



Neem Leaf Glycoprotein Restrains VEGF Production by Direct Modulation of HIF1 α -Linked Upstream and Downstream Cascades

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Neem Leaf Glycoprotein (NLGP) is a natural immunomodulator, have shown sustained tumor growth restriction as well as angiogenic normalization chiefly by activating CD8⁺ T cells. Here, we have investigated the direct role of NLGP as a regulator of tumor microenvironmental hypoxia and associated vascular endothelial growth factor (VEGF) production. We observed a significant reduction in VEGF level in both *in vivo* murine tumor and *in vitro* cancer cells (B16Mel, LLC) and macrophages after NLGP treatment. Interestingly, NLGP mediated VEGF downregulation in tumor cells or macrophages within hypoxic chamber was found at an early 4 h and again at late 24 h in mRNA level. Our data suggested that NLGP prevented hypoxia-induced strong binding of HIF1 α with its co-factors, CBP/p300 and Sp3, but not with Sp1, which eventually limit the binding of HIF1 α -transcriptional complex to hypoxia responsive element of VEGF promoter and results in restricted early VEGF transcription. On the otherhand, suppressed phosphorylation of Stat3 by NLGP results reduction of HIF1 α at 24 h of hypoxia that further support sustained VEGF down-regulation. However, NLGP fails to regulate VHL activity as observed by both *in vivo* and *in vitro* studies. Therefore, this study for the first time reveals a mechanistic insight of NLGP mediated inhibition of angiogenesis by suppressing VEGF, which might help in vascular normalization to influence better drug delivery.

Keywords: hypoxia, VEGF, HIF1 α , STAT3, tumor-microenvironment, NLGP

SUMMARY

NLGP downregulates VEGF at 4 h transcriptionally and both VEGF and HIF1 α at 18 h translationally. Co-factors of HIF1 α -transcriptional-complex downregulated at 4 h in hypoxia by NLGP. Downregulation of pStat3 by NLGP downregulates HIF1 α resulting in a sustained VEGF reduction in hypoxia.

INTRODUCTION

Malignant solid tumor progression needs aberrant angiogenic stimuli for rapid neovascularization to maintain nutrient and oxygen supply to proceed beyond 2 mm³ (1, 2). Among various pro-angiogenic factors, vascular endothelial growth factor (VEGF) is the most influential factor responsible for promoting tumor angiogenesis despite the nature and origin of tumor. Thus, VEGF targeting has efficacy in several cancer management (3, 4). Rapid tumor cell proliferation creates hypoxia or oxygen deficit (5) and stabilizes HIF1 α , the main VEGF regulator along with growth factors and oncogenes (6, 7). Within the tumor microenvironment (TME), tumor cells themselves along with non-tumor stromal cells serves as the source of VEGF (8).

HIFs are basic helix-loop-helix transcription factors belonging to the PAS (PER/aryl hydrocarbon receptor nuclear translocator) family. It has two subunits, an oxygen-sensitive α -subunit and a constitutively expressed β -subunit (9). In normoxia, α -subunit undergoes post-transcriptional modifications with prolyl-hydroxylase (PHD) and von-Hippel-Lindau (VHL) leading to proteosomal degradation via E3 ubiquitin ligase (10). However, in hypoxia, HIF1 α becomes stable and translocate to the nucleus where it forms a heterodimer with HIF1 β and many other co-factors to activate more than 124 significant genes (11). In addition to hypoxia, several other factors are potent stimulator of either HIF1 α or VEGF or both. Growth factors, cytokines etc. by increase HIF1 α protein synthesis via activation of PI3K/AKT (12) or ERK/MAPK pathways (13) or STAT3 signaling pathway (14, 15). Interestingly, recent work suggested a co-operative role of STAT3 and HIF1 α in VEGF upregulation (16). VEGF expression can also be induced by HIF1 α independent manner by STAT3, AP1, Sp1, and cAMP etc. (16, 17).

Targeting VEGF by many agents, including anti-VEGF monoclonal antibodies, have been developed showing promising effect *in vitro*, but their effects *in vivo* settings or in cancer patients are limited due several adverse effects, such as hypertension, gastrointestinal-perforation, bleeding, impairment of wound healing etc. (18). On the other hand, several plant based natural molecules or anti-oxidants show promises in reducing VEGF but their mechanisms are largely unknown. Neem leaf glycoprotein (NLGP), a non-toxic immune-modulator, show sustained tumor growth restriction in multiple murine cancer settings primarily by activating CD8⁺ cytotoxic T cells (19, 20). We also reported normalization of aberrant angiogenesis in murine carcinoma and melanoma hosts in an immune dependent manner (21). Therefore, this is of immense interest to study whether and how NLGP restricts VEGF synthesis and secretion from tumor resident cells.

Herein, we show that NLGP primarily targets VEGF synthesis by disrupting the binding of HIF1 α with its co-factors, which ultimately prevents binding of HIF1 α - transcriptional complex to the HRE region of VEGF. Additionally, NLGP prevents Stat3 activation and STAT3-dependent HIF1 α transcription. Both of these events simultaneously mitigate VEGF secretion from tumor and non-tumor stromal cells.

MATERIALS AND METHODS

Antibodies and Reagents

DMEM-high glucose medium and Fetal bovine serum (FBS) were obtained from Invitrogen (NY, USA). Purified anti-mouse antibodies (VEGF, HIF1 α , Sp1, Sp3, p300, CBP, pAKT, pERK, STAT3, pSTAT3) and Stat3 siRNA were procured from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-mouse/rabbit fluorescence conjugated secondary antibodies (FITC and PE conjugate) were purchased from Sigma Aldrich (St. Louis, US). RT-PCR primers were designed and procured from Eurofins, Bangalore, India. Trizol reagent for RNA isolation and Revert AidTM cDNA synthesis kit were procured from Invitrogen (Carlsbad, CA, USA) and Fermentas (Waltham, MA, USA), respectively.

