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RESEARCH ARTICLE

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Targeting *SELPLG*/P-selectin glycoprotein ligand 1 in preclinical ARDS: Genetic and epigenetic regulation of the *SELPLG* promoter

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Abstract

We previously identified a missense single nucleotide polymorphism rs2228315 (G>A, Met62Ile) in the selectin-P-ligand gene (*SELPLG*), encoding P-selectin glycoprotein ligand 1 (PSGL-1), to be associated with increased susceptibility to acute respiratory distress syndrome (ARDS). These earlier studies demonstrated that *SELPLG* lung tissue expression was increased in mice exposed to lipopolysaccharide (LPS)- and ventilator-induced lung injury (VILI) suggesting that inflammatory and epigenetic factors regulate *SELPLG* promoter activity and transcription. In this report, we used a novel recombinant tandem PSGL1 immunoglobulin fusion molecule (TSGL-Ig), a competitive inhibitor of PSGL1/P-selectin interactions, to demonstrate significant TSGL-Ig-mediated decreases in *SELPLG* lung tissue expression as well as highly significant protection from LPS- and VILI-induced lung injury. In vitro studies examined the effects of key ARDS stimuli (LPS, 18% cyclic stretch to simulate VILI) on *SELPLG* promoter activity and showed LPS-mediated increases in *SELPLG* promoter activity and identified putative promoter regions associated with increased *SELPLG* expression. *SELPLG* promoter activity was strongly regulated by the key hypoxia-inducible transcription factors, HIF-1α, and HIF-2α as well as NRF2. Finally, the transcriptional regulation of *SELPLG* promoter by ARDS stimuli and the effect of DNA methylation on *SELPLG* expression in endothelial cell was confirmed. These findings indicate *SELPLG* transcriptional regulation by clinically-relevant inflammatory factors with the significant TSGL-Ig-mediated attenuation of LPS and VILI highly consistent with PSGL1/P-selectin as therapeutic targets in ARDS.

KEYWORDS
ARDS, P- selectin, *SELPLG* promoter activity, TSGL-Ig, VILI

INTRODUCTION

The acute respiratory distress syndrome (ARDS) is a heterogenous disorder triggered by a complex interplay of genetic factors and diverse environmental factors including sepsis, bacterial or viral infections including coronavirus disease 2019 (COVID-19), major traumatic injury with massive transfusion, and mechanical ventilation.[1,2] The SARS-CoV-2/COVID-19 pandemic has dramatically highlighted multiple unmet needs in the care for patients with ARDS including the lack of key genetic insights into disease susceptibility and health disparities, the absence of validated diagnostic, predictive, and prognostic biomarkers, and lack of effective Food and Drug Administration (FDA)-approved pharmacotherapies. The heterogeneity of the ARDS phenotype, complexity of the dysregulated inflammatory signals, and the absence of predictive biomarkers have contributed to numerous failed clinical trials of promising therapies.

Over the past 50 years, several candidate gene or genetic association studies have identified specific variants that contribute to ARDS susceptibility[3–8] and survival.[9] In a genome-wide association study in Blacks, we reported that a missense single nucleotide polymorphisms (SNP) rs2228315 (G>A, Met62Ile) located in the selectin-P-ligand gene (*SELPLG*) which encodes for P-selectin glycoprotein ligand 1 (PSGL-1), was associated with increased susceptibility to ARDS.[10] Preclinical validation studies with murine models showed increased *SELPLG* expression with lipopolysaccharide (LPS)- and ventilator-induced lung injury (VILI) which was significantly attenuated by either *SELPLG* knock down or by PSGL-1 inhibition via a neutralizing antibody.[10]

Interactions between PSGL-1 (primarily expressed on polymorphonuclear leukocytes) and its counterreceptor, P-selectin (primarily expressed on platelets and activated endothelial cells [EC]), play a critical role in leukocyte trafficking, platelet aggregation, and thrombosis, all key features of ARDS pathobiology.[11] Consistent with this observation, the infusion of a FDA-approved P-selectin-binding humanized monoclonal antibody, Crizanlizumab,[12] dramatically reduced lung injury in a rat model of ARDS[13] and significantly reduced the rate of vaso-occlusive crises in sickle cell diseases.[14] In the current study, we employed Tandem P-selectin glycoprotein ligand-1 immunoglobulin (TSGL-Ig), a novel recombinant tandem PSGL1 immunoglobulin fusion molecule and competitive inhibitor of PSGL1/P-selectin interactions,[15,16] to further investigate the role of PSGL1 in preclinical ARDS models of LPS combined with VILI. TSGL-Ig carries two P-selectin sulfated-glycopeptide-binding domains resulting in a dimer with 4 P-selectin binding sites per molecule of TSGL-Ig compared to 2 sites for regular monoclonal antibody. TSGL-Ig was shown to prevent ischemia-reperfusion injury,[15] and lung vaso-occlusion in sickle cell disease mice.[16] However, the functions of TSGL-Ig in sepsis-induced ARDS and VILI are unknown. In addition to novel studies utilizing the novel, immunoglobulin fusion molecule, TSGL-Ig, we examined the transcriptional regulation of *SELPLG* promoter in response to ARDS stimuli (LPS, 18% cyclic stretch [CS]), and genetic and epigenetic regulation of *SELPLG* by ARDS relevant transcription factors.

