Activation of NLRP3 inflammasome complex potentiates venous thrombosis in response to hypoxia

Neha Gupta[a], Anita Sahu[a], Amit Prabhakar[a], Tathagata Chatterjee[b], Tarun Tyagi[a], Babita Kumari[a], Nilofar Khan[a], Velu Nair[c], Nitin Bajaj[d], Manish Sharma[a,1], and Mohammad Zahid Ashraf[a,1]

[a]Genomics Division, Defence Institute of Physiology and Allied Sciences, Defence Research and Development Organization, Timarpur, Delhi 110054, India; [b]Army Hospital (Research & Referral), New Delhi 110010, India; [c]Armed Forces Medical College, Pune 411040, India; and [d]Command Hospital Chandimandir, Chandigarh 134107, India

Edited by Gregg L. Semenza, Johns Hopkins University School of Medicine, Baltimore, MD, and approved March 24, 2017 (received for review December 14, 2016)

Venous thromboembolism (VTE), caused by altered hemostasis, remains the third most common cause of mortality among all cardiovascular conditions. In addition to established genetic and acquired risk factors, low-oxygen environments also predispose otherwise healthy individuals to VTE. Although disease etiology appears to entail perturbation of hemostasis pathways, the key molecular determinants during immediate early response remain elusive. Using an established model of venous thrombosis, we here show that systemic hypoxia accelerates thromboembolic events, functionally stimulated by the activation of nucleotide binding domain, leucine-rich-containing family, pyrin domain containing 3 (NLRP3) inflammasome complex and increased IL-1β secretion. Interestingly, we also show that the expression of NLRP3 is mediated by hypoxia-inducible factor 1-alpha (HIF-1α) during these conditions. The pharmacological inhibition of caspase-1, in vivo knockdown of NLRP3, or HIF-1α other than IL-1β-neutralizing antibodies attenuated inflammasome activation and curtailed thrombosis under hypoxic conditions. We extend the significance of these preclinical findings by studying modulation of this pathway in patients with altitude-induced venous thrombosis. Our results demonstrate distinctive, increased expression of NLRP3, caspase-1, and IL-1β in individuals with clinically established venous thrombosis. We therefore propose that an early proinflammatory state in the venous milieu, orchestrated by the HIF-induced NLRP3 inflammasome complex, is a key determinant of acute thrombotic events during hypoxic conditions.

thrombosis | hypoxia | HIF-1α | NLRP3 inflammasome | IL-1β

Epidemiological studies during recent years have unprecedentedly highlighted venous thromboembolism (VTE) as a key comorbidity factor during several life-threatening medical conditions. In addition to clinical complications such as cancer (1), cardiovascular diseases (2), surgery (3), and trauma (4), hypoxia as experienced during ascent to high altitude has emerged as another predisposing factor for VTE (5–8). A significantly higher incidence of deep vein thrombosis and pulmonary embolism (8, 9), portal vein thrombosis (10), cerebral venous thrombosis (11), transient ischemic attacks, and stroke (12) has been observed at high to extreme altitude (13). Despite clinical relevance, a caveat in our basic understanding of early molecular events underlying hypoxia-induced venous thrombosis poses a major bottleneck for effective design of interventional approaches.

Recent studies have highlighted a strong link between hypoxia responses and inflammation, involving activation of multiple cell types including lymphocytes, platelets, and endothelium (14, 15). Plausibly, in addition to direct mechanism involving hypoxia-induced modulation of hemostasis and coagulation factors (5, 16), pleiotropic modalities such as sterile inflammation could be involved. The role of this axis and key mediators in hypoxia-induced hypercoagulation, however, remains to be experimentally validated. The activation of coagulation pathways per se has been shown to promote inflammation in the system (17, 18). This secondary inflammation can engage in a positive feed-forward loop to aggravate the prothrombotic phenotype. Thus, the cause or consequence relationship between inflammation and hypercoagulation during hypoxia remains far from being resolved.

We recently showed that hypobaric hypoxia promotes a prothrombotic propensity through the involvement of a crucial cysteine protease, calpain (19). In continuing work, we blended an unbiased systems-level approach with targeted pharmacological inhibition and in vivo siRNA-mediated knockdown strategies to show that the activation of nucleotide binding domain, leucine-rich-containing family, pyrin domain containing 3 (NLRP3) inflammasome complex augments thrombus formation in response to hypoxia. We reinforced significance of this key preclinical finding from the animal model by demonstrating the activation of the NLRP3 gene in patients, who developed thrombosis at high altitudes. We also present evidence that the activation of this complex is an early response to hypoxia and is critically regulated by hypoxia-inducible factor 1-alpha (HIF-1α).

Results

For all experiments described here, we have used uniform group nomenclature. After inferior vena cava (IVC) ligation (also refer to *SI Materials and Methods*), the group of animals kept in normal atmosphere conditions was designated as thrombotic (T), whereas the ligated ones kept under simulated hypobaric hypoxia were identified as hypoxia thrombotic (HT). Sham surgery controls kept under normal environmental conditions were designated as normoxic (N), and those kept in simulated hypoxia were identified as hypoxic (H).

Significance

Hypoxia predisposes otherwise healthy individuals to venous thrombosis, but the underlying mechanism has been unclear. Our study revealed a causal role for nucleotide binding domain, leucine-rich-containing family, pyrin domain containing 3 (NLRP3) inflammasome and IL-1β during hypoxia-induced venous thrombosis. We further show a direct association between NLRP3 and hypoxia-inducible factor 1-alpha (HIF-1α) during these conditions. Specific interventions within the hypoxia–HIF-1α–NLRP3–IL-1β axis in the venous milieu significantly reduced venous thrombosis in our animal model. Notably, we also observed modulation of similar pathways in patients diagnosed with altitude-induced venous thrombosis. Our study thus revealed thrombosis at high altitude to be centrally regulated by a complex network of coagulatory and inflammatory processes, critically linked through HIF-1α.

