HIF-1α REGULATES CD55 EXPRESSION IN AIRWAY EPITHELIUM

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DEDICATION

This thesis is dedicated to my loving parents, Hemant and Jagruti Pandya, and my wonderful sister Binal Pandya. Thank you for your continuous role in supporting me throughout my academic achievements. While this thesis reflects my hard work and contribution to biomedical research, I would not be where I am today without my family's constant love, encouragement, and their hard work to ensure that I was taken care of while I focused on pursuing my PhD.

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Pankita Hemant Pandya

HIF-1α REGULATES CD55 EXPRESSION IN AIRWAY EPITHELIUM

Rationale: CD55 down-regulation on airway epithelium correlates with local complement activation observed in hypoxia-associated pulmonary diseases. Therefore, we hypothesized that induction of hypoxia inducible factor 1 alpha (HIF-1α) in hypoxic airway epithelium, mediates CD55 down-regulation.

Methods: Chetomin and HIF-1α siRNA inhibited HIF-1α in hypoxic SAECs (1% O_2), and mice lungs (10% O_2). DMOG mediated HIF-1α stabilization in normoxic SAECs and mice lungs (21% O_2). Transduction of SAECs with AdCA5 also stabilized HIF-1α. *CD55* and *CA9* transcripts were measured by RT-PCR. CD55 and HIF-1α protein expression was assessed by western blots. *In vivo*, immunohistochemistry (IHC) confirmed CD55 and HIF-1α expression. C3a and C5a levels in bronchoalveolar lavage fluid (BALF) were measured by ELISA.

Results: HIF-1α was induced in 6 hour hypoxic SAECs (p<0.05), but *CD55* transcripts were repressed (p<0.05). CD55 protein was down-regulated by 72 hours (p<0.05). *CA9* transcripts were elevated by 48 -72 hours (p<0.05 and p<0.01, respectively). *In vivo*, *CD55* transcripts and protein were down-regulated by 24 hours post-hypoxia (p<0.01) which corresponded to complement activation (p<0.05) in BALF. However, *CA9* was increased (p<0.01). Chetomin (100nM) treatment in 6 hour hypoxic SAECs, recovered *CD55* transcripts (p<0.01) and protein (p<0.05), but down-regulated *CA9* (p<0.05). Similarly, *in vivo* chetomin (1mg/ml) treatment recovered CD55 protein (p<0.01) and down-

regulated *CA9* (p<0.01). Silencing HIF-1α (50nM) in hypoxic SAECs restored *CD55* transcripts by 6 hours (p<0.05), and protein expression by 24 hours (p<0.05). However, *CA9* was repressed (p<0.01). *In vivo* silencing of HIF-1α (50μg) restored CD55 protein expression (p<0.05) but down-regulated *CA9* (p<0.05). Stabilizing HIF-1α in normoxic SAECs via DMOG (1μM), down-regulated CD55 transcripts and protein (p<0.01), but increased *CA9* within 6-24 hours (p<0.05 and p<0.01, respectively). HIF-1α induction by DMOG (1mg/ml) in normoxic mice lungs down-regulated *CD55* transcripts (p<0.01) and protein (p<0.01), but increased *CA9* (p<0.05). Induction of HIF-1α in AdCA5 (50 PFUs/cell) transduced normoxic SAECs, resulted in CD55 protein down-regulation (p<0.05), but increased *CA9* (p<0.001).

Conclusions: HIF-1α down-regulates CD55 on airway epithelium. Targeting this mechanism may be a potential therapeutic intervention for attenuating complement activation in hypoxic pulmonary diseases.

David S. Wilkes, M.D. Chair

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LIST OF ABBREVIATIONS

AdCA5	Adenoviral Vector Encoding Constitutively Activated HIF-1α
AdLACZ	Adenoviral Vector Encoding Beta-galactosidase
AHR	Airway Hyperresponsiveness
APC	Antigen Presenting Cell
ARD1	Arrest Defective-1
ARNT	Aryl Hydrocarbon Nuclear Translocator
BALF	Bronchoalveolar Lavage Fluid
BCP	1-bromo-3-chloropropane
BCR	B Cell Receptor
BHLH-PAS	Basic Helix-Loop-Helix-Per-ARNT-Sim Family
BPE	Bovine Pituitary Extract
BSA	Bovine Serum Albumin
β-ME	Beta-mercaptoethanol
CA9	Carbonic Anhydrase IX
CBP	Creb Binding Protein
CD19	Cluster of Differentiation 19
CD40	Cluster of Differentiation 40