MATERIALS AND METHODS

Reagents and antibodies

LPS 127-08 from *Escherichia coli* were purchased from Sigma-Aldrich. The Fugene HD transfection reagent for the Luciferase reporter assay and the reagents for measuring dual luciferase activity (Luciferase Assay reagent II and Stop & Glo reagent) were purchased from Promega. B-Actin-horseradish peroxidase antibody (cat #A3854-200UL) from Sigma-Aldrich. TSGL-Ig was obtained from Quell Pharma LLC. FG-4592, KC7F2, PT-2385, Bixin, Brusatol were purchased from Selleck Chemicals LLC.

Animals

Male C57BL/6J mice aged 8–12 weeks (Jackson Laboratories) were used for all experiments. All mice were maintained at room temperature without pathogen and 12 h of light/dark circadian rhythm. Mice feed, litter and drinking water bottles were autoclaved, and the animals were free to eat and drink water. Body weight was measured once a week using a digital electronic balance. All animal-related ethics review, procedures and experiments were approved by the Institutional Animal Care and Use Committee of University of Arizona (animal protocol number 13-490) and were performed in accordance with relevant guidelines and regulations and in compliance with Animal Research: Reporting on in Vivo Experiments (ARRIVE) guidelines.

LPS/VILI "two-hit" preclinical ARDS mouse model and delivery of TSGL-Ig

Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (5 mg/kg) given by intraperitoneal injection and then intubated with a 20-G angiocatheter which was used for LPS instillation (*E. Coli* 0127: B8, 0.1 mg/kg). After 18 h, mice were re-anesthetized,

intratracheally reintubated and connected to mechanical ventilation (SAR-1000 mechanical ventilator—CWE, Inc.) using tidal volumes (V_T) of 20 mL/kg, respiratory rate of 90 breaths/min, and positive end-expiratory pressure of 0 cmH2O for 4 h as previously described.[17,18] Supplemental ketamine and xylazine were provided to ensure adequate anesthetic depth during mechanical ventilation. Spontaneously breathing (SB) control mice received intratracheal PBS instead of LPS and following 18 h period, allowed to breathe spontaneously on room air (SB) for 4 h before harvesting. TSGL-Ig (1 mg/Kg), a novel recombinant tandem PSGL1 immunoglobulin fusion molecule which is a competitive inhibitor of PSGL1/P-selectin interactions[15,16] was delivered intravenously concomitantly with LPS challenge before initiation of VILI.

Bronchoalveolar lavage (BAL) analysis

BAL fluid analysis retrieval, protein analysis and cell count analysis, including polymorphonuclear cell (PMN) determinations, were performed as described previously.[10]

Quantitative lung histology

After harvesting, the lungs were fixed in 10% formalin underwent hematoxylin and eosin (H&E) staining as previously described.[17,18] Histological images, four random lung fields/slide, three slides/mouse were graded by a pathologist blinded to study groups. Images were selected for ImageJ software analysis of the area percentage of H&E staining. The percent area was determined by ratio of area of lung solid tissue with inflammatory consolidation to the total lung area/field.

Real-time PCR assay for lung tissue *SELPLG* expression

RNA was isolated from lung tissue with the RNeasy plus min kit from QIAGEN 74136. A master mix of RLT plus was made with 10 μL of beta-mercaptoethanol (from Sigma Aldrich [m6250]) per milliliter of RLT plus buffer (part of the QIAGEN kit). A total of 20 mg of each tissue was then homogenized individually in 600 μL of the mixture per sample. Next, complementary DNA was synthesized with the SuperScript VILO IV kit (11754050; ThermoFisher Scientific). Finally, gene expression levels of ACTB (Taqman Mm00607939_s1), *SELPLG* (Taqman Mm01204601_m1) were assayed by RT-PCR with Taqman Gene Expression Mastermix (4369016). Finally, we compared *SELPLG* messenger RNA (mRNA) levels relative to β-actin levels in lung tissue homogenates by real-time PCR in SB and LPS + VILI-challenged mice.