Author contributions: M.S. and M.Z.A. designed research; N.G., A.S., A.P., T.C., T.T., B.K., and N.B. performed research; N.K. and V.N. contributed new reagents/analytic tools; N.G., A.S., A.P., M.S., and M.Z.A. analyzed data; and N.G., M.S., and M.Z.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence may be addressed. Email: mohammadzashraf@gmail.com or manishks77@gmail.com.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1620458114/-/DCSupplemental.

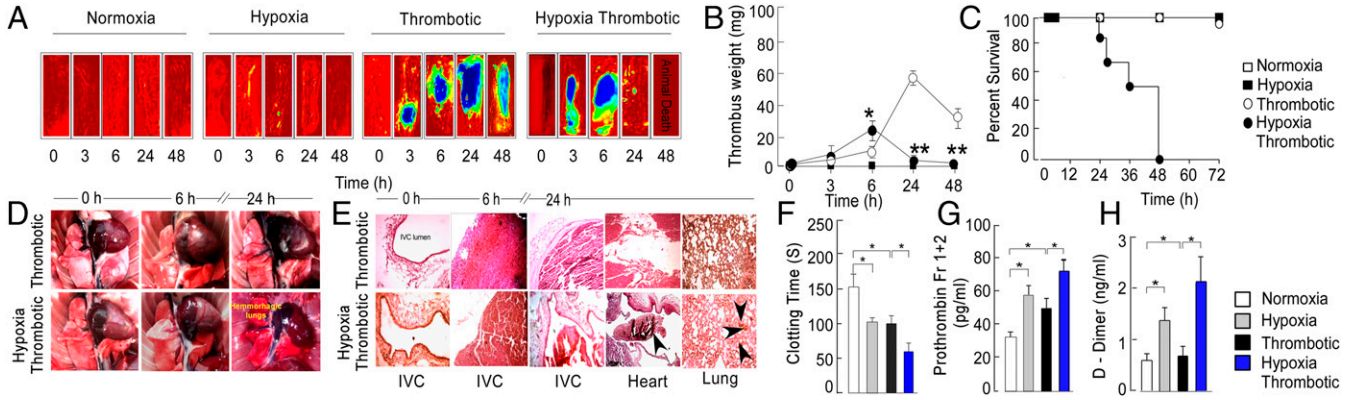

Fig. 1. Systemic hypoxia accelerates venous thromboembolism. (*A*) Representative heat maps representing IVC in situ. (*B*) Thrombus weight. (*C*) Percentage survival rate calculated using Log-rank (Mantel-Cox) test ($P < .001$). (*D*) Representative in situ images of gross lungs obtained from thrombotic ($n = 5$) and hypoxia thrombotic groups ($n = 7$). (*E*) Representative hematoxylin-eosin-stained cross-sectional images of IVC, lungs, and heart from indicated groups. Estimation of (*F*) clotting time, (*G*) secretory prothrombin fragment 1+2, and (*H*) D-dimer at the 6-h point. $*P < 0.05$ and $**P < 0.01$.

Hypoxic Exposure Aggravates Venous Thrombosis, Leading to Fatal Thromboembolic Consequences. We first established temporal kinetics of thromboembolic events in four groups of animals. In situ examination at various intervals (3, 6, 24, and 48 h) revealed significantly larger thrombus in the HT group (Fig. 1 *A* and *B*). Further, thrombus weight peaked earlier in the HT group (6 h). We observed a reduction in thrombus weight (Fig. 1*B*) with extended hypoxic exposure (≥24 h), along with a higher mortality rate in this group (Fig. 1 *C* and Fig. S1*A*). The thrombus from the HT group was denser compared with that for the T group at early times, suggesting exaggerated thrombogenesis with hypoxia exposure (Fig. S1 *A* and *B*). Further, the lumen of IVC in the HT group appeared to be completely obstructed after 6 h of hypoxic exposure with prominent recanalization compared with the T group (Fig. S1*A*), suggesting an early onset of thrombolytic process as well. Thrombotic animals in normoxia (T) showed progressive increase in thrombus formation (until 24 h), but negligible mortality (Fig. 1 *A*–*C* and Fig. S1*A* and *B*).

Necroptic examination in the HT group suggested mortality to be associated with hemorrhagic lungs (Fig. 1*D*), whereas no sign of bleeding or thrombus could be noticed in the lungs of animals from the NT group. Thromboembolus was found in the heart and lungs of animals in the HT group (24 h), with concomitant evidence for resolution from the IVC (Fig. 1 *D* and *E* and Fig. S1*A*), suggesting a shift of hemostatic equilibrium toward fibrinolytic pathway at this point. We therefore investigated both thrombotic and fibrinolytic axes in these animals.

We recorded parameters associated with prothrombotic conditions, including clotting time, prothrombin fragment 1+2 (corresponding to cleavage of prothrombin by factor Xa), and D-dimers in all groups. The clotting time was significantly shortened in the HT group (Fig. 1*F*). Further, a higher level of prothrombin fragment 1+2 (20) and D-dimer (Fig. 1 *G* and *H*) supported an accelerated thrombogenic activity in this group.

We also observed increased expression of free plasminogen activation inhibitor-1 (PAI-1) (Fig. S1*C*) in HT animals. The levels of fibrinolytic enzyme urokinase plasminogen activator (uPA) decreased, whereas tissue plasminogen activator (tPA) levels (Fig. S1 *D* and *E*) increased in thrombotic animals. Fibrinolytic molecules were also elevated in the H group, suggesting that hypoxia per se could modulate hemostasis pathways. These results suggested that systemic hypoxia exacerbated hypercoagulation, with compensatory hyperfibrinolysis that likely culminated in disseminated intravascular coagulation, leading to sudden animal death.

Evidence for Modulation of Hypoxia Response Pathways During Pathogenesis of Venous Thrombosis. Our observation that systemic hypoxia accelerated thrombotic cascade prompted us to investigate the functional involvement of hypoxia response pathways. As shown in Fig. 2 *A*–*C*, similar to nonligated animals exposed to hypoxia, the expression of HIF-1α and its target genes [vascular endothelial growth factor (*VEGF*) and erythropoietin (*EPO*)] was significantly increased in thrombotic groups (highest in HT group), supporting the possibility that the hypoxic milieu is an integral component involved during perturbation of hemostasis.

