate tumor-derived endothelium

Our previous studies have demonstrated the influence of LDR on the activation of tumor endothelium for permitting the tumor infiltration of immune effector cells in the rejection of established tumors. This provoked us to analyze whether gamma irradiation would influence angiogenic signaling in tumor endothelium directly or not. To test this, we irradiated both tumor-derived endothelial cells (SVR-1 cells) and primary HUVEC with 2 Gy of irradiation and analyzed the expression of various angiogenic proteins in tumor-derived endothelial cells. We found that irradiation of these tumorigenic SVR-1 cells inhibited the levels of eNOS in both SVR-1 (Figures 3A and 3B) and HUVEC cells (Figure 4), confirming reduced levels of eNOS in irradiated insulinoma.[14] This could be due to reduced levels of HSP90 protein, a major chaperone of eNOS proteins in the activated endothelium in irradiated tumors.[14,15] Interestingly, LDR enhanced the expression of caveolin-1 proteins in SVR-1 cells, in line with our previous findings.[14,15] However, radiation did not modify the expression of pAKT significantly in these endothelial cells. These results suggested that LDR has the potential to mitigate eNOS signaling, which is largely influenced by VEGF and other tumor-derived angiogenic factors in endothelium that likely contributes to their increased permeability to endothelium in stromal tissues.

We have previously demonstrated that the LDR-driven iNOS+ M1 phenotype[14] is decisive for tumor vasculature normalization, enhanced tumor infiltration of T cells, and subsequent tumor immune rejection in RipTag5mice.[14,15] On the basis of these results, we anticipated that iNOS+ macrophages would influence angiogenic signaling in tumorigenic endothelium. To test this presumption, we cultured SVR-1 cells in the presence of conditioned media from irradiated CD11b+ mouse primary macrophages that had acquired an M1 phenotype postirradiation[14] and analyzed the expression of eNOS proteins. Following our expectation, conditioned media from 2 Gy irradiated primary macrophages by their virtue of the M1 phenotype[14] inhibited the expression of eNOS in SVR-1 (Figure 3C-D), and HUVEC cells (Figure 4A-D). These results further confirmed the angiostatic impact of LDR-primed iNOS+ macrophages. Further, low-dose gamma irradiation directly or via LDR-primed macrophages reduced the levels of S1P and VEGF-induced angiogenic proteins in HUVEC cells. This is likely due to the presence of various Th1 effector cytokines (eg, IFN-γ and TNF-α) in the conditioned media of 2 Gy-exposed macrophages.[14] Thus, these data not only corroborated our previous studies[15] where depletion of iNOS proteins in insulinoma bearing RipTag5 mice enhanced tumor-related inflammation, T cell infiltration,[15] and inhibited pro-inflammatory responses, but also reinforced the involvement of iNOS proteins in modulating the angiogenic responses in tumors, which is one of the key hurdles in cancer therapy.

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REFERENCES

1. Jain RK, Duda DG, Willett CG, et al. Biomarkers of response and resistance to antiangiogenic therapy. *Nat Rev Clin Oncol.* 2009;6:327–338.
2. Jain RK. A new target for tumor therapy. *N Engl J Med*. 2009;360: 2669–2671.
3. Jain RK. Antiangiogenesis strategies revisited: from starving tumors to alleviating hypoxia. *Cancer Cell*. 2014;26:605–622.
4. Kheshtchin N, Arab S, Ajami M, et al. Inhibition of HIF-1alpha enhances anti-tumor effects of dendritic cell-based vaccination in a mouse model of breast cancer. *Cancer Immunol Immunother*. 2016;65: 1159–1167.
5. Lim KH, Ancrile BB, Kashatus DF, Counter CM. Tumour maintenance is mediated by eNOS. *Nature*. 2008;452:646–649.
6. Bouzin C, Brouet A, De Vriese J, Dewever J, Feron O. Effects of vascular endothelial growth factor on the lymphocyte-endothelium interactions: identification of caveolin-1 and nitric oxide as control points of endothelial cell anergy. *J Immunol*. 2007;178:1505–1511.
7. Liu J, Zhang J, Wang X, et al. HIF-1 and NDRG2 contribute to hypoxia-induced radioresistance of cervical cancer Hela cells. *Exp Cell Res*. 2010;316:1985–1993.
8. Hong SS, Lee H, Kim KW. HIF-1alpha: a valid therapeutic target for tumor therapy. *Cancer Res Treat*. 2004;36:343–353.
9. Lapeyre-Prost A, Terme M, Pernot S, et al. Immunomodulatory activity of VEGF in cancer. *Int Rev Cell Mol Biol*. 2017;330:295–342.
10. Thomas AA, Fisher JL, Hampton TH, et al. Immune modulation associated with vascular endothelial growth factor (VEGF) blockade in patients with glioblastoma. *Cancer Immunol Immunother*. 2017;66: 379–389.
11. Voron T, Marcheteau E, Pernot S, et al. Control of the immune response by pro-angiogenic factors. *Front Oncol*. 2014;4:70.
12. Yoshida GJ. Metabolic reprogramming: the emerging concept and associated therapeutic strategies. *J Exp Clin Cancer Res*. 2015;34:111.
13. Cairns RA, Papandreou I, Sutphin PD, Denko NC. Metabolic targeting of hypoxia and HIF1 in solid tumors can enhance cytotoxic chemotherapy. *Proc Natl Acad Sci USA*. 2007;104:9445–9450.
14. Prakash H, Klug F, Nadella V, Mazumdar V, Schmitz-Winnenthal H, Umansky L. Low doses of gamma irradiation potentially modifies immunosuppressive tumor microenvironment by retuning tumor-associated macrophages: lesson from insulinoma. *Carcinogenesis*. 2016;37:301–313.
15. Klug F, Prakash H, Huber PE, et al. Low-dose irradiation programs macrophage differentiation to an iNOS(+)/M1 phenotype that orchestrates effective T cell immunotherapy. *Cancer Cell*. 2013;24: 589–602.
16. Hanahan D. Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature*. 1985;315:115–122.
17. Shevchenko I, Karakhanova S, Soltek S, et al. Low-dose gemcitabine depletes regulatory T cells and improves survival in the orthotopic Panc02 model of pancreatic cancer. *Int J Cancer*. 2013;133:98–107.
18. Guleng B, Tateishi K, Ohta M, et al. Blockade of the stromal cell-derived factor-1/CXCR4 axis attenuates in vivo tumor growth by