EC culture, LPS stimulation, and in vitro CS

Human pulmonary artery ECs (Lonza) were plated onto BioFlex silicone elastomer six-well plates coated with type I collagen and were cultured in endothelial growth medium (EGM-2) containing 10% fetal bovine serum (Lonza) in 5% CO_2 at 37°C and 95% humidity to achieve contact-inhibited monolayers. For mechanical stress studies, BioFlex plates were placed on a Flexcell Strain Tension System (FX-3000; Flexcell International) kept in a 5% CO_2 incubator at 37°C and 95% humidity. Plates were stretched to produce either 18% elongation at a frequency of 0.5 Hz, 30 cycles/min. As we have previously reported, 18% CS corresponds to pathologically relevant levels of mechanical stress that result in phenotypic EC monolayer changes, increased susceptibility to barrier-disruptive agonists, but with preserved monolayer integrity even after prolonged exposure (48 h).[19]

Prediction of *SELPLG* transcription factor binding sites (TFBS)

In silico predicted TFBS and potential effects of SNPs on TF binding were identified via positional weight matrices searches using TRANSFAC Match (http://www.biobase-international.com/) and Genomatix MatInspector 8.0 (http://www.genomatix.de/).

Gene cloning, mutagenesis, and 5′-deletion mutations

Gene cloning, mutagenesis, and luciferase activity assays were performed as previously described.[19] Briefly, 2100 bp DNA fragments of the *SELPLG* promoter region (−2000 bp to +100 bp from the transcription start site [TSS]) were synthesized by GenScript (08854; Piscataway) based on sequence NM_003006 and confirmed by sequencing. Amplicons were fused to a pGL3-basic reporter vector (Promega). To further study the putative *cis*-elements, a series of mutant plasmids were constructed sparing the TSS by PCR amplification with primers. The DNA fragments containing the truncated region were inserted into the *Xho*I and *Mlu*I sites of pGL3, and the relevant regions of the final constructs were confirmed by sequencing.

Dual luciferase reporter gene assay

EC were cotransfected with *SELPLG* promoter plasmid constructs containing firefly luciferase reporter and TK renilla vector using Fugene HD transfection reagent (Promega). After exposure to inflammatory factors (LPS, 18% CS) or demethylation reagents 5′-Aza, the transfected cells were collected with passive lysis buffer, and dual luciferase activity was measured using Luciferase Assay reagent II and Stop & Glo reagent (Promega) according to the manufacturer's protocol. Normalized luciferase activity was expressed as ratio of firefly and renilla luciferases activities. To assess the effects of LPS, mechanical stress on human *SELPLG* promoter activity, we exposed human pulmonary artery ECs transfected with pGL3-B luciferase containing a human *SELPLG* promoter sequence (up to +2 kb) to either LPS, 18% CS (4 h), or the combination of LPS and 18% CS.

Statistical analysis

Continuous data were compared using nonparametric methods. Where applicable, standard one-way analysis of variance (ANOVA) was used, and groups were compared using the Newman–Keuls test. Two-way ANOVA was used to compare the means of data from two or more different experimental groups. If a significant difference was present by ANOVA ($p < 0.05$), the least significant differences test was performed post hoc. Statistical tests were performed using GraphPad Prism (version 7.00 for Windows; GraphPad Software, www.graphpad.com). Statistical significance was considered at $p < 0.05$.

RESULTS

Inhibition of P-selectin/PSGL-1 interactions (TSGL-Ig) attenuates lung injury in a "two-hit" LPS/VILI murine model

H&E staining of lung tissues from LPS/VILI-exposed mice demonstrated significant lung tissue structural damage combined with severe alveolar inflammation including lung edema, PMN infiltration, diffuse hemorrhage, and alveolar septal thickening. By contrast, mice pretreated with TSGL-Ig (1 mg/kg) exhibited significantly attenuated LPS/VILI-stimulated histologic damage (Figure 1a). Consistent with the above findings, the ImageJ quantification showed a significant increase in area of lung tissue inflammation with LPS/VILI compared to controls and a reduction of area of inflammation with TSGL-Ig pretreatment ($p < 0.01$) (Figure 1b). Similarly, pretreatment of mice with TSGL-Ig resulted in a significant reduction in LPS/VILI-induced BAL cell counts ($p < 0.01$) (Figure 1c), and LPS/VILI-induced BAL protein levels ($p < 0.01$) (Figure 1d).

TSGL-Ig attenuates lung tissue expression of *SELPLG* in a "two-hit" mouse model

To test gene expression of *SELPLG* in LPS with mechanical ventilation-induced ALI and effects of TSGL-Ig on *SELPLG* in LPS/VILI, we measured mRNA levels by qRT-PCR. Significant upregulation of *SELPLG* lung tissue expression (*SELPLG* mRNA levels relative to β-Actin) was observed by real-time PCR of lung tissue homogenates from mice exposed to combined LPS and VILI compared to SB controls (Figure 2a) ($p < 0.01$) with pretreatment with TSGL-Ig significantly attenuating this upregulated *SELPLG* expression (Figure 2a) ($p < 0.05$). These results support a critical role for *SELPLG* expression in murine LPS/VILI responses, and the potential therapeutic role of blocking P-selectin/PSGL-1 interactions with TSGL-Ig.