We studied the expression of 84 well-documented hypoxia-responsive genes, using real-time PCR analysis under specific conditions (Fig. S2*A*). We observed nearly 77% of these genes to be differentially expressed after 6 h of exposure to hypoxia (Fig. S2*B*); 76% of these genes were differentially regulated by IVC ligation alone (T), and 86% by IVC ligation followed by exposure to hypoxia (HT) (Fig. S2*B*). Further, the majority of genes up-regulated by hypoxia were present in the T dataset (Fig. S2*C*). The expression value of most of these genes was uniformly highest in the HT group, further suggesting exacerbation of responses by systemic hypoxia (Fig. S2 *D* and *E*).

Genome-Wide Expression Analysis Suggests Hierarchical Activation of Intense Inflammatory Response During Hypoxia-Induced Thrombus Formation. We next used RNA sequencing to obtain an unbiased cross-sectional snapshot of changes in gene expression in all groups. Nearly 606 unique transcripts (Entrez Gene) were significantly modulated by at least ±twofold in the H group (Dataset S1). The T and HT groups showed a much higher number of differentially expressed genes (4,966 and 4,664, Entrez Gene, respectively; Dataset S1). As evident from Fig. 3*A*, 487 hypoxia-specific genes (H) were also differentially expressed in thrombotic groups (T and HT). A significant number of differentially expressed genes were unique to T and HT groups (which did not appear in the H group), which was a clear indication that specific additional pathways were perturbed during thrombotic (T and HT) conditions (Fig. 3*A* and Fig. S3*A*).

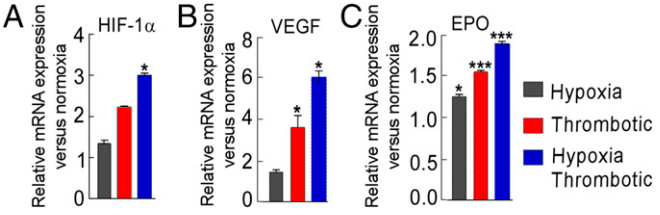

Fig. 2. Functional role of hypoxia response pathways during thrombosis. Relative expression of HIF-1α (*A*), VEGF (*B*), and EPO (*C*) at the 6-h point. 18S rRNA was used as internal control, and expression was normalized to that of the normoxic group (N). Mean ± SEM ($n = 6$) is shown. $*P < 0.05$; $***P < 0.001$.

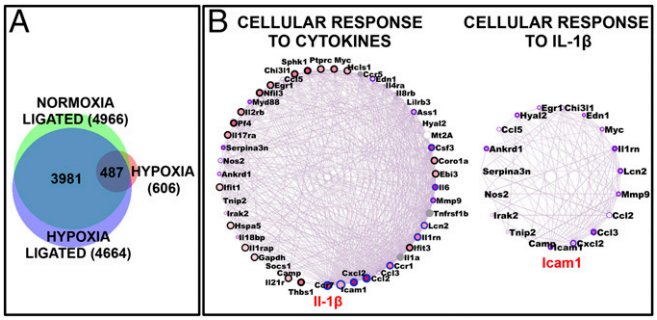

Fig. 3. Genome-wide expression and network analysis during thrombosis. Transcriptome sequencing was performed on RNA isolated from IVC of animals from all four groups, followed by data mining. (*A*) Intersection among differentially expressed genes, represented using Venn diagram. (*B*) Key networks (degree-sorted circle view) representative of inflammatory responses (significantly enriched from up-regulated genes in thrombotic group). Highest-degree nodes (IL-1β and ICAM 1) are indicated in red.

We extracted nonoverlapping biological processes, significantly modulated in the HT group, using GeneMania (21) (Fig. 3*B* and Fig. S3 *B* and *C*). The pathways enriched from the set of down-regulated genes principally indicated perturbation of metabolic processes characteristic of hypoxic stress (Fig. S3*C*). The processes enriched from up-regulated genes (Fig. S3*B*) suggested activation of angiogenesis, oxidative stress response, and calcium ion homeostasis in addition to other hypoxia response pathways. The dataset also revealed modulation of key processes regulating thrombotic propensity, including coagulation and platelet activation (Fig. S3*B*). We also observed processes representing strong and hierarchical activation of immune response in the HT group, as represented by networks related to early immune response such as cytokine signaling, activation of innate immune response, immunological synapse, and activation of adaptive immune system (involving T lymphocytes) (Fig. S3*B*). The subsequent analysis of these data yielded two lines of evidence suggesting that IL-1β could be the central regulator of such immune response in these animals. First, we observed a distinct network of genes regulating cellular responses to IL-1β (Fig. 3*B*). Second, multiple measures of centrality in immune response to specific subnetworks presented IL-1β as an important "bottleneck" gene, whose deletion led to collapse of the biological network. As can be readily observed from Fig. 3*B*, IL-1β is the most connected (highest degree) node or hub gene in the network related to cellular responses to cytokines.

Activation of NLRP3 Inflammasome in Response to Hypoxia-Induced Thrombosis. We next measured levels of IL-1β secreted in the localized IVC milieu after 6 h of hypoxia exposure and, in agreement with our transcriptome data, observed a significant increase in concentration of IL-1β in the T and HT groups (Fig. 4*A*). The concomitant activation of two signaling pathways is critical for the secretion of functionally active IL-1β: the first signal ensures transcriptional up-regulation of IL-1β, and the second leads to the assembly of a pro-IL-1β-processing inflammasome complex. Our transcriptome data (Fig. S4*A*) were distinctively evident for the activation of these arms in thrombotic groups. Real-time PCR analysis confirmed significant increase in the expression of IL-1β (Fig. 4*B*), caspase-1 (Fig. 4*C*), and NLRP3 (Fig. 4*D*) transcripts in the HT group. Further, caspase-1/ICE activity and NLRP3 expression were significantly elevated in the IVC milieu (tissue homogenate; Fig. 4 *E* and *F*) and plasma (Fig. S4 *B* and *C*) of animals under these conditions. Taken together, these data suggest an early activation of the NLRP3 inflammasome pathway in thrombotic animals. In addition to IL-1β, NLRP3 inflammasome also regulates IL-18 secretion. Consistent with this proposition, we also observed a significant increase in IL-18 levels (Fig. 4*G*) in IVC of animals in thrombotic groups (T and HT).