Maintenance of Cell Lines

B16F10 murine melanoma cells (B16Mel) were purchased from the National Center for Cell Sciences (NCCS), Pune, India. Lewis Lung Carcinoma (LL/2 (LLC1) were purchased directly from American Type Cell Culture (ATCC[®] CRL1642TM, Manassas, VA, USA). Macrophages were collected from peritoneal cavity of C57BL/6J mice and tumor conditioned using B16Mel tumor lysate. Cells were maintained at 70% confluency in complete DMEM high glucose media supplemented with 10% (v/v) heat inactivated FBS, 2mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C with the supply of 5% CO₂. Authentication is done using STR method in the cell banks. All cells were maintained for 10 to 12 passages and all handling procedure was done according to guidelines provided by ATCC. B16Mel cells were tested for mycoplasma contamination using mycoplasma detection kit (EZdetectTM PCR Kit for Mycoplasma Detection; based on 16s-23s rRNA spacer region, Himedia, India). All experiments were done within 6 months of purchase.

Mice and Tumor Inoculation

Inbred female C57BL/6J mice (age, 4–6 weeks, average body weight 21 g) were obtained and maintained as described (21). All experiments were performed in accordance with the guidelines provided by the Institutional Animal Care and Ethics Committee (Approval No. IAEC-1774/RB-7/2016/3).

Neem Leaf Glycoprotein

Neem leaf glycoprotein (NLGP) was prepared from neem leaves (*Azadirachta indica*), by the method as described previously (21). A standard protocol was followed as described (22).

Generation of Hypoxic Environment *in vitro*

B16Mel cells were kept in a hypoxia chamber (Stem Cell Technologies, Canada) to mimic artificial tumor hypoxic environment. The later was generated by passing a hypoxia gas mixture of 5% O₂, 85% N₂, and 10% CO₂, at 20psi pressure for 4 min. According to manufacturer's protocol, this time is required to completely replace the atmospheric gas inside the chamber with the desired gas mixture.

Histology and Immunohistochemistry

Tumors were fixed to stain with VEGF according to method described (19). In some cases, VEGF positive regions were selected and dissected using laser capture microscopy.

Cytokine Detection Assay

Cytokines secreted from B16Mel cells within culture supernatant both in hypoxia and normoxia w/wo NLGP, were measured by ELISA as described (19).

Co-localization Studies

B16Mel cells were grown in chamber slides in hypoxia, w/wo NLGP, fixed with paraformaldehyde and permeabilized in 0.1% Triton X-100. After methodical washing with PBS-Tween 20 and PBB (0.5% BSA + PBS) blocking was done using 2% BSA. Primary antibodies for Sp1, Sp3, CBP, p300 and HIF1 α were added in dilution range 1:200 to 1:500 and incubated in a moist chamber, overnight. Secondary antibody was added and incubated for 1 h. Slides were finally mounted with DAPI and images were acquired using Leica DM 1000, Fluorescent Microscope (Leica, BM 4000B, Germany).

Nuclear and Cytosolic Extraction

B16Mel cells were scraped using chilled PBS, centrifuged and cell pellet was re-suspended in ice-cold EMSA buffer to incubate for 1 h at 4°C. Nuclear and cytosolic fractions were carried out according to the protocol described (19).

RT-PCR

Cellular RNA was isolated using Trizol (Invitrogen, Camarillo, CA) and random hexamers were used to generate corresponding cDNA (First Strand cDNA Synthesis Kit; Fermentas, Hanover, MD). Amplification was done according to protocol described (22).

Co-immunoprecipitation and Western Blot

B16Mel cells were subjected to hypoxia w/wo NLGP for 4 h, and Co-IP of Sp1/3, CBP, p300 with HIF1 α was performed as described (23).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were conducted following the manufacturer's protocol (Millipore, Darmstadt, Germany). B16Mel cells (1×10^6) were subjected to normoxia and hypoxia w/wo NLGP as described (23) was followed. DNA was extracted using phenol/chloroform to conduct PCR using promoter specific primers: HRE region in the promoter of VEGF (HIF1 α binding site): sense 5'-CCACAGTGCATACGTGGGCTC-3', antisense 5'-GGTGTACAGTATGCACCCGAG-3'.

si-RNA Mediated STAT3 Silencing

STAT3 specific si-RNA (Santacruz Biotechnology, Dallas, TX) was added in 70% confluent B16Mel culture as described (23). Finally, expression of *stat3* and *hif1 α* was checked both in untreated, NLGP treated and siRNA transfected B16Mel cells by RT-PCR.

Flow-Cytometric Staining

Flow-cytometry of pAKT, pERK and pSTAT3 was done as described (24).

Statistical Analysis

All reported results represent the mean \pm SD of data obtained in either six (for *in vivo* analysis) or three to six (*in vitro* assays) independent experiments. Statistical significance was established by unpaired *t*-test using INSTAT 3 Software (Graphpad Inc., USA), with differences between groups attaining a *p*-value < 0.01 considered as significant.