CD46	Cluster of Differentiation 46
CD55	Cluster of Differentiation 55
CD59	Cluster of Differentiation 59
CD80	Cluster of Differentiation 80
CD81	Cluster of Differentiation 81
CD97	Cluster of Differentiation 97
CHIP	Chromatin Immunoprecipitation Assay
CLAD	Chronic Lung Allograft Dysfunction
CO_2	Carbon dioxide
$CoCL_2$	Cobalt (II) Chloride
CRD	Carbohydrate Recognition Domain
CRPs	Complement Regulatory Proteins
CRRY	Complement Receptor Type-1 Related Gene Y
CR1	Complement Receptor 1
CR2	Complement Receptor 2
CT	Computed Tomography
Ct	Critical Threshold
C-TAD	Carboxy-terminal Transactivation Domain

C1INH	C1 Inhibitor
C3aR	Complement Component C3a Receptor
C4BP	C4 Binding Protein
C5aR	Complement Component C5a Receptor
DAF	Decay Accelerating Factor
DAF 1	Decay Accelerating Factor 1
DAF 2	Decay Accelerating Factor 2
DAMPS	Danger Associated Molecular Patterns
DFO	Desferrioxamine
DMOG	Dimethyloxaloylglycine
DMSO	Dimethyl Sulfoxide
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immunosorbant Assay
EMT	Epithelial to Mesenchymal Transition
EVLP	Ex Vivo Lung Perfusion
FBS	Fetal Bovine Serum
Fc Region	Fragment Crystallizable Region
FIH-1	Factor Inhibiting HIF-1α

FOXP3	Forkhead Box Protein 3
GPI	Glycosylphosphatidylinositol
HDACs	Histone Deacetylases
HDAC 1	Histone Deacetylase 1
HDAC 2	Histone Deacetylase 2
HDAC 3	Histone Deacetylase 3
HDAC 4	Histone Deacetylase 4
HDAC 5	Histone Deacetylase 5
HDAC 7	Histone Deacetylase 7
hEGF	Human Epidermal Growth Factor
HIF-1α	Hypoxia Inducible Factor – 1 Alpha
HIF-1β	Hypoxia Inducible Factor - 1 Beta
HIF-2α	Hypoxia Inducible Factor – 2 Alpha
HIF-3α	Hypoxia Inducible Factor – 3 Alpha
HRE	Hypoxia Responsive Elements
HRP	Horseradish Peroxidase
HSP-70	Heat Shock Protein 70
IHC	Immunohistochemistry

IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IKKβ	IκB Kinase-Beta
IL-1β	Interleukin 1 Beta
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-9	Interleukin 9
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-17A	Interleukin 17A
IL-17F	Interleukin 17F
IL-21	Interleukin 21
IL-22	Interleukin 22
IL-23	Interleukin 23
IL-31	Interleukin 31
IL-35	Interleukin 35

IPAS	Inhibitory PAS Domain Protein
IPF	Idiopathic Pulmonary Fibrosis
IRI	Ischemia Reperfusion Injury
iTreg	Induced T Regulatory Cells
K532	Lysine Amino Acid 532
MAC	Membrane Attack Complex
MAPK	Mitogen-Activated Protein Kinases
MASP	Mannose-Binding Protein-Associated Serine Protease
MASP 2	Mannose-Binding Protein-Associated Serine Protease 2
MBL	Mannose-Binding Lectin
MCP	Membrane Cofactor Protein
MHC I	Major Histocompatibility Complex Class I
MHC II	Major Histocompatibility Complex Class II
MMPs	Matrix Metalloproteinases
MMP-7	Matrix Metalloproteinase 7
mTOR	Mammalian Target of Rapamycin
NFκB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NK Cells	Natural Killer Cells

NO	Nitric Oxide
NODS	Nucleotide Oligomerization Domain
n. s.	Not Statistically Significant
N-TAD	Amino-termini Transactivation Domain
nTreg	Natural T Regulatory Cells
N803	Asparagine Amino Acid 803
OAD	Obstructive Airway Disease
OB	Obliterative Bronchiolitis
ODDD	Oxygen Dependent Degradation Domain
O_2	Oxygen (diatomic gas)
PAMPS	Pathogen Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PFUs	Plaque Forming Units
PGE2	Prostaglandin E_2
PH	Pulmonary Hypertension
PHDs	Prolyl Hydroxylases
PHD 1	Prolyl Hydroxylase 1
PHD 2	Prolyl Hydroxylase 2