We observed increased recruitment of neutrophils (elastase-positive foci) and macrophages (mac-387–positive regions) in the IVC of animals from the HT group (Fig. S4*D*). The increase in myeloperoxidase activity and monocyte chemotactic protein-1 levels (Fig. S4 *E* and *F*) further supported recruitment of neutrophils and macrophages during thrombosis.

The secretion of IL-1β and activation of IL-1 receptor signaling initiates a cascade of events leading to modulation of expression of numerous other cytokines. We therefore investigated changes in the levels of cytokines, using cytokine-specific antibody arrays. We observed an increase in the secretion of proinflammatory cytokines in thrombotic animals (Fig. 4*H* and Fig. S4*G*). Taken together, we concluded that hypoxic signal culminates in the activation of NLRP3 inflammasome and active IL-1 signaling that eventually results in a prothrombotic state.

Hypoxia Response Pathways Functionally Regulate Expression of NLRP3 and IL-1β During Thrombosis. To investigate the relationship between hypoxia and inflammasome activation, we first used a pharmacological inhibitor of hypoxia response pathway, CAY10585, which inhibits accumulation and transcriptional activity of HIF-1α and thus, decreases the expression of HIF-1α target genes (22). As shown in Fig. S5*A*, CAY10585 treatment led to decreased expression of HIF-1α and its target genes (*VEGF* and *EPO*). We also observed conspicuous differences in the expression of 84 hypoxia pathway genes (modulated by hypoxia; Fig. S2 *A*–*E*) in CAY10585-treated groups (Fig. S5*B*).

We next tested whether CAY10585 modulated expression of NLRP3 pathway genes in our model system. As shown in Fig. 5 *A* and *B*, the expression of NLRP3 and IL-1β transcripts was significantly lowered in the animals pretreated with this inhibitor. These observations suggested a key role of hypoxia in regulating the expression of NLRP3 inflammasome components.

To establish the functional relevance of inhibition by CAY10585, we next recorded thrombus weight, prothrombin fragment 1+2, and D-dimer in the presence of this inhibitor. We observed a reduction in thrombus weight (Fig. 5*C*), level of prothrombin fragment 1+2 (Fig. 5*D*), and D-dimer (Fig. 5*E*) in the HT group, pretreated with this inhibitor and thus clearly

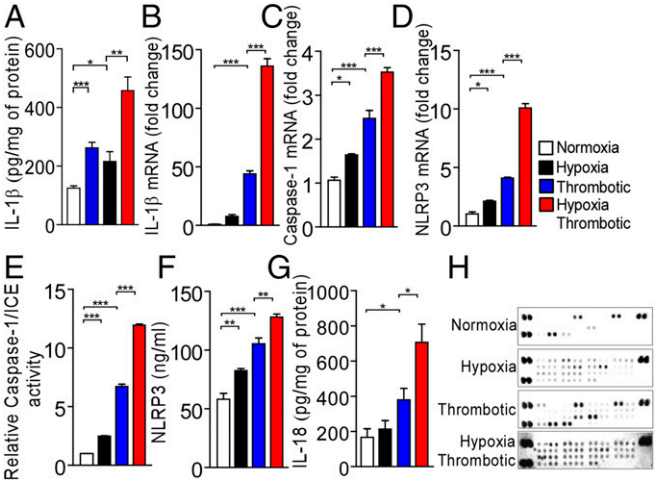

Fig. 4. Activation of NLRP3 inflammasome during hypoxia-induced thrombosis. (*A*) Estimation of IL-1β by ELISA in tissue homogenates after 6 h of exposure. (*B*–*D*) Relative levels of expression of IL-1β (*B*), Caspase-1 (*C*), and NLRP3 (*D*) transcripts (real-time PCR) at indicated points in the HT group. 18S rRNA was used as an internal control, and expression was normalized to that of animals in normoxic group. (*E*) Relative Caspase-1/ICE activity in indicated groups. Estimation of NLRP3 (*F*) and IL-18 (*G*) levels by ELISA after 6 h exposure. (*H*) Estimation of cytokines (using cytokine arrays) from tissue homogenates from all four groups. The results are representative of a minimum of three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

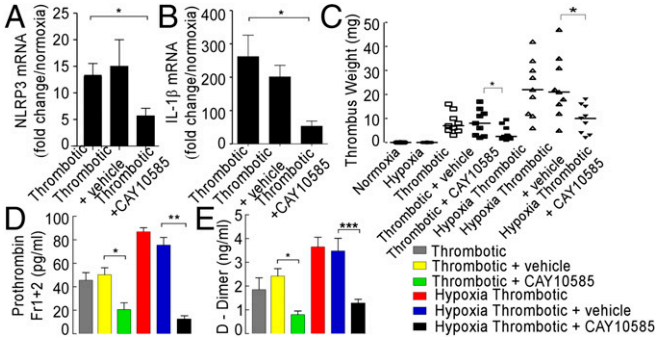

Fig. 5. Hypoxia-induced proinflammatory state is regulated by transcriptional activity of HIF-1α. HIF inhibitor, CAY10585 (100 μg/kg) was administered (intravenous) before IVC ligation and hypoxic challenge for 6 h. Relative expression of (*A*) NLRP3 and (*B*) 1L-1β transcripts (real-time PCR). (*C*) Dot plot showing medians of thrombus weight, (*D*) Levels of prothrombin fragment 1+2, (*E*) D-dimer (ELISA) estimation in plasma samples. Mean ± SEM is shown ($n \geq 6$). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

supporting a functional role of hypoxia response pathways in the activation of the NLRP3 inflammasome and thrombogenesis.