RESULTS

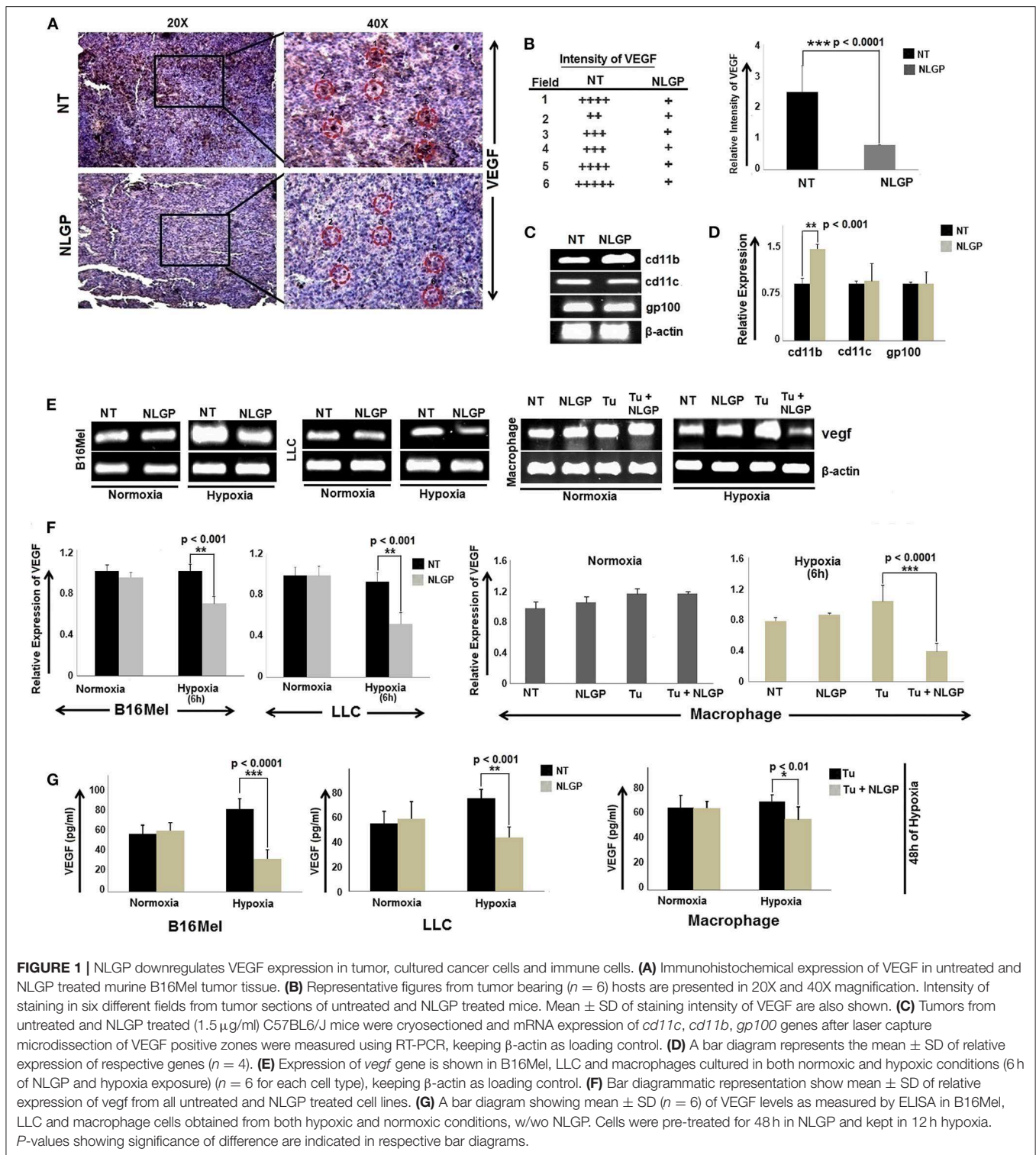
NLGP Mediated Tumor Growth Restriction Is Associated With Downregulation of VEGF in Immune and Tumor Cells

In continuation to our previous research in understanding the mechanism of NLGP mediated tumor growth restriction, we have reported that therapeutic NLGP treatment significantly reduces the availability of VEGF in tumor hosts, in an immune dependent (CD8⁺ T cells and IFN γ) manner (21). To analyze the detailed molecular mechanism behind NLGP mediated modulation of VEGF, here, first we studied the cellular source of VEGF, those are targeted by NLGP. Laser-capture-microdissection of tumor tissues followed by RT-PCR analysis suggested that *in vivo* NLGP treatment showed no change in melanoma (gp100) and dendritic cell (DC) (CD11c) marker, but an increase in macrophage marker (CD11b) (**Figure 1C**). Thus, observed reduction in VEGF as shown in **Figure 1A** might be due to NLGP's inhibition on VEGF secretion from B16Mel and macrophage cells (**Figures 1A–D**). This observation is suggestive that VEGF reduction is not due to direct killing of the tumor cells but rather NLGP could modulate tumor cells to restrict VEGF production.

Based on this *in vivo* result, next we studied VEGF expression of B16Mel and LLC cells and in macrophages under normoxia and hypoxia with NLGP treatment. RT-PCR and ELISA suggested a significant decrease in *vegf* gene as well as protein expression after 6 h and 48 h of NLGP treatment, respectively, in all cells studied. The extent of VEGF reduction was prominent under hypoxic condition with no change in normoxia (**Figures 1E–G**).