PHD 3	Prolyl Hydroxylase 3
PIPLC	Phosphotidylinostyl Phospholipase C
PI3K	Phosphatidylinositol 3 Kinase (is this right alphabetically)
PNH	Paroxysmal Nocturnal Hemoglobinuria
PRR	Pattern Recognition Receptors
PVDF	Polyvinylidene Fluoride
pVHL	Von-Hippel Lindau Protein
P402	Proline Amino Acid 402
P564	Proline Amino Acid 564
rHRE	Reverse Hypoxia Response Elements
RISC	RNA Induced Silencing Complex
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
RORγt	RAR-related Orphan Receptor Gamma t
RPM	Revolutions Per Minute
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
SABM	Small Airway Epithelial Cell Basal Medium

SAECs	Small Airway Epithelial Cells
SAGM	Small Airway Epithelial Cell Growth Medium
SCR	Short Consensus Repeats
SEM	Standard Error Mean
shRNa	Short-hairpin Ribonucleic Acid
siRNA	Small Interfering Ribonucleic Acid
SIRT3	Sirtuin-3
TBS-T	Tris-Buffered Saline with Tween-20
TCR	T Cell Receptor
Tfh Cells	T Follicular Helper Cells
TGF-β	Transforming Growth Factor- Beta
Th1 Cells	T Helper 1 Cells
Th2 Cells	T Helper 2 Cells
Th9 Cells	T Helper 9 Cells
Th17 Cells	T Helper 17 Cells
TLRS	Toll-Like Receptors
TNF-α	Tumor Necrosis Factor- Alpha
Treg Cells	T Regulatory Cells

2-OG	2-Oxoglutartarte

I. INTRODUCTION

A. Immune Responses

The Immune System

Immune responses can be classified as an intricate network of biochemical processes that protect against threats made towards an individual's normal homeostasis. These threats can be in several forms ranging from foreign antigens such as pathogenic microbes (pathogens) to allergenic antigens (1), (2). Physical barriers such as epithelial membranes provide the first line of defense against such threats (3). The immune system is comprised of two other defense mechanisms commonly referred to as innate and adaptive immunity to provide sufficient protection and defense against these attacks (4).

Innate Immune Responses

Innate immunity is defined as immediate, non-specific, host defenses against foreign antigens (5). This form of immune response involves several fast-acting soluble bioactive molecules such as defensins, chemokines, lipid mediators, and complement proteins (2). Interestingly, complement proteins also contribute to innate immunity (2), (6). Although, immunological memory is not associated with innate responses, this form of immune-regulation can still discriminate between specific groups of pathogens through toll-like receptors (TLRs), cytosolic nucleotide-binding oligomerization domains (NODs), or other receptors that recognize pathogen or danger associated molecular patterns

(PAMPS or DAMPS) on antigens (3). Cell-mediated protection in innate immunity is also achieved through mechanisms involving phagocytic cells (macrophages, monocytes, and neutrophils), natural killer cells (NK cells), and cells that release inflammatory mediators (eosinophils, mast cells, and basophils). Phagocytic cells facilitate engulfing of pathogens through complement-mediated opsonization, or by binding to the pathogen through their scavenger or pattern recognition receptors (PRRs) (3). The phagocytosed pathogen is subsequently enclosed in a phagosome which can fuse with lysosomal granules and kill the pathogen by lysosomal enzymes (3). NK cells protect against pathogens by monitoring major histocompatibility complex class I (MHC I), which are present on all nucleated cells (3), (4). If MHC I is present in its normal state on the cell surface, then the inhibitory signal is activated on NK cells, thereby, preventing cellular destruction (3), (4). However, if altered or abnormal MHC I are recognized by NK cells, then the killing signal is activated, and NK cells induce apoptosis (3), (4). Granulocytes such as eosinophils, mast cells, and basophils play a role in innate immunity by releasing inflammatory mediators such as leukotrienes that have chemotactic functions to further enhance the immune response by recruiting more immune cells to the site of inflammation (3). Mechanisms involved in innate immune responses serve as a precursor to initiate activation of more specific form of immunity known as the adaptive immune response.