Evidence for a Functional Role of HIF-1α During Hypoxia-Induced NLRP3 Expression. We next tested whether HIF-1α, the central regulator of hypoxia responses (23), was involved in hypoxia-induced expression of NLRP3 and thrombogenesis. We used an in vivo siRNA approach. As shown in Fig. 6*A*, animals in the HT group (6 h), treated with HIF-1α siRNA, showed a significant reduction in the accumulation of the HIF transcript. The expression of NLRP3 (Fig. 6*B*), IL-1β (Fig. 6*C*), and caspase-1 (Fig. S6*A*) transcripts was significantly reduced in these groups. Further, caspase-1/ICE activity (Fig. S6*B*) and NLRP3 protein levels (Fig. S6*C*) were also diminished in the plasma of these groups. The animals in siRNA-treated groups showed significant reduction in thrombus weight (Fig. 6*D*), with an increase in clotting (Fig. 6*E*) and prothrombin time (PT; Fig. S6*D*). The knock-down of HIF-2α in our experiments led to a significant increase in HIF-1α expression, a likely compensatory response (Fig. 6*A*). Concurrent with an increase in expression of HIF-1α, the expression of NLRP3, IL-1β, and caspase-1 was also elevated, along with increase in thrombus weight, clotting time, and PT (Fig. 6 *A*–*E* and Fig. S6*D*).

We next analyzed the NLRP3 promoter for putative HIF-responsive elements/sites. Our in silico analysis returned three sequences closely matching HIF-responsive element consensus (Fig. S6*E*). We therefore performed chromatin immunoprecipitation experiments, using two different HIF-1α antibody clones and probes spanning NLRP3 promoter. As shown in Fig. 6*F*, we consistently observed recruitment of HIF-1α at one of these sites [−975 w.r.t transcription start site (TSS)], implicating functional involvement of HIF-1α in regulating NLRP3 expression during HT conditions.

NLRP3 Inflammasome Axis Inhibition Curtails Hypoxia-Induced Thrombosis. To establish whether the activation of NLRP3 inflammasome played a causal role in the initiation and propagation of thrombosis in our model system, we used three different inhibition strategies and, subsequently, performed in situ thrombus examination, recorded thrombus weight and length, and checked in vivo levels of prothrombin fragment 1+2 and D-dimer, in addition to monitoring the aggregation of platelets isolated from these animals.

We first knocked down NLRP3 transcript in the animals, using in vivo grade siRNA (10 mg/kg body weight), aiming to check the assembly of the NLRP3 inflammasome complex. We observed a significant reduction in caspase-1/ICE activity (Fig. S7*A*), in addition to reduced thrombus (Fig. 7 *A*–*C*), in IVC of the HT group treated with NLRP3 siRNA. We also observed a significant reduction in the levels of prothrombin fragment 1+2 and the D-dimer (Fig. 7 *D* and *E*, respectively). These results suggested a causal role for NLRP3 during thrombosis. The platelets isolated from animals pretreated with NLRP3 siRNA, before IVC ligation, also showed reduced aggregation in response to ADP (used as physiological agonist) (Fig. 7*F*). This observation suggested an upstream role of NLRP3 inflammasome to platelet activation, which is a vital step in the thrombogenic cascade.

In the next set of experiments, we used SML0499 to inhibit the catalytic activity of caspase-1, required for the production of active IL-1β from its proform. The in situ thrombus examination, thrombus weight and length, prothrombin fragment 1+2, D-dimer, and ex vivo platelet aggregation assay using ADP are shown in Fig. S7 *B*–*G*. The cumulative results from all these assays showed that inhibition of caspase-1 activity reduced thrombogenesis under hypoxic conditions.

Finally, we injected specific antibodies against active IL-1β and thus limited its bioavailability, essential for signaling via cognate IL-1 receptors. As presented in Fig. S7 *H*–*M*, these animals also showed a reduction in thrombus formation, as evident from a similar set of parameters described earlier. Taken together, these results (Fig. 7 *A*–*F* and Fig. S7 *B*–*M*) demonstrated an indispensable role for NLRP3 inflammasome-mediated active IL-1β generation in hypoxia-induced thrombus formation. The expression analysis in specific cell types [peripheral blood mononuclear cells (PBMNs), platelets, and vessel wall] suggested that both NLRP3 and IL-1β increased significantly in the PBMNs, apart from thrombus isolated from a localized (ligated) venous site (Fig. S8).

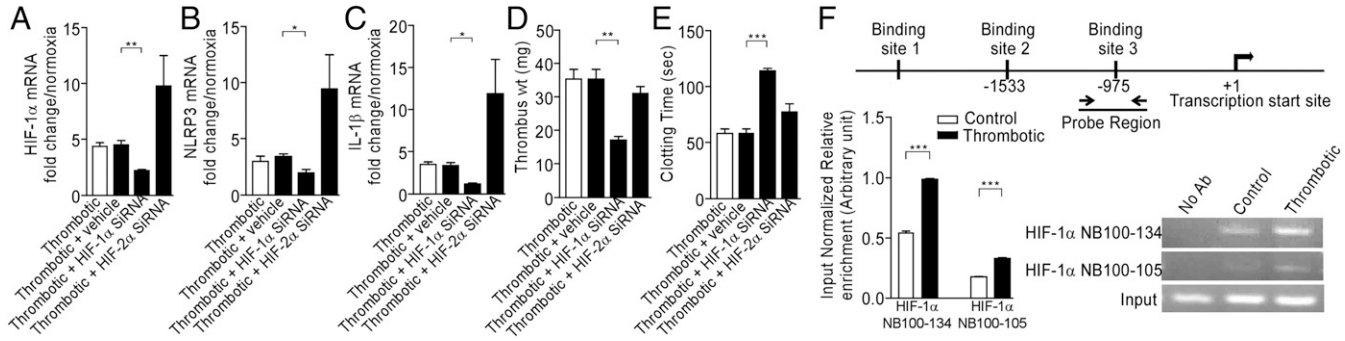

Fig. 6. HIF-1α regulates NLRP3 expression and thrombogenesis. Animals were treated with in vivo grade HIF-1α and HIF-2α siRNA. After RNA isolation from indicated groups, real-time PCR was performed for (*A*) HIF-1α, (*B*) NLRP3, (*C*) IL-1β. Thrombus weight (*D*) and clotting time (*E*) were also recorded. (*F*) Chromatin immunoprecipitation with two different HIF-1α antibody (indicated) and primer pairs spanning putative sites (indicated). The enrichment of NLRP3 promoter region in chromatin immunoprecipitation experiments was quantitated and plotted to obtain the bar graph (mean ± SEM) shown in the figure. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