SELPLG promoter activity increases in response to LPS and mechanical stress

To interrogate the mechanisms underlying the fluctuation of *SELPLG* expression in our preclinical LPS/VILI models, we transfected *SELPLG* promoter reporters into ECs and exposed them to LPS or/and 18% CS. A 1.5-fold increase in *SELPLG* promoter activity (expressed by relative ratio of firefly/renilla luciferase units) was observed with LPS when compared to vehicle controls ($^*p < 0.05$ vs. controls). For 18% CS, there was a twofold increase in *SELPLG* promoter activity when compared to static controls ($^*p < 0.05$ vs. controls). The combination of LPS and 18% CS caused a 3.5-fold increase in *SELPLG* promoter activity compared to static controls ($^*p < 0.05$ vs. controls) (Figure 2b).

SELPLG promoter regions that potentially alter LPS-induced promoter activity

In silico analysis of *SELPLG* identified multiple sites for transcription factor binding and regulatory elements: shear stress-response element (~−2000 bp upstream of TSS), STAT5 (−1000 bp), nuclear factor kappa B (NFκB) (−600 bp), and a mechanical response element (MSRE) (−400 bp), an antioxidant response element (ARE)

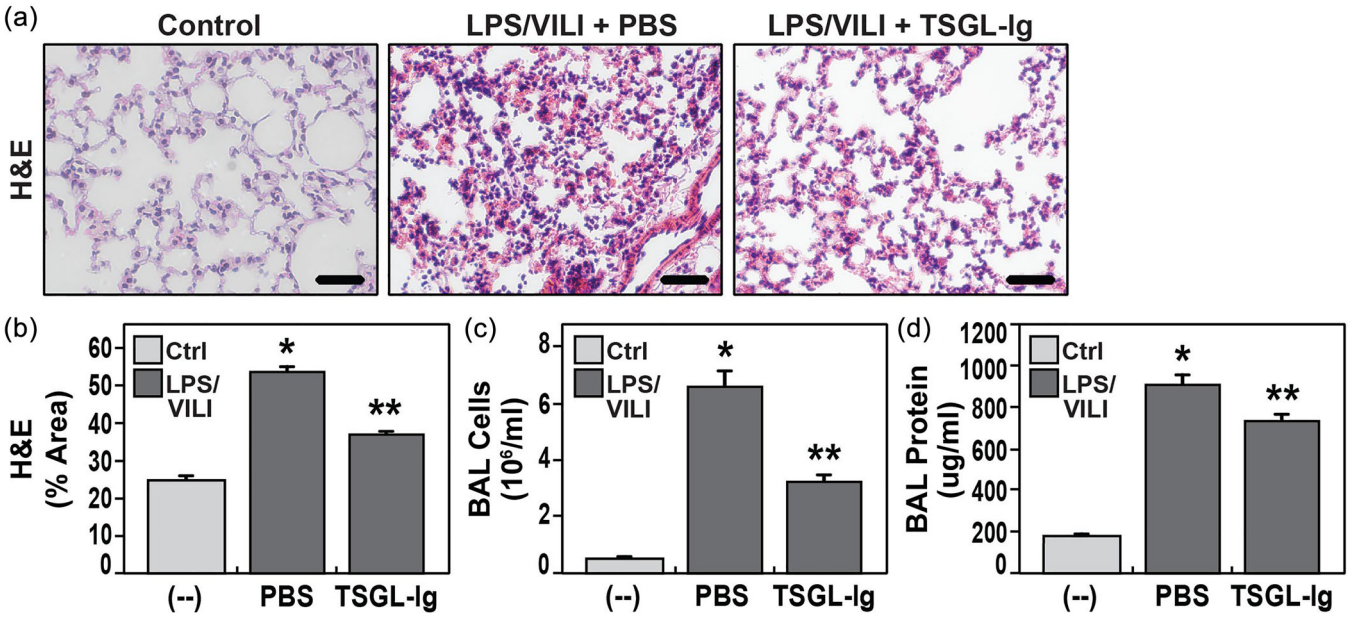

FIGURE 1 (a, b) Inhibition of P-Selectin/PSGL-1 interactions with TSGL-Ig significantly attenuates murine lung injury in the preclinical "two-hit" LPS/VILI model. H&E lung tissue staining shows significant interstitial and alveolar inflammation with significant neutrophil infiltration and alveolar edema compared to control C57BL/6 mice. Mice receiving TSGL-Ig had significant reduction in interstitial and alveolar inflammation. Changes in lung tissue inflammation with "two-hit" LPS/VILI injury (compared to controls) and TSGL-Ig treatment (compared to combo-PBS) were quantitatively summarized by ImageJ software (*$p < 0.001$ control vs. combo-PBS, **$p < 0.01$ combo-PBS vs. combo-TPSGL-Ig). (c) Changes in BAL cells with "two-hit" LPS/VILI injury (compared to controls) and TSGL-Ig treatment (compared to combo-PBS) (*$p < 0.001$ control vs. combo-PBS, **$p < 0.01$ combo-PBS vs. combo-TPSGL-Ig). (d) Changes in BAL proteins with "two-hit" LPS/VILI injury (compared to controls) and TSGL-Ig treatment (compared to combo-PBS) (*$p < 0.001$ control vs. combo-PBS, **$p < 0.01$ combo-PBS vs. combo-TPSGL-Ig). H&E, hematoxylin and eosin; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PSGL-1, P-selectin glycoprotein ligand 1; TSGL-Ig, Tandem P-Selectin glycoprotein ligand-1 immunoglobulin.