Adaptive Immune Responses

In contrast to the non-specific and rapid onset of innate immunity, adaptive immunity has evolved mechanisms to target pathogens by immune responses with specific immunological memory. This process of targeting pathogens or other foreign antigens by specific immune responses, occurs gradually over time due to differentiation of naïve lymphocytes to mature effector T cells and antibody secreting B cells, as well as, antigen-specific clonal expansion (3). Both B and T cells originate in the bone marrow (3). However, during the maturation process the B cells remain in the bone marrow, while the T cells migrate to the thymus (3). At the termination of the maturation process, these lymphocytes migrate to peripheral lymphoid organs where costimulatory signals and MHC class II (MHC II) molecules on antigen presenting cells (APCs), such as B cells, dendritic cells, or macrophages and present antigen to T cell receptors (TCR) of $CD4^+$ T cells for initiation of cell-mediated immunity (3). A naïve T cell that encounters an antigen in a peptide-MHC complex is capable of proliferating and differentiating into effector $CD4^+$ T cells that target that specific antigen(3), (7).

Depending on the transcriptional machinery, the microenvironment, and the antigen-specific costimulatory signals involved in $CD4^+$ T cell activation, these T cells can differentiate into several T helper cell subtypes (Figure 1) including: T helper 1 (Th1), T helper 2 (Th2), T helper 9 (Th9), T helper 17 (Th17), follicular helper T cells (Tfh), and regulatory T cells (Tregs) (8). These T helper cell subtypes differ in their ability to produce cytokines and in the type of immune response they elicit. Th1 cells release interferon-gamma (IFN-γ) and interleukin-

2 (IL-2). They are known to play a role in autoimmunity, as well as, targeting intracellular pathogens (8). Th2 cells are characterized by release of the interleukins IL-4, IL-5, IL-10, IL-13, and IL-31, which help target extracellular parasites, and promote allergic diseases such as asthma (8), (9). Th9 cells are characterized by production of interleukin-9 and 10 (IL-9 and IL-10). This cell subset mediates immune responses associated with allergic asthma, intestinal inflammation, colitis, and microbial infections (10). Interleukins 17A, 17F, 21, and 22 (IL-17A, IL-17F, IL-21, and IL-22) are released by Th17 cells, which contribute to autoimmune diseases, and mount a response against extracellular bacteria or fungi (8). Tfh cells are associated with the release of interleukin 6 (IL-6) and IL-21, and participate in humoral immunity by activating antigen-specific B cells within the follicular areas of the lymphoid tissue (8). Tregs exist as thymus-derived (tTreg) and peripherally derived Tregs (pTreg). In contrast to tTregs which readily express transcription factor forkhead box protein 3 (FOXP3) and are released from the thymus, pTregs are induced in the periphery to become Tregs due to presence of transforming growth factor-beta (TGF-β) (8). Despite the differences in their development, both forms of Tregs produce cytokines IL-10, TGF-β and interleukin 35 (IL-35) to help attenuate inflammation (11).