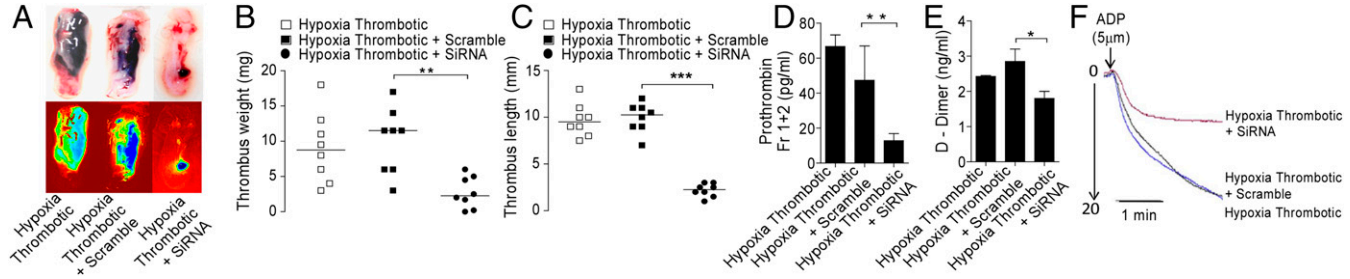

Fig. 7. Knock-down of NLRP3 curtails hypoxia-Induced thrombosis. NLRP3 was knock-down using in vivo grade siRNA complexes and specific parameter studied. (*A*) Photomicrographs of thrombosed IVC; (*B*) thrombus weight; (*C*) thrombus length (median indicated); (*D*) Prothrombin fragments 1+2; (*E*) D-dimer, and (*F*) platelet aggregation assay in groups (indicated in figure), 6 h postinduction. All datasets are representative of a minimum of three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Evidence for the Involvement of the NLRP3 Inflammasome in Human Patients with Altitude-Induced Thrombosis. We next sought to investigate the potential involvement of the NLRP3 inflammasome in clinically confirmed cases of VTE ($n = 18$) occurring in response to the hypoxic environment. The demographic, clinical, and specific genetic parameters of patients with VTE are presented in Fig. S9. We observed a relatively higher number of patients lacking thrombophilic traits [including deficiency of protein C, protein S, and ATIII, in addition to activated protein C (APC) resistance], major SNPs [factor V Leiden, prothrombin, tissue factor pathway inhibitor (TFPI), fibrinogen-β, methylene tetrahydrofolate reductase (MTHFR), and PAI-1], and other additional risk factors (including lipid profile, homocysteine, and blood glucose levels) known to be associated with a predisposition to VTE (Fig. S9). These observations likely suggested that VTE episodes in these individuals were potentially triggered by environmental conditions (hypoxia) prevailing at altitudes.

To test the likely involvement of the NLRP3 inflammasome pathway, we next studied relative expression of key genes of this pathway in these patients. As shown in Fig. 8 *A–D*, we observed an increase in NLRP3, caspase-1, IL-1β, and IL-18 mRNA expression in patients compared with healthy age-matched controls. Furthermore, caspase-1/ICE activity was significantly elevated along with increased levels of NLRP3 (protein) and IL-1β in the patient samples (plasma, Fig. 8 *E–G*). This dataset supported involvement of the NLRP3 inflammasome pathway in the pathogenesis of VTE in individuals exposed to the hypoxic challenge.

Fig. 8*H* schematically depicts the scheme of events causally underlying activation of thrombosis in hypoxic environments.

Discussion

The present study revealed a causal role of strong inflammatory response involving NLRP3 and IL-1β in activating hypoxia-induced thrombogenic cascade in the venous milieu. Of critical note is the fact that HIF-1α, known to regulate a plethora of human diseases (24), emerged as the key node connecting hypoxia responses to proinflammatory state via its ability to regulate the expression of NLRP3 (transcript) under these conditions. Conceivably, the evidence for a direct connection between HIF-1α and NLRP3 is likely to have general implications, especially as a target for intervention in other pathological conditions emanating from hypoxia and the proinflammatory state.

The biological activation of IL-1β requires parallel activation of pathways, culminating in transcriptional up-regulation of IL-1β, increased expression of NLRP3, and enzymatic activation of caspase-1 (25). Some recent reports suggested platelets as a likely source of IL-1β (26–29), produced by virtue of a stored repertoire of molecules (mRNA, inflammasome components) and cue-dependent processing/secretion during thrombogenesis. Thus, an important question pertaining to the possibility of platelet-origin IL-1β in sustaining an intense phenotype, such as that observed in our study, remains paradigmatic. Our present dataset provides some additional information in this regard. We observed up-regulation of IL-1β transcript in addition to other inflammasome components in mononuclear cells (PBMNs), apart from thrombus isolated from localized (ligated) venous site, and this up-regulation could be prevented using HIF inhibitor, CAY10585, or HIF-1α-specific siRNA. It thus is reasonable to assume that the immune cells are likely to play an important role in regulating the intensity of venous thrombosis, likely through de novo cue-dependent transcriptional up-regulation of IL-1β and inflammasome pathway genes (NLRP3, caspase-1). Finally, the fact that we also observed a concomitant increase in the relative expression of NLRP3, caspase-1, IL-1β, and IL-18 transcripts in peripheral blood cells of volunteers who developed VTE at high altitudes lends strong support to this proposition.