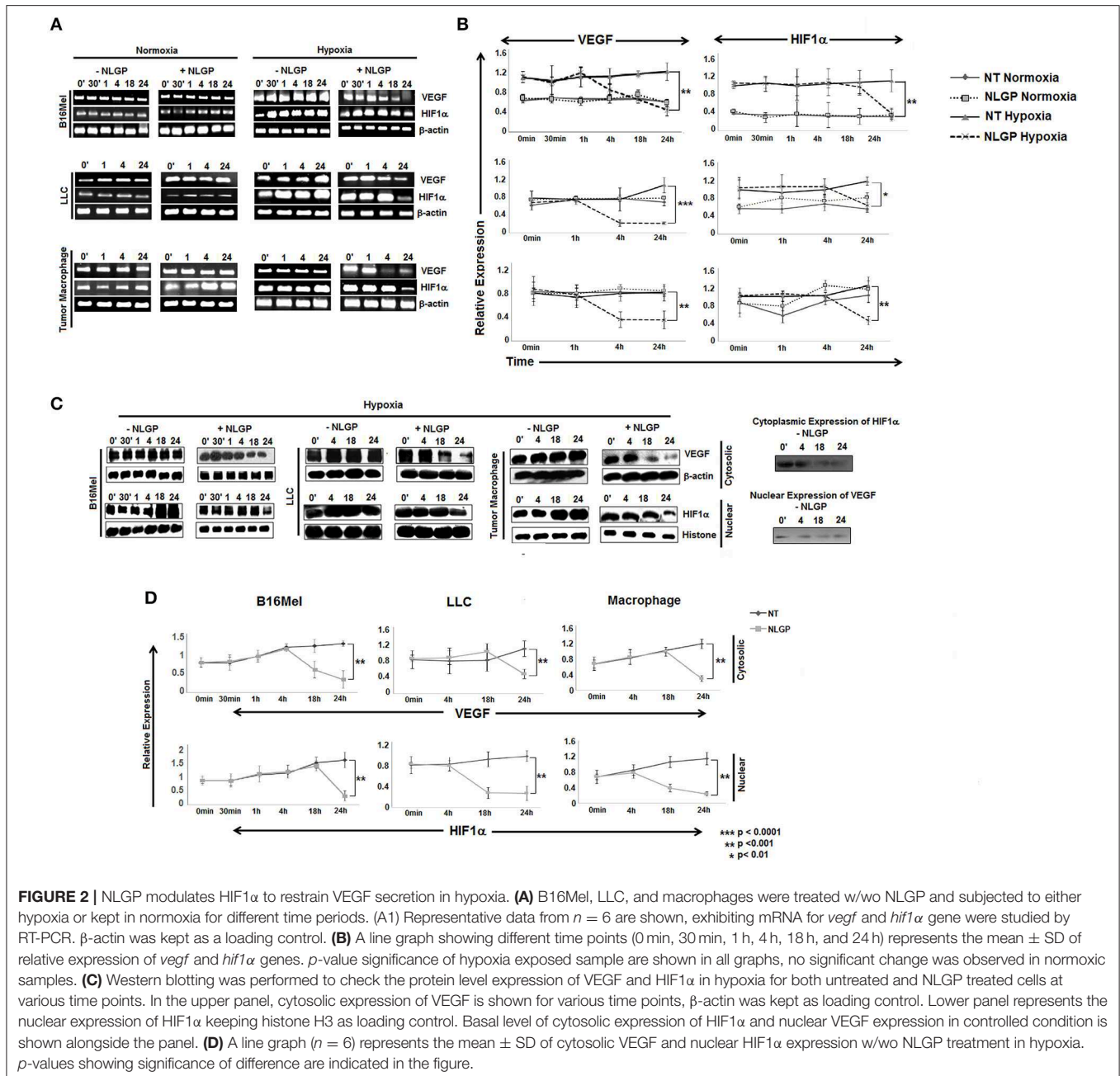
NLGP Modulates HIF1 α in Tumor Cells to Restrain VEGF Production

Given the direct effect of NLGP in reduction of VEGF in hypoxic condition, next we checked the involvement of HIF1 α , the main regulator of VEGF and also the exact time point at which VEGF is downregulated. B16Mel, LLC and macrophage cells were exposed to normoxic and hypoxic conditions for various time points (0 min, 30 min, 1 h, 4 h, 18 h, and 24 h) in presence or absence of NLGP (1.5 μ g/ml). RT-PCR analysis suggested downregulation of *vegf* started at 4 h and reached a maximum decrease at 24 h (**Figures 2A,B**), whereas downregulation of *hif1 α* was observed only at 24 h. Western blot analysis suggested a downregulation of total VEGF started at 18 h of NLGP treatment under hypoxia



(Figures 2C,D; Supplementary Material). As hypoxia stabilizes HIF1 α protein and promotes its translocation to nucleus from cytoplasm, next we studied its expression in both cases. Although cytosolic HIF1 α expression remains low in all the conditions but hypoxia increases HIF1 α expression in nucleus of B16Mel

cells, while, NLGP treatment reduced stable HIF1 α expression at 24 h (Figures 2C,D). Therefore, collectively these data suggest that NLGP does not affect HIF1 α expression in either protein or mRNA level at 4 h to control their VEGF production in tumor cells.



NLGP Neither Affects Hypoxia-Induced HIF1 α Stabilization nor O₂/VHL-Dependent HIF1 α Degradation