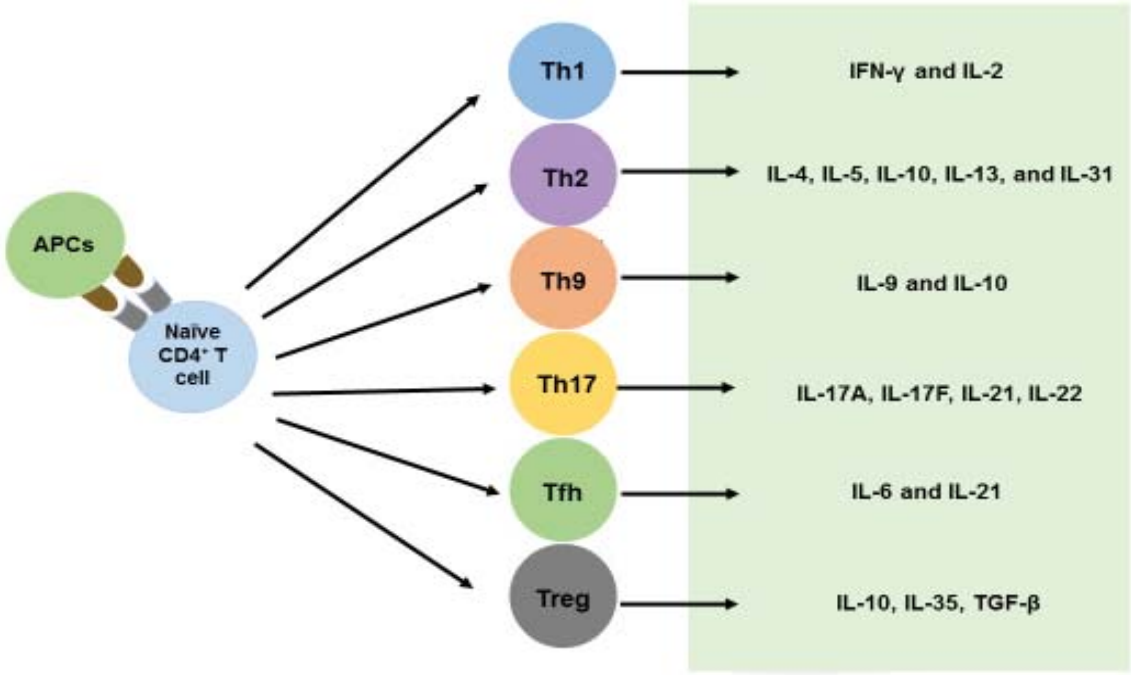

Figure 1. Effector T Helper Cell Subsets. Stimulation of Naïve $CD4^+$T helper cells by APCs, results in formation of various effector T helper cells. Each T cell subset can be characterized by the release of specific cytokines. Adapted from O'Shea and Paul; Science, 2010 (12).

Similar to the role of MHC II in regulating T cell mediated immunity, MHC class I molecules present on all nucleated cells can also activate T cells. However they induce activation of $CD^{+}8$ T cells, commonly known as cytotoxic T cells (7). Cells bearing "non-self antigens," are targeted by $CD8^{+}$ T cells for cytotoxic attack via release of granzymes and perforins (3), resulting in compromised integrity of cellular membranes. Cell death induced by $CD8^{+}$ T cells are specific to cells expressing the antigen bound to MHC I. Thus, sparring adjacent cells from programmed cell death (3).

Adaptive immune responses also involve antibody-mediated protection (3), (6). Naïve B cells are activated in the presence of antigens, and by costimulatory signals from T helper cells. This enables these naïve B cells to differentiate into antigen-specific antibody secreting plasma cells (3). The antibody secreting plasma cells are major factors in adaptive humoral immune response. Through somatic hypermutation, affinity maturation, and class switching, various antibody isotypes are produced. There are five immunoglobulin (Ig) or antibody isotypes (Table 1 and Figure 2): IgD, IgM, IgG, IgE, and IgA. All naïve B cells have cell surface expression of IgD and IgM (3), but IgD is present in very low amounts in the blood (3). IgM exists in a pentameric form, and is the first antibody to be secreted (3). However, the monomeric IgG is the most abundant antibody found in the blood (3). Low levels of IgE, a monomeric antibody, exist in blood and extracellular fluid. However, binding of IgE to its receptors on mast cells induce the release of histamines, which modulate allergic reactions or target parasites (3), IgA is found in the

dimeric form in bodily secretions such as tears or saliva (3), (13). Notably, IgG and IgA antibodies can be further classified into various subclasses (3), (14).

Antibody	Forms	Characteristics
IgD	Monomer	Very low amounts in blood; Present on all naïve B cells; Unknown function
IgM	Pentamer/Monomer	First secreted antibody; Present on all naïve B cells; Activates complement
IgG	Monomer	Most abundant antibody in blood; Activates complement
IgE	Monomer	In blood; Can bind to receptors and release histamines from mast cells
IgA	Dimer/ Monomer	In bodily secretions such as saliva or tears; Neutralization activity