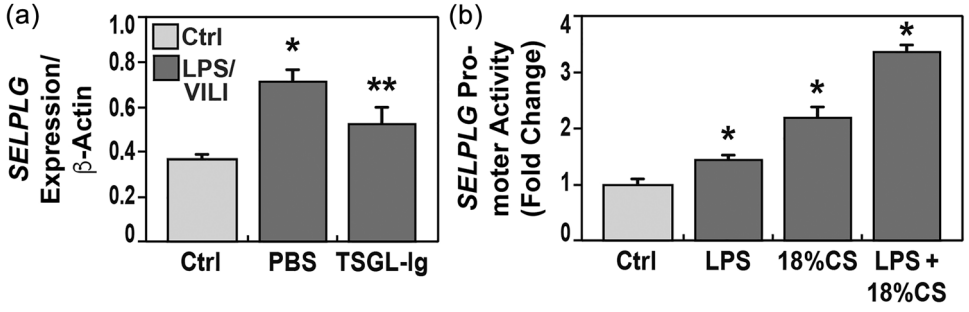

FIGURE 2 *SELPLG* expression levels and promoter activity in response to LPS and mechanical stress. (a) Lung tissue homogeneates from a "two-hit" LPS/VILI injury had significantly higher *SELPLG* expression (as measured by *SELPLG* mRNA levels relative to β-actin) when compared to controls (*$p < 0.01$). Pretreatment with TSGL-Ig significantly attenuated the expression of *SELPLG* when compared to the "two-hit" LPS/VILI untreated lung tissue (**$p < 0.05$). (b) 2100 bp DNA fragments of the *SELPLG* promoter region (−2000 bp to +100 bp from the transcription start site) were synthesized by GenScript (NJ 08854; Piscataway) based on sequence NM_003006 and confirmed by sequencing. Amplicons were fused to a pGL3-basic reporter vector (Promega). The pGL3 plasmids containing *SELPLG* promoters were transfected into HPAECs with phRL-TK. Then transfected cells exposed to vechicle, LPS 100 ng/mL, 18% CS, or combinations of LPS with 18%CS for 4 h). Luciferase activities were measured using Dual Luciferase Assay System (Promega). *SELPLG* promoter activities was measured by relative ratio of firefly/renilla luciferase units, and normalized by vehicle controls. Compared to static controls, LPS, 18% CS, or LPS combined with 18% CS induced ~1.5-, ~2- and ~3.5-fold increases in activity (each *$p < 0.05$ vs. controls, $n = 6$ each). CS, cyclic stretch; LPS, lipopolysaccharide; mRNA, messenger RNA; VILI, ventilator-induced lung injury.

(−300 bp). In addition, four promoter SNPs were found to potentially influence *SELPLG* promoter activity: SNP −1663 [G>A] residing within a CpG island, −1010 [delTT] in proximity to a STAT5-binding site, −671 [A>G] in proximity to a NFκB-binding site, and −478 [C>T] in proximity to MSRE (Figure 3a).

To clarify *SELPLG* promoter regulation, ECs were transfected with either the full length *SELPLG* promoter vector or one of eight serial deletion vectors followed by exposure to LPS or vehicle. Without LPS, the basic promoter activity of *SELPLG* was significantly increased by nest deletion of full- length promoter from −2000 bp to −800 bp, and then dramatically decreased by deletion of promoter from −800 bp to 0 bp (pGL3). It is indicated basic promoter inhibitory/repressors binding region (−2000 to −800) and core promoter/activators binding region (−800 to 0). Upon exposure to LPS, *SELPLG* promoter activities were significantly increased >2-folds (−2000 to −1700) or 1.5-folds (−1400 to −500) ($p < 0.05$, respectively), which indicated two significant LPS response regions existed in *SELPLG* promoter (Figure 3b).

Epigenetic regulation of *SELPLG* promoter activity by methylation/demethylation

Recent reports indicated that epigenetic alterations may be the key factor in the pathogenesis of sepsis[20] and ARDS.[21] To assess the epigenetic regulation of *SELPLG* expression by methylation, transfected full-length *SELPLG* promoters in ECs were exposed to the demethylation reagent 5′-Aza. These studies demonstrated 5′-Aza exposure to significantly increase *SELPLG* promoter activity (*$p < 0.01$), whereas the control pGL-3B vector without the *SELPLG* promoter did not respond to 5′-Aza (Figure 4a).