inhibiting angiogenesis in a vascular endothelial growth factor-independent manner. *Cancer Res*. 2005;65:5864–5871.

19. Giustarini D, Rossi R, Milzani A, Dalle-Donne I. Nitrite and nitrate measurement by Griess reagent in human plasma: evaluation of interferences and standardization. *Methods Enzymol*. 2008;440:361–380.
20. Prakash H, Nadella V, Singh S, Schmitz-Winnenthal H. CD14/TLR4 priming potentially recalibrates and exerts anti-tumor efficacy in tumor associated macrophages in a mouse model of pancreatic carcinoma. *Sci Rep*. 2016;6:31490.
21. Matak P, Heinis M, Mathieu JR, et al. Myeloid HIF-1 is protective in *Helicobacter pylori*-mediated gastritis. *J Immunol*. 2015;194: 3259–3266.
22. Bhandari T, Nizet V. Hypoxia-inducible factor (HIF) as a pharmacological target for prevention and treatment of infectious diseases. *Infect Dis Ther*. 2014;3:159–174.
23. Werno C, Menrad H, Weigert A, et al. Knockout of HIF-1alpha in tumor-associated macrophages enhances M2 polarization and attenuates their pro-angiogenic responses. *Carcinogenesis*. 2010;31: 1863–1872.
24. Doedens AL, Stockmann C, Rubinstein MP, et al. Macrophage expression of hypoxia-inducible factor-1 alpha suppresses T-cell function and promotes tumor progression. *Cancer Res*. 2010;70: 7465–7475.
25. Piret JP, Mottet D, Raes M, Michiels C. CoCl2, a chemical inducer of hypoxia-inducible factor-1, and hypoxia reduce apoptotic cell death in hepatoma cell line HepG2. *Ann N Y Acad Sci*. 2002;973:443–447.
26. Ardyanto TD, Osaki M, Tokuyasu N, Nagahama Y, Ito H. CoCl2-induced HIF-1alpha expression correlates with proliferation and apoptosis in MKN-1 cells: a possible role for the PI3K/Akt pathway. *Int J Oncol*. 2006;29:549–555.
27. Matak P, Heinis M, Mathieu JR, et al. Myeloid HIF-1 is protective in *Helicobacter pylori*-mediated gastritis. *J Immunol*. 2015;194: 3259–3266.
28. Kempf VA, Lebiedziejewski M, Alitalo K, et al. Activation of hypoxia-inducible factor-1 in bacillary angiomatosis: evidence for a role of hypoxia-inducible factor-1 in bacterial infections. *Circulation*. 2005; 111:1054–1062.
29. Guo X, Xue H, Shao Q, et al. Hypoxia promotes glioma-associated macrophage infiltration via periostin and subsequent M2 polarization by upregulating TGF-beta and M-CSFR. *Oncotarget*. 2016;7: 80521–80542.
30. Chai CY, Chen WT, Hung WC, et al. Hypoxia-inducible factor-1alpha expression correlates with focal macrophage infiltration, angiogenesis and unfavourable prognosis in urothelial carcinoma. *J Clin Pathol*. 2008;61:658–664.
31. Li N, Li Y, Li Z, et al. Hypoxia inducible factor 1 (HIF-1) recruits macrophage to activate pancreatic stellate cells in pancreatic ductal adenocarcinoma. *Int J Mol Sci*. 2016;17:779.
32. Joshi S, Singh AR, Zulcic M, Durden DL. A macrophage-dominant PI3K isoform controls hypoxia-induced HIF1alpha and HIF2alpha stability and tumor growth, angiogenesis, and metastasis. *Mol Cancer Res*. 2014;12:1520–1531.
33. Sluimer JC, Gasc JM, van Wanroij JL, et al. Hypoxia, hypoxia-inducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis. *J Am Coll Cardiol*. 2008;51:1258–1265.
34. Dehne N, Brune B. HIF-1 in the inflammatory microenvironment. *Exp Cell Res*. 2009;315:1791–1797.
35. Takeda N, O'Dea EL, Doedens A, et al. Differential activation and antagonistic function of HIF-{alpha} isoforms in macrophages are essential for NO homeostasis. *Genes Dev*. 2010;24:491–501.
36. Yang KS, Lim JH, Kim TW, et al. Vascular endothelial growth factor-receptor 1 inhibition aggravates diabetic nephropathy through eNOS signaling pathway in db/db mice. *PLoS ONE*. 2014;9:e94540.
37. Jin ZG, Ueba H, Tanimoto T, Lungu AO, Frame MD, Berk BC. Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. *Circ Res*. 2003;93:354–363.

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