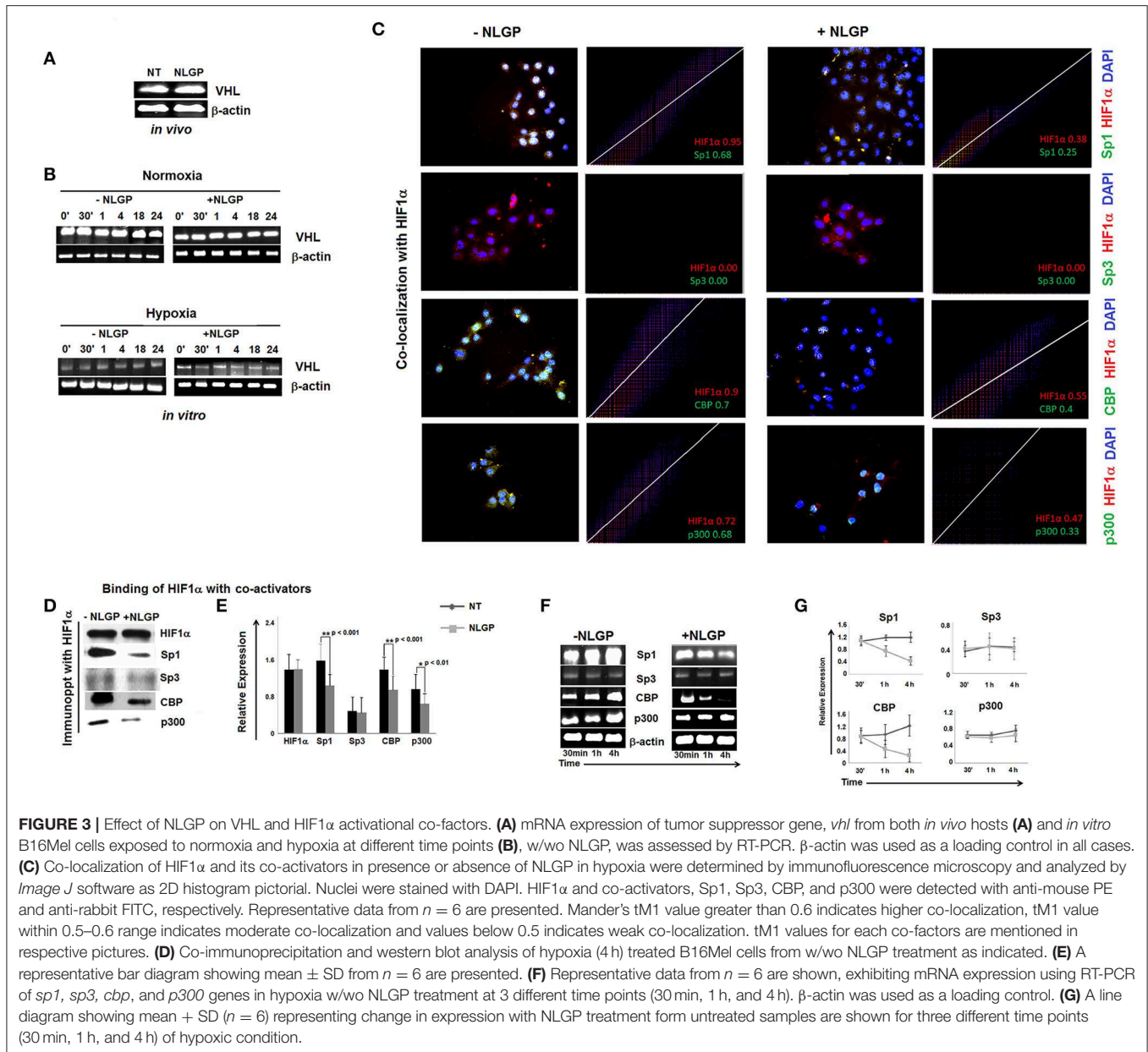
NLGP mediated downregulation of VEGF gene transcription was achieved after 4 h. However, NLGP is unable to reduce stable HIF1 α expression in B16Mel cells at this time point, which was observed only at 24 h in both transcriptional and translational levels. This result clearly pointed out that NLGP treatment does not affect the HIF1 α stabilization in hypoxic condition.

Next, we look at VHL expression, an important tumor suppressor gene. In presence of O₂, VHL ubiquitinates HIF1 α for its subsequent proteosomal degradation. However, neither

in vivo (Figure 3A) nor *in vitro* (Figure 3B) NLGP treatment shows any effect in VHL expression in tumor tissues or in B16Mel cells, respectively, and suggested that NLGP mediated VEGF downregulation is not associated with VHL-dependent HIF1 α degradation (Figures 3A,B).

NLGP Targets the Binding of HIF1 α to Its Co-activator and Prevents Formation of Active HIF1 α Transcriptional Complex

HIF1 α binds with HIF1 β and other co-activators (like, Sp1, CBP/p300, etc.) to form active transcriptional complex after translocating to the nucleus and bind with hypoxia responsive

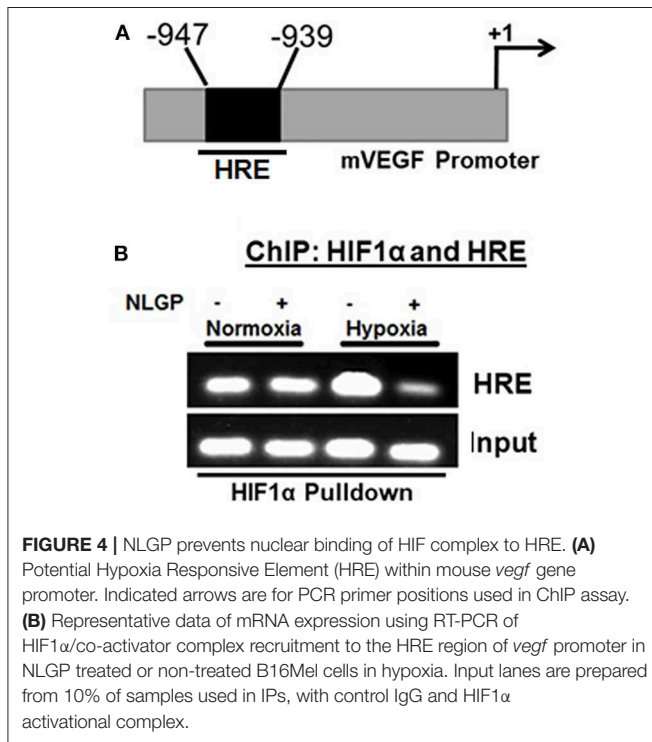


elements (HRE) of VEGF promoter (25). As NLGP treatment does not show any effect on HIF1 α stabilization, we assessed the binding of HIF1 α with its co-activator(s). Accordingly, B16Mel cells were exposed to hypoxia w/o NLGP (1.5 μ g/ml) for 4 h and co-localization and co-immunoprecipitation assays were performed. Co-localization assay of B16Mel cells grown-on chamber slide in hypoxic environment demonstrated that NLGP significantly prevents co-localization of HIF1 α with Sp1, CBP, and p300. Analysis of co-localization by *Image J* software of HIF1 α with Sp1, CBP, and p300 shows higher Mander's tM1 value for all hypoxia treated cohorts (>0.6) compared to NLGP-treated cohorts (<0.5) (Figure 3C). Co-immunoprecipitation assay also supported that NLGP treatment is able to reduce protein-protein interaction of HIF1 α with its co-activators (Figures 3D,E; Supplementary Material),