Regulation of *SELPLG* promoter activity by hypoxia-inducible factors (HIFs)

Since hypoxia is the hallmark of ARDS, we next assessed the effects of key HIFs, HIF1α and HIF2α, on *SELPLG* promoter activity. As HIFs are degraded by HIF prolyl hydroxylase domain-2 (PHD2), we used the PHD2 inhibitor, FG-4592, which reduces HIF degradation to increase HIF-1α and HIF-2α levels. EC treatment with the PHD2 inhibitor, FG-4592, significantly increased *SELPLG* promoter activity (twofold, *$p < 0.05$ vs. control, $n = 6$). Specific inhibitors of either HIF1α (KC7F2 40 μM) or HIF2α (PT2385 1 μM) significantly attenuated FG-4592-induced increases in *SELPLG* promoter activity effects (**$p < 0.01$ vs. FG-4592) (Figure 4b).

Regulation of *SELPLG* promoter activity by NRF2 modulators

In ARDS, a large amount of reactive oxygen species are generated and cause damage to the structure and

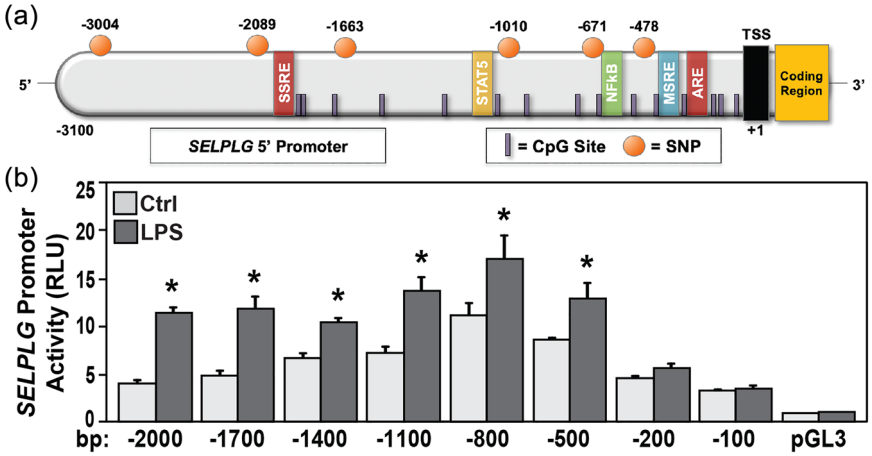

FIGURE 3 Functional analysis of *SELPLG* promoter regions, response elements and single nucleotide polymorphisms (SNPs). (a) In silico models for *SELPLG* promoter identifying SNPs that are potentially relevant in altering *SELPLG* promoter activity and their proximity to key transcription factor binding sites (TFBSs). (b) Defining *SELPLG* promoter regions responsive to LPS-induced increase of promoter activity. Truncated *SELPLG* promoters were generated from serial deletion mutation of full-length promoter and constructed into pGL3-basic vector. HPAECs transfected by plasmids containing full-length or truncation of the promoter were exposed to vehicle or LPS 100 ng/mL for 4 h. The basic *SELPLG* promoter activities were significant compared to pGL3-basic with peak at −800 bp. LPS significantly increased activities of −2000, −1700, −1400, −1100, −800, and −500 *SELPLG* promoters, compared to control ones without LPS (each *$p < 0.05$, $n = 4$ each). LPS, lipopolysaccharide; SELPLG, selectin-P-ligand gene.