Table 1. Antibody Isotypes

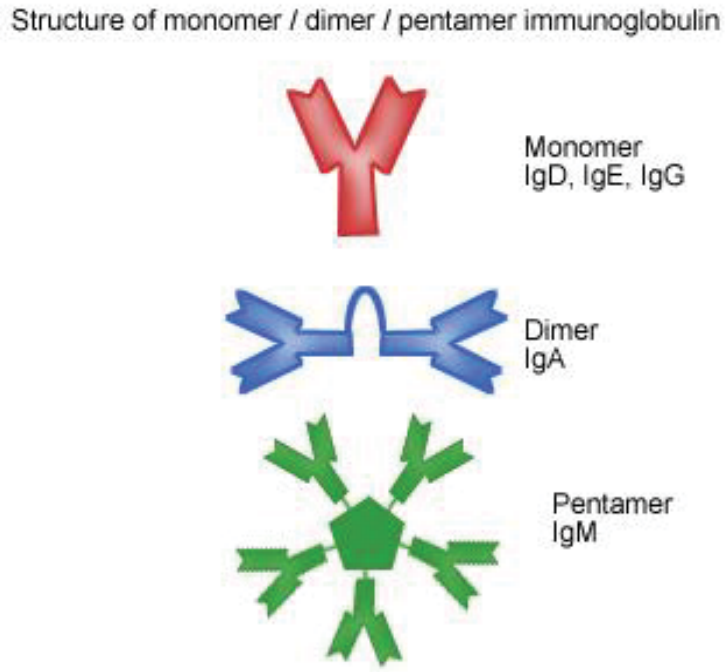

Figure 2. Antibody Isotype Classification. Five major antibody isotypes exist. These antibodies can be structurally classified as being a monomer, dimer, or pentamers. Retrieved from Medscape (15).

Interdependence of Innate and Adaptive Immunity

While innate and adaptive immunity are distinguished as two separate entities of the immune system (Figure 3), a more precise understanding of these responses suggests that the effectiveness of their defense mechanisms depend on their co-existence and interaction with one another (5). Several components of the immune system link innate and adaptive immune response to provide a more efficient mechanism of protection. Examples of such components that bridge the two immune responses include dendritic cells which serve as APCs in adaptive immunity, but also play a role in phagocytosis of pathogens when they invade epithelial membranes within innate immunity (16). Other APCs that bear the phagocytic ability, like macrophages, can also link innate and adaptive immune responses by releasing cytokines referred to as acute phase proteins (3). These acute phase proteins can also induce innate responses such as complement activation (17), but also initiate adaptive immune responses by stimulating dendritic cells for migration to lymph nodes where it can interact and activate T cells (3). Recent studies have also shed light on how NK cells, previously classified as strict innate immune cells, can also play a role in adaptive immune response through properties such as clonal expansion (18). Complement proteins also link adaptive and innate immune responses. Activation of complement proteins in innate response can also modulate adaptive immunity by lowering the threshold for B cell activation, or by binding to their respective receptors on T cells or APCs for immune regulation (19). Several

proteins of the complement system that regulate complement activity, have also been reported to have an impact on T cell differentiation (20), (21).

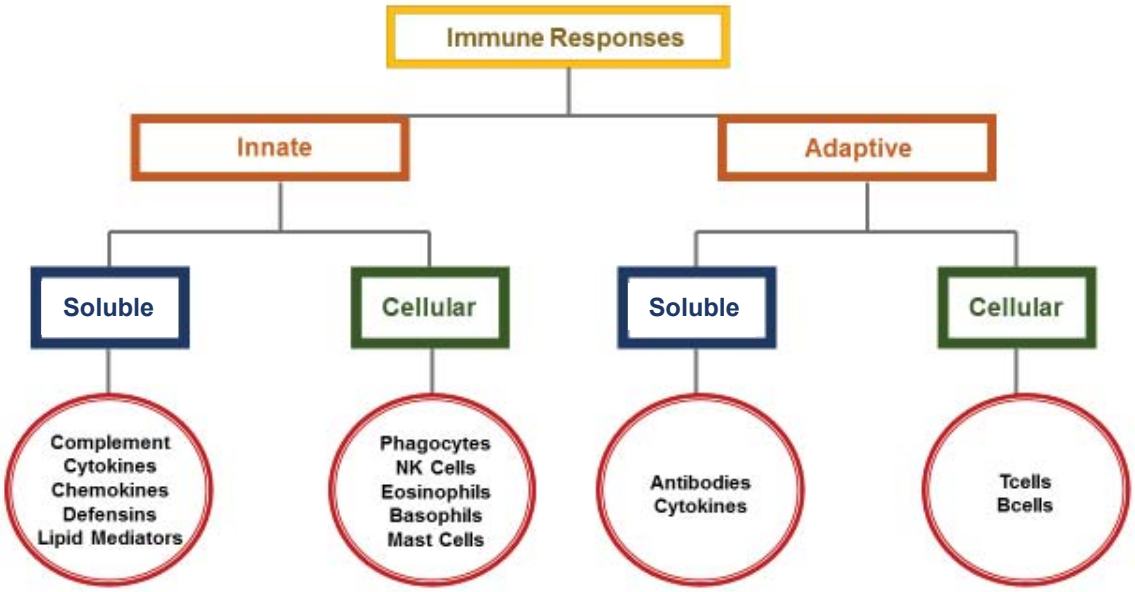

Figure 3. Branches Of The Immune System. The immune system can be characterized by innate and adaptive immune responses. Each one of these responses consist of their specific soluble and cellular factors. Adapted from Westover, et al; Autism, 2011 (22).