whereas the binding between HIF1 α and Sp3 (a competitive inhibitor of Sp1) showed no change in NLGP treated B16Mel cells (Figures 3C,D). Interestingly, NLGP treatment also downregulates transcriptional expression of both Sp1, CBP and p300 in B16Mel cells under hypoxic condition after 4 h (Figures 3E,G). Therefore, the overall data suggest that NLGP is able to prevent the formation of active HIF1 α transcriptional complex required for VEGF transcription.

NLGP Prevents Binding of HIF1 α Transcriptional Complexes to VEGF Promoter in Hypoxia

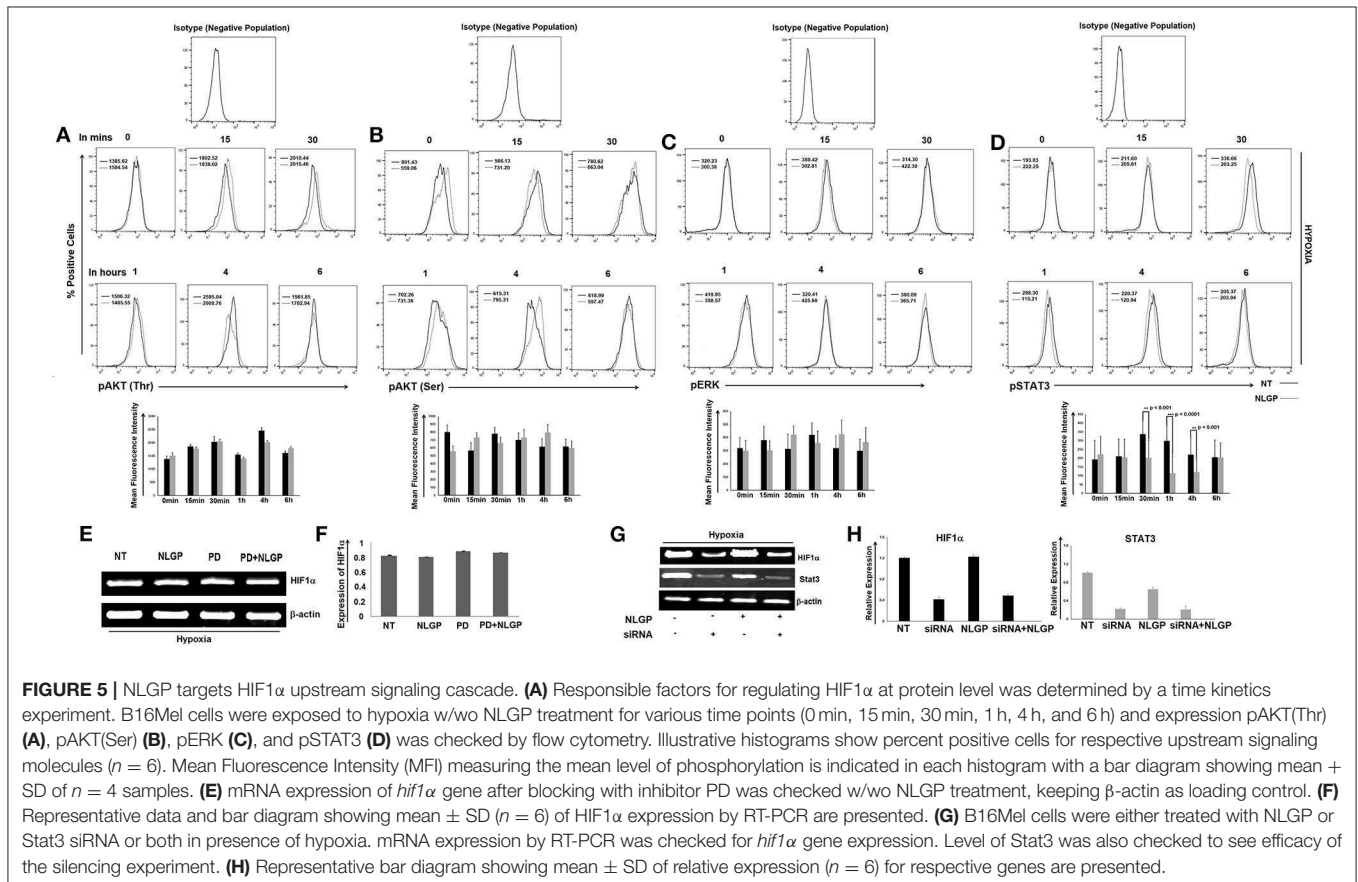
As NLGP prevents the nuclear translocation of HIF1 α and the formation of active HIF1 α complex, next we assessed its



binding with cognate HRE sequence at VEGF promoter using chromatin immune-precipitation (ChIP) assay. Results obtained from ChIP assay suggested that hypoxia remarkably promoted the binding of HIF1 α with VEGF promoter in B16Mel cells whereas, NLGP treatment caused a significant reduction in binding of HIF1 α with VEGF promoter in hypoxic condition (Figures 4A,B).