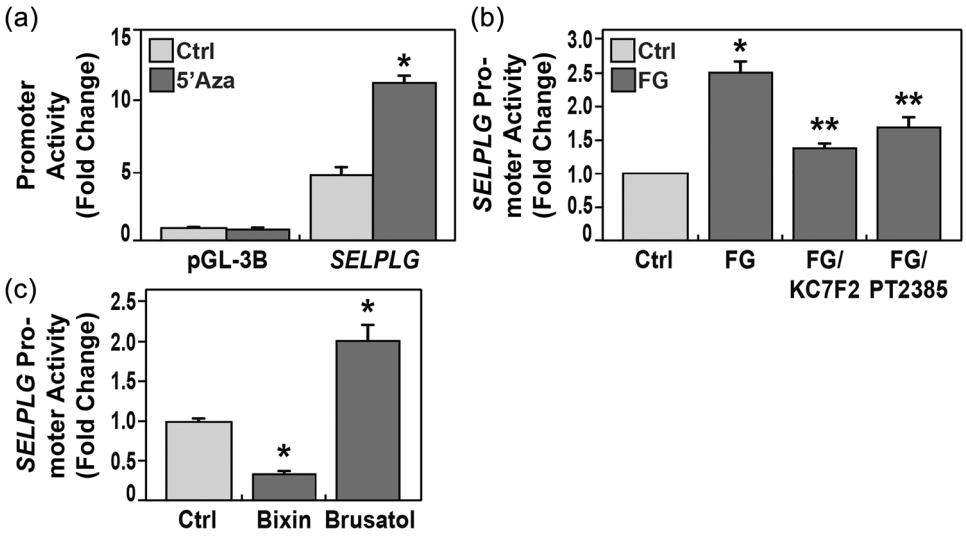

FIGURE 4 Regulation of *SELPLG* promoter activities by demethylation, hypoxia inducible factors (HIFs) and antioxidant NRF2. (a) Demethylation increased *SELPG* promoter activities. HPAECs were transfected with plasmids containing full length *SELPLG* promoters and exposed to demethylation reagent 5-Aza-2′-deoxycytidine (5′-Aza) for 24 h and dual luciferase activities were measured. 5′-Aza significantly increased *SELPLG* promoter activities (*$p < 0.01$ vs. controls, $n = 6$). (b) HIF1α/HIF2α significantly increased *SELPLG* promoter activity. *SELPLG* promoter reporters were transfected into HPAECs and exposed to PHD2 inhibitor FG-4592 100 μM for 8 h. FG-4592 significantly increased *SELPLG* promoter activity (*$p < 0.01$ vs. control, $n = 6$ each). Upon exposure to FG-4592 with specific HIF1α inhibitor KC7F2 40 μM or HIF2α inhibitor PT2385 1 μM, effects of FG-4592 on *SELPLG* promoter activities were significantly attenuated (**$p < 0.01$ vs. FG-4592), respectively. (c) Antioxidant regulator NRF2 significantly decreased *SELPLG* promoter activities. HPAECs with transfected *SELPLG* promoter were exposed to NRF2 activator Bixin 4 μM or NRF2 inhibitor Brusatol 40 nM for 2 h. *SELPLG* promoter activities were significantly decreased by NRF2 activator Bixin (**$p < 0.01$ vs. Ctrl) and augmented by NRF2 inhibitor Brusatol (*$p < 0.01$ vs. Ctrl), respectively. PHD2, prolyl hydroxylase domain-2; SELPLG, selectin-P-ligand gene.

function of cells.[22] Cellular protection against oxidative stress is mainly conferred by NRF2, a transcription factor that promotes the expression of genes that counteract oxidative stress and inflammation.[23] We showed that *SELPLG* promoter activity is significantly decreased by the NRF2 activator, Bixin 4 μM (*$p < 0.05$ vs. control), and increased by the NRF2 inhibitor, Brusatol 40 nM (*$p < 0.05$ vs. control) (Figure 4c). It is implicated that NRF2 significantly repressed the *SELPLG* promoter activities.

DISCUSSION

Interactions between P-selectin and its ligand, PSGL-1, are important in the trafficking of leukocytes across activated ECs to the site of inflammation[24] and is observed in acute inflammatory conditions such as sepsis,[25,26] sickle cell crises,[27–30] and COVID-19 and non-COVID-19 ARDS.[11,31] In the lungs, the transmigration of PMNs from the vascular lumen through gaps between the activated ECs into the alveolar space follows a series of steps that include interactions between the selectins and ligands.[32] PSGL-1, the most important and extensively characterized selectin ligand plays a critical role in the early phase of leukocyte rolling onto the activated EC by binding to P-Selectin and forming a "molecular Velcro" that increases adhesion and subsequent transmigration.[32,33]

This study expands our previous findings that *SELPLG* SNPs are significantly linked to ARDS risk/severity (Met62Ile) and *SELPLG* expression is markedly increased in murine models of LPS and VILI-induced lung injury.[10] We used the LPS/VILI two-hit model to simulate the clinical scenario of ARDS subjects with severe sepsis progressing to respiratory failure and mechanical ventilation with susceptibility to VILI.[34] We now demonstrate that the novel inhibitor of P-selectin/PSGL-1 interactions, TSGL-Ig, decreases *SELPLG* lung tissue expression and attenuates LPS/VILI-induced lung injury. TSGL-Ig carries two P-selectin sulfated-glycopeptide-binding domains in a tandem configuration on a single polypeptide chain that are fused to an inactivated Fc domain of human IgG1, resulting in a dimer with four P-selectin binding sites per molecule of TSGL-Ig which enhances selectin-binding properties.[15] TSGL-Ig was previously shown to prevent ischemia-reperfusion injury of orthotopic liver transplants in mice by attenuating leukocyte sequestration in the liver microcirculation,[15] and significantly attenuated

intravenous oxy-hemoglobin triggered lung vaso-occlusion in sickle cell disease mice.[16]