The hypoxic milieu in vivo has been proposed as a critical regulator of sterile inflammation and consequent pathological effects. The mechanistic basis appears to include modulation of intrinsic mitochondrial redox homeostasis (30), in addition to activation of toll-like-receptors and various danger signals such as ATP release from necrotic cells (31). The issue of hypoxia-induced

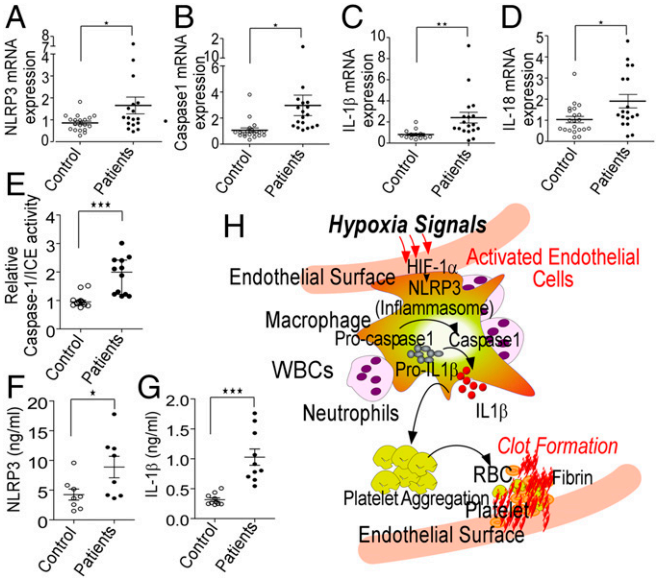

Fig. 8. Evidence for involvement of NLRP3 inflammasome components in patients with altitude-induced venous thrombosis. (*A–D*) RNA was isolated from PBMNs from the blood samples of patients ($n = 18$), and real-time PCR for indicated genes was performed. β-actin was used as an internal control. The relative expression of NLRP3 (*A*), caspase-1 (*B*), 1L-1β (*C*), and IL-18 (*D*) transcripts. (*E*) Caspase-1/ICE activity in plasma samples from patients and controls ($n = 12$). Estimation of NLRP3 (*F*) and IL-1β (*G*) levels in plasma samples from patients and controls ($n \geq 8$) Median values for individual groups are also shown. $*P < 0.05$; $**P < 0.01$. (*H*) Diagrammatic representation of an inferred scheme of events during hypoxia-induced thrombosis.

inflammasome activation and the proinflammatory state breeding pathophysiological outcomes at high altitude encompasses conflicting studies and opinions (15, 32). Our transcriptome data revealed dense gene networks related to strong proinflammatory responses, involving both innate and adaptive immune cells (Fig. 3). Further, as described here, hypoxia-induced thrombosis could be circumvented by inflammasome inhibition, suggesting an early role of inflammation in this process. We also showed that inhibition of hypoxia response pathways (using pharmacological inhibitor or siRNA) prevented transcriptional up-regulation of NLRP3 and IL-1β with significant antithrombotic effects. Taken together, these results posit that hypoxia regulates significant pathological effects via its ability to promote the proinflammatory state.

Virtually all mechanistic understanding, elucidated to date, pertaining to specific forms of thrombosis keep complying with Virchow's Triad, although in somewhat kaleidoscopic molecular patterns regulating individual hypercoagulable states (33). In keeping with the essence of this fact, venous and arterial thrombosis also appears to entail principally similar events, but conspicuously divergent origins. Although early endothelial injury is an established modus operandi of arterial thrombosis, it appears to be dispensable during early stages of venous forms that precipitate under diverse conditions and stimuli. A recent study showed that the NLRP3 inflammasome inhibitor, Arglabin, curtailed the atherogenic effect of high-fat diet in ApoE2.K_i mice (34). In view of such information, it is tempting to speculate that activation of NLRP3 inflammasome complex could constitute a unifying molecular cornerstone between diverse pathological states and various forms of thrombosis.

Hypercoagulable state is also known to predispose an individual to elevated risk for pulmonary embolism, which is a bigger clinical challenge and often more fatal, arising from increased thrombus dissemination. We too observed a somewhat similar phenomenon in our animal model with disseminated intravascular coagulation or hypercoagulation concomitant with elevated fibrinolytic activity, under hypoxic condition (Fig. 1). Conversely, a thrombotic state could also manifest as a result of skewing of homeostasis toward hypercoagulation due to a less-effective fibrinolytic system. Taken together, such arguments define an apparent paradigm for clinical significance and an area of future investigation. In view of our results, we posit that the strength of biological cues propagating individual pathways (coagulatory-fibrinolytic) critically regulate resultant effects with phenotypic manifestation such as localized thrombosis, consumptive coagulopathy, or pulmonary embolism.

In summary, our study revealed an important target, NLRP3 inflammasome, for hypoxia-induced venous thrombosis in addition to reinforcing an intriguing complexity involving intricately interacting coagulatory, thrombolytic, and inflammatory hubs at its core.

Materials and Methods

Detailed materials and methods are included in *SI Materials and Methods*.

Animal Experiments. All experiments were conducted in compliance with guidelines of the Committee for Purpose of Control and Supervision of Experiments on Animals, Government of India. Male Sprague–Dawley rats, weighing 250–300 g, were used and exposed to hypobaric hypoxia, using environmental chamber simulating 429 torr. The IVC ligation model for in vivo thrombosis was used as previously described by us (19).

Human Studies. Human studies were conducted in strict compliance with the ethical standards of Indian Council of Medical Research. Informed consent was obtained from the subjects as per Declaration of Helsinki. Young male patients with VTE (n = 18) evacuated from high-altitude regions to tertiary care facilities (Command Hospital Chandimandir, Chandigarh or Army Hospital, New Delhi) were enrolled. Equal numbers of healthy, age-matched male subjects with no prior history for VTE were included as controls.