NLGP Targets Stat3-Dependent Transcription of HIF1 α to Reduce VEGF

Although NLGP treatment downregulates VEGF primarily by targeting HIF1 α binding with VEGF promoter, it also causes a significant downregulation of HIF1 α in transcription level at 24 h. Several transcription factors or oncogenic stimuli are reported to have HIF1 α promoting activity like Stat3, NF- κ B, AKT, ERK etc. To study the target molecule(s) in our settings, we have checked the activation status of different HIF1 α -linked up-stream transcription factors in NLGP-treated B16Mel cells w/o hypoxia by flow-cytometry and western blot analysis. Both the assays suggested that after 30 min of NLGP treatment there was a significant downregulation of pStat3 expression, which further showed a decrease at 1 h and maintained a reduced pStat3 expression till 4 h in hypoxia-exposed tumor cells (Figures 5A–D). However, other factors like AKT, ERK show no significant changes after NLGP treatment



(Figures 5A–C), even after blocking them (Figures 5E,F). But, inhibition of Stat3 by Stat3-specific siRNA treatment significantly abrogated this NLGP mediated downregulation of HIF1 α after 24 h (Figures 5G,H).

DISCUSSIONS

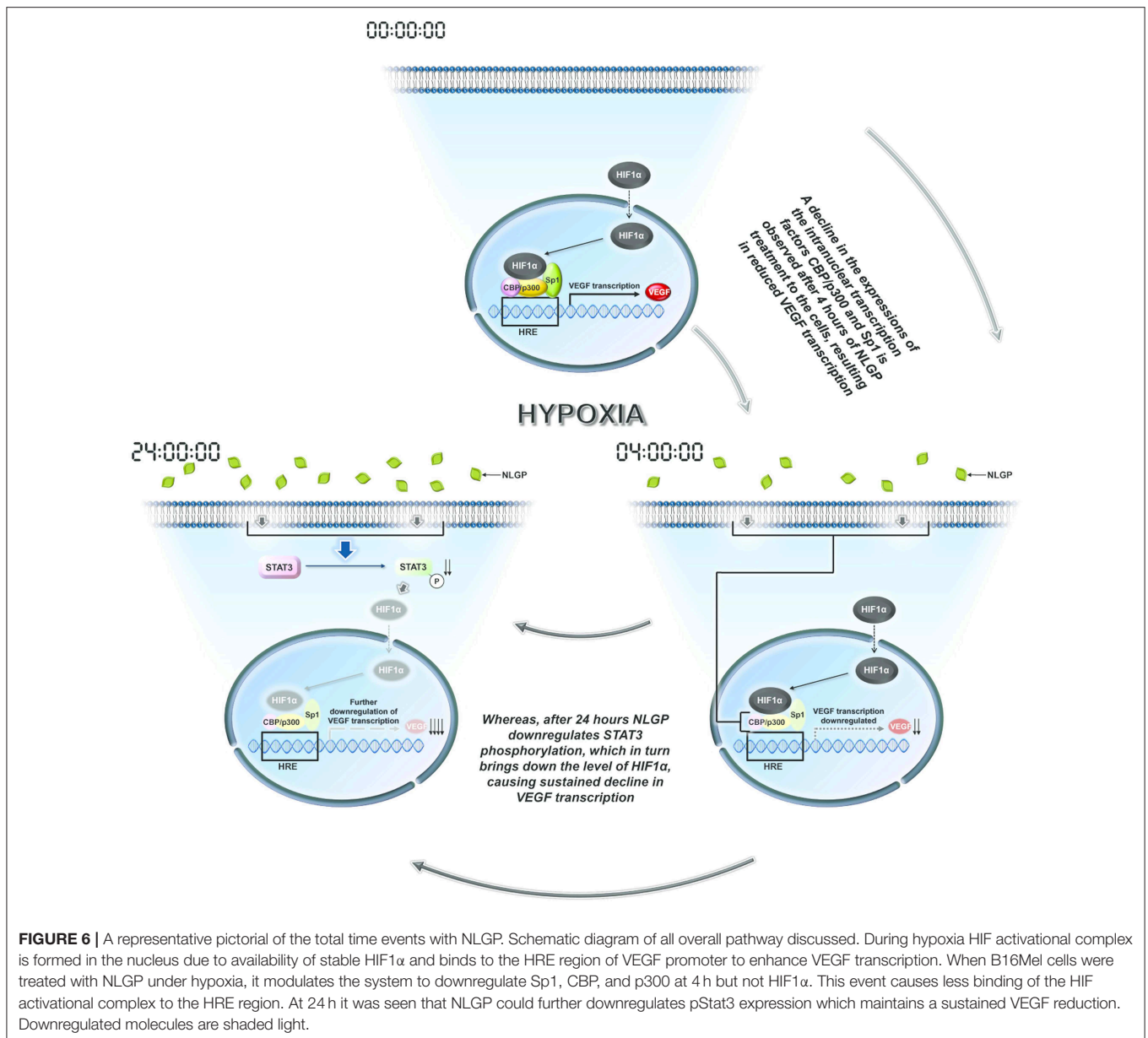
Angiogenesis plays a critical role in tumor growth by inducing VEGF secretion, which ultimately creates hypoxia and fosters tumor-promoting events and metastasis (26). Therefore, angiogenesis inhibitors specifically VEGF antagonist gained special interest in cancer management. However, these synthetic drugs generate toxicity as well as resistance. While non-toxic plant-based molecules seem more promising, detailed molecular mechanisms of action of plant based anti-angiogenic molecules are largely unknown. We reported a non-toxic immunomodulator, NLGP, having sustained effect on tumor growth restriction (27–30) and vascular normalization (21). These are primarily mediated by (i) activation of cytotoxic CD8⁺ T cells (20, 27, 29) and their interaction with CD4⁺ helper T cells (31), (ii) downregulation of immunosuppressive cytokines (IL-6, IL-10, TGF β , etc.) (20, 24, 28, 32), (iii) decrease in suppressor cell (Treg, TAM, DC2, MDSC) accumulation and their functions (32–34), (iv) generation of central and effector memory T cells (19). However, this molecule failed to induce any direct cytotoxic effect to the tumor cells (35). Here, for the first time we have shown that NLGP can directly modulate tumor cell property, which might switch the hostile TME.