We demonstrated that *SELPLG* promoter activity increases with LPS and mechanical stretch. Through serial progressive 5′ to 3′ unidirectional deletions and promoter activity assays, we identified possible *SELPLG* promoter regions that might affect basic and LPS-induced enhanced *SELPLG* transcription activities. These LPS-responsive promoter regions contain in silico binding sequences for known TFs involved in LPS transcriptional regulation in EC including NFκB[35] JAK-STAT, and JNK stress kinase pathways.[36] Epigenetic factors regulate *SELPLG* promoter activity as demonstrated by increased *SELPLG* promoter activity with DNA methylation with 5′-Aza. Key transcription factors such as HIFs and NRF2 modulate *SELPLG* transcription which strongly support promoter regulation. Hypoxemia is a critical pathological hallmark of ARDS/ALI and via HIFs, HIF-1α and HIF-2α, hypoxia is a potent stimulus for amplification of ARDS inflammatory cascades.[37–39] Both HIF-1α and HIF-2α are multifunctional and have been implicated as contributing to ARDS severity. HIF-1α drives LPS-induced IL-1β,[40] tumor necrosis factor, IL-12, and VEGF expression[41] and nmMLCK expression.[39] We have shown HIF-2α, and to a lesser extent HIF-1α, are involved in transcriptional regulation of the ARDS/VILI DAMP, eNAMPT[42] and cytoskeletal element gene, *MYLK*.[39] Our current studies demonstrate that HIF-1α and HIF-2α accumulation (by PHD2 inhibition) also increase *SELPLG* promoter activity. Nrf2-deficient mice have demonstrated an increased severity of ARDS with enhanced lung inflammation.[43] NRF2-mediated protection in models of ARDS/ALI has been attributed largely to the induction of antioxidant genes[44] and downregulate proinflammatory mediators.[45] In our previous study, NRF2-driven repression of *MYLK* expression attenuated inflammatory lung injury.[46] Here we demonstrated that *SELPLG* promoter activities were significantly repressed by NRF2 activation and enhanced by NRF2 inhibition. In a recent report, TSGL-Ig increased thioredoxin, glutamate-cysteine ligase, NAD(P)H quinone dehydrogenase 1 and HIF-1a in liver ECs and indicated that cytoprotective effects of TSGL-Ig partially mediated by Nrf2.[15]

Previous studies suggest aberrant DNA methylation of lung tissues may be involved in the pathophysiology of LPS-induced ALI/ARDS[47] and we have previously demonstrated excessive mechanical stress and LPS to reduce *NAMPT* and *MYLK* promoter DNA methylation to increased gene transcription.[39,48] Our current study confirmed the epigenetic regulation of the *SELPLG* promoter via demethylation.

A limitation of our work is that we did not investigate the detailed signal pathways involved in how TSGL-Ig attenuates the severity of ARDS/ARDS beyond the obvious inhibition of P-selectin signaling. In addition, although we explored the promoter regulation by ARDS-relevant factors, we did not identify the exact TFs binding regions involved in *SELPLG* regulation or determine how *SELPLG* demethylated in ARDS/ALI, and other epigenetic regulations including histone modification and miRNAs on *SELPLG* transcription activities during ARDS/ARDS. These issues need to be further investigated. Finally, as we investigate relevant signaling pathways, future studies will determine TSGL-Ig pharmacokinetic and pharmacokinetic studies in small animal models and optimal TSGL-Ig dosing in large animal models of ARDS.

In summary, we confirmed that TSGL-Ig-mediated reductions in PSGL-1/P-selectin interactions significantly attenuates acute lung injury in an ARDS/ALI mice model. Exploration of the genetic and epigenetic mechanisms of *SELPLG* regulation under ARDS/VILI inflammatory conditions confirmed promoter regulation of its expression. These studies add to our previous preclinical studies utilizing genetically engineered *Selplg*$^{-/-}$ KO mice and human GWAS studies.[10] These studies further confirm that *SELPLG* and its coding protein, PSGL-1, are attractive therapeutic targets to significantly attenuate the severity of inflammatory lung injury including ARDS and VILI.

AUTHOR CONTRIBUTIONS

Specific contributions include—conceptualization: Christian Bime, Joe G. N. Garcia, Xiaoguang Sun; *Data curation*: Xiaoguang Sun, Saad Sammani, Matthew Hufford; *Formal analysis*: Xiaoguang Sun, Saad Sammani, Belinda L. Sun; *Funding acquisition*: Christian Bime, Joe G. N. Garcia; *Investigation*: Xiaoguang Sun, Saad Sammani, Matthew Hufford; *Methodology*: Carrie L. Kempf; *Project administration*: Christian Bime, Joe G. N. Garcia; *Resources*: Christian Bime, Joe G. N. Garcia; *Supervision*: Christian Bime, Joe G. N. Garcia; *Validation*: Xiaoguang Sun; *Visualization*: Sara M. Camp, Xiaoguang Sun; *Writing-original draft*: Xiaoguang Sun, Christian Bime; *Writing-reviewing & editing*: Xiaoguang Sun, Belinda L. Sun, Joe G. N. Garcia, Christian Bime. All authors have approved the final draft manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

All research involving animals submitted for publication was approved by an animal care and use committee with oversight of the facility in which the studies were conducted.

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