Statistics. Data are presented as mean ± SEM. The statistical significance of differences was evaluated using unpaired t test or Mann-Whitney test. Bonferroni post hoc test was done for multiple group comparison, using Prism 5 (GraphPad) software. The statistical significance of differences were represented as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

ACKNOWLEDGMENTS. We acknowledge Dr. Shashi B Singh, Directorate General Armed Forces Medical Services, Dr. Srishti Gupta, Dr. Iti Garg, and Dr. R. J. Tirpude for their support. This study was funded by Defence Research and Development Organization Project SL-10/DIP-255.

1. Zwicker JI, et al. (2009) Tumor-derived tissue factor-bearing microparticles are associated with venous thromboembolic events in malignancy. *Clin Cancer Res* 15:6830–6840.
2. Zhou X, et al. (2010) Incidence and risk factors of venous thromboembolic events in lymphoma. *Am J Med* 123:935–941.
3. Chan MY, Andreotti F, Becker RC (2008) Hypercoagulable states in cardiovascular disease. *Circulation* 118:2286–2297.
4. Demers C, et al. (1998) Incidence of venographically proved deep vein thrombosis after knee arthroscopy. *Arch Intern Med* 158:47–50.
5. Bendz B, Rostrup M, Sevre K, Andersen TO, Sandset PM (2000) Association between acute hypobaric hypoxia and activation of coagulation in human beings. *Lancet* 356:1657–1658.
6. Lapostolle F, et al. (2001) Severe pulmonary embolism associated with air travel. *N Engl J Med* 345:779–783.
7. Smallman DP, McBratney CM, Olsen CH, Slogic KM, Henderson CJ (2011) Quantification of the 5-year incidence of thromboembolic events in U.S. Air Force Academy cadets in comparison to the U.S. Naval and Military Academies. *Mil Med* 176:209–213.
8. Ward M (1975) *Mountain medicine: A clinical study of cold and high altitude* (Crosby Lockwood Staples, London).
9. Zangari M, et al. (2013) Could hypoxia increase the prevalence of thrombotic complications in polycythemia vera? *Blood Coagul Fibrinolysis* 24:311–316.
10. Anand AC, Saha A, Kumar R, Sharma V, Jha SK (2000) Portal system thrombosis: A new dimension of high altitude illnesses. *Trop Gastroenterol* 21:172–173.
11. Cheng S, Chng SM, Singh R (2009) Cerebral venous infarction during a high altitude expedition. *Singapore Med J* 50:e306–e308.
12. Jha SK, Anand AC, Sharma V, Kumar N, Adya CM (2002) Stroke at high altitude: Indian experience. *High Alt Med Biol* 3:21–27.
13. Gupta N, Ashraf MZ (2012) Exposure to high altitude: A risk factor for venous thromboembolism? *Semin Thromb Hemost* 38:156–163.
14. Mannucci PM, Gringeri A, Peyvandi F, Di Paolantonio T, Mariani G (2002) Short-term exposure to high altitude causes coagulation activation and inhibits fibrinolysis. *Thromb Haemost* 87:342–343.
15. Eltzschig HK, Carmeliet P (2011) Hypoxia and inflammation. *N Engl J Med* 364:656–665.
16. Rider P, et al. (2012) The transcription of the alarmin cytokine interleukin-1 alpha is controlled by hypoxia inducible factors 1 and 2 alpha in hypoxic cells. *Front Immunol* 3:290.
17. Mileno MD, et al. (1995) Coagulation of whole blood stimulates interleukin-1 beta gene expression. *J Infect Dis* 172:308–311.
18. Reitsma PH, Rosendaal FR (2004) Activation of innate immunity in patients with venous thrombosis: The Leiden Thrombophilia Study. *J Thromb Haemost* 2:619–622.
19. Tyagi T, et al. (2014) Altered expression of platelet proteins and calpain activity mediate hypoxia-induced prothrombotic phenotype. *Blood* 123:1250–1260.
20. Páramo JA (2010) Prothrombin fragments in cardiovascular disease. *Adv Clin Chem* 51:1–23.
21. Warde-Farley D, et al. (2010) The GeneMANIA prediction server: Biological network integration for gene prioritization and predicting gene function. *Nucleic Acids Res* 38:W214–220.
22. Lee K, et al. (2007) (Aryloxyacetylamino)benzoic acid analogues: A new class of hypoxia-inducible factor-1 inhibitors. *J Med Chem* 50:1675–1684.
23. Semenza GL (2007) Life with oxygen. *Science* 318:62–64.
24. Semenza GL (2000) HIF-1 and human disease: One highly involved factor. *Genes Dev* 14:1983–1991.
25. Martinon F, Mayor A, Tschopp J (2009) The inflammasomes: Guardians of the body. *Annu Rev Immunol* 27:229–265.
26. Lindemann S, et al. (2001) Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. *J Cell Biol* 154:485–490.
27. Denis MM, et al. (2005) Escaping the nuclear confines: Signal-dependent pre-mRNA splicing in anucleate platelets. *Cell* 122:379–391.
28. Hottz ED, et al. (2013) Platelets mediate increased endothelium permeability in dengue through NLRP3-inflammasome activation. *Blood* 122:3405–3414.
29. Brown GT, Narayanan P, Li W, Silverstein RL, McIntyre TM (2013) Lipopolysaccharide stimulates platelets through an IL-1β autocrine loop. *J Immunol* 191:5196–5203.
30. Usui F, et al. (2015) Inflammasome activation by mitochondrial oxidative stress in macrophages leads to the development of angiotensin II-induced aortic aneurysm. *Arterioscler Thromb Vasc Biol* 35:127–136.
31. Weinberg SE, Sena LA, Chandel NS (2015) Mitochondria in the regulation of innate and adaptive immunity. *Immunity* 42:406–417.
32. Wanderer AA (2011) Hypoxia and inflammation. *N Engl J Med* 364(20):1976.
33. López JA, Chen J (2009) Pathophysiology of venous thrombosis. *Thromb Res* 123:S30–S34.
34. Abderrazak A, et al. (2015) Anti-inflammatory and antiatherogenic effects of the NLRP3 inflammasome inhibitor arglabin in ApoE2.Ki mice fed a high-fat diet. *Circulation* 131:1061–1070.
35. Trapnell C, et al. (2012) Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* 7:562–578.