A substantial reduction in uncontrolled angiogenesis is demonstrated recently after NLGP treatment in tumor bearing mice by downregulating intratumoral VEGF (21). Accordingly, the present work is initiated to understand how NLGP reduces VEGF within TME where tumor and tumor associated stromal cells including immune cells are major VEGF sources (36) and therefore could be potential targets of NLGP. To better understand the cellular target of NLGP, we looked inside a tumor tissue architecture and laser capture microdissection aided that NLGP can reduce VEGF secretion by modulating both macrophages and tumor cells. As reported earlier, NLGP significantly reduces of M2-type macrophages (24) and suppressor MDSCs (34). NLGP cannot directly kill macrophages or tumor cells thus it could modify the VEGF promoting factors like hypoxia (21). To understand the cellular and molecular mechanisms more precisely, we opt for *in vitro* culture of B16Mel, LLC, and macrophage cells, where we observed significant reduction of VEGF in tumor cells and macrophages after NLGP treatment as early as 4 h at mRNA level with more prominent result in hypoxia than normoxia, suggesting hypoxia as a major factor that NLGP targets. HIF1 α a major angiogenesis inducer in tumor cells (carcinoma and melanoma) both *in vitro* and *in vivo*. Hence, those conditions known to activate HIF1 α can upregulate VEGF production by recruiting other cofactors forming HIF activation complex (37). Therefore, possible mechanisms by which NLGP inhibits VEGF gene expression could include (i) Promotion of degradation of HIF1 α protein, (ii) Reduction of HIF1 α protein synthesis, (iii) Prevention of nuclear translocation of HIF1 α , (iv) Hindrance in active HIF1 α complex formation

by preventing binding between HIF1 α and partner(s), (v) Direct block in binding of HIF1 α complex to HRE region of VEGF promoter. As we carried out our experiments in hypoxia, which stabilizes HIF1 α , we did not find any changes in expression of HIF1 α mRNA and protein up to 24 h with NLGP treatment, so the first possibility is obsolete. Furthermore, degradation of HIF1 α protein requires obligatory presence of VHL (38), NLGP has no effect *in vivo* or *in vitro* over VHL expression. The second possibility at least for early (at 4 h) is ruled out since NLGP can only reduce HIF1 α protein expression after 18 h. NLGP treatment failed to reduce the accumulation of nuclear HIF1 α and can downregulate the same only after 24 h, excluding the third possibility for early VEGF downregulation. Next, we look further into the binding capacity of HIF1 α with other cofactors, like Sp1, CBP, p300, and we found that NLGP prevents co-localization of HIF1 α with CBP, p300 and Sp1 but not with Sp3, which can competitively target HIF1 α and Sp1 binding. Sp1 and Sp3 compete for binding to the HRE region as they share more than 90% sequence homology (39–41). Consequently, we observed significant less binding of active HIF1 α complex with HRE region. Moreover, this effect is more global as NLGP treatment shows downregulated expression of other HIF1 α target genes (PDK1 and EPO) (data not shown).

Many plant-based molecules show inhibitory effects toward VEGF mainly by modulating HIF1 α protein synthesis or degradation (42–49). On the other hand, NLGP initially target and destabilizes HIF1 α -HIF1 β -Sp1-CBP-p300 active transcriptional complex formation at 4 h, while at late hours it downregulates HIF1 α protein synthesis. Several signaling pathways are responsible for downregulation of HIF1 α , like AKT, ERK, MAPK, STAT3, etc. The status of AKT and ERK phosphorylation in B16Mel cells did not show any NLGP induced changes in either normoxia or hypoxia. However, NLGP causes significant reduction in Stat3 phosphorylation under hypoxia. Consistent with our previous reports, we observed downregulation of pStat3 by NLGP and siRNA mediated knock-down of stat3, both of which mimics NLGP's effect. Recent studies suggest that HIF1 α is regulated by STAT3 as the later increases the half-life of the transcription factor in both human and mouse melanoma cells (50). STAT3 can directly bind to HIF1 α promoter and upregulates its expression post-translationally (51). A pictorial shown in **Figure 6** demonstrates how NLGP regulates VEGF in hypoxic TME at 4 and 24 h.

A significant reduction of HIF1 α -VEGF signaling axis within tumor by NLGP treatment has immense importance even from immunological perspective, where efficacy of NLGP is already proven. Moreover, effect of NLGP is dependent on CD8⁺ T cells and reduced hypoxia-and related factors may promote susceptibility of tumor cells toward T and NK cell mediated killing via induction of autophagy as well as more efficient functioning of several immune cells by downregulating CTLA4 on T cells and PDLs on tumor cells. However, here we are unable to explore how NLGP interact with macrophage or tumor cells to reduce stat3, which may eventually prevent the formation of active HIF1 α -transcriptional complex. This study adds new insight into the potential mechanism of NLGP's activity besides immunomodulation which may



intensify its prospective acceptance as a novel strategy for cancer management.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

All experiments were performed in accordance with the guidelines provided by the (Chittaranjan National Cancer Institute, Kolkata) Institutional Animal Care and Ethics Committee (Approval No. IAEC-1774/RB-7/2016/3).

AUTHOR CONTRIBUTIONS

AS, ABo, and RB: experimental design of the study, planning and execution, analysis of data, and manuscript preparation. AS, PN, and SD: performed majority research. ABh, NG, TG, IG, and SB: performed minor research work and helped in providing resources. ABo and RB: supervised the project and acquired funds. All authors approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fonc.2020.00260/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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