B. Complement System

Complement Proteins

Known for “complementing the activity of antibodies,” these heat labile complement proteins in normal plasma and serum were first discovered in the late nineteenth century by Jules Bordet (23), (24). While the liver serves to be the primary site for synthesis of complement proteins (25), local production of complement proteins has also been reported in tissues such as the pulmonary epithelium (airway and alveolar type II epithelial cells) (26). To date, the complement system consists of more than 30 soluble or membrane-bound proteins (27). The wide array of proteins comprising the complement system range from the C3a and C5a complement receptors (C3aR and C5aR), to soluble or membrane-bound regulatory proteins that modulate their activation. These complement receptors and the membrane-bound regulatory proteins are expressed on various cell types including phagocytes, epithelial cells, and T cells (28), (29), (30). Other complement proteins are serine proteases that can be present as zymogens until they are cleaved into their active forms by enzymatic precursors upstream in the signaling cascade (31), (32). Their activation can initiate further downstream proteolytic cleavage of complement proteins within the pathway to elicit an immune responses. Activation of these complement proteins can occur by the serine proteases already present in the complement system or by the presence of these proteases in other pathways such as the coagulation system (33). Mechanisms by which these activated complement proteins facilitate immune responses against the pathogens involve opsonization,

pore formation, or their role as chemoattractants to recruit other immune cells to the site of inflammation (34). Initiation of the complement cascade can occur by three different pathways (Figure 4): classical, lectin, and alternative pathways (1).

Complement Pathways

Activation of complement by the classical pathway results from the formation of immune complexes which involve antigen fixing antibodies such as IgM or IgG binding to pathogen surfaces (35). This interaction allows for the complement protein, C1q, to bind to the (Fc) portion of the antibody. Thus allowing the serine proteases, C1r and C1s, to bind to C1q and become activated. Through their autocatalytic activity, complement proteins C1r and C1s become activated and mediate the formation of C3 convertase by cleaving complement proteins C4 and C2 into C4b and C2a, respectively. Activated complement fragments, C4b and C2a, can associate together on the pathogen surface to form the C3 convertase (1), (36), (37), (38). This C3 convertase can subsequently cleave C3 protein into two fragments: the anaphylatoxin C3a fragment and C3b, which goes on to form the C5 convertase. Similar to the activity of C3 convertase, C5 convertase mediates the proteolytic cleavage of complement protein C5 into C5a and C5b. C5a is an anaphylatoxin, and C5b contributes to formation of the membrane attack complex (MAC) which leads to cell lysis (1), (36), (37), (38).

The lectin pathway is initiated when mannose binding lectin (MBL) binds to carbohydrates present on pathogen surface, necrotic cells, late apoptotic blebs (36), (39). MBL contains a carbohydrate recognition domain (CRD) that allows it to function as a soluble receptor binding to mannose rich glycans on pathogens (36), (39). This binding to the pathogenic surfaces, activates mannose associated serine proteases (MASPs) resulting in downstream complement cascade similar to classical pathway (1), (36). In the same manner, the lectin pathway also results in formation of MAC, and death of the pathogen.

The alternative pathway varies from the classical and lectin pathways in that it activates the complement cascade by a “tickover” process (36), (40). This involves spontaneous hydrolysis of the thioester bond in C3 protein to give it a unique conformation compared to C3 observed in the classical and lectin pathways (40). This altered structure of the hydrolyzed C3 can bind to soluble Factor B, which results in the proteolytic cleavage of this protein by the serine protease, Factor D (36). Binding of hydrolyzed C3 to the cleaved fragments of Factor B can form the C3 convertase of the alternative pathway. In this manner, the alternative pathway acts as an “amplification loop,” because continuous low level C3 hydrolysis results in formation of more C3 convertases (36), (41). The alternative pathway is also capable of utilizing C3b from either the lectin or classical pathway to activate the “tickover” process (36), (41). Despite the differences in activation and formation of C3 convertases among the three complement pathways, they all result in the downstream formation of the MAC and in cellular or pathogenic lysis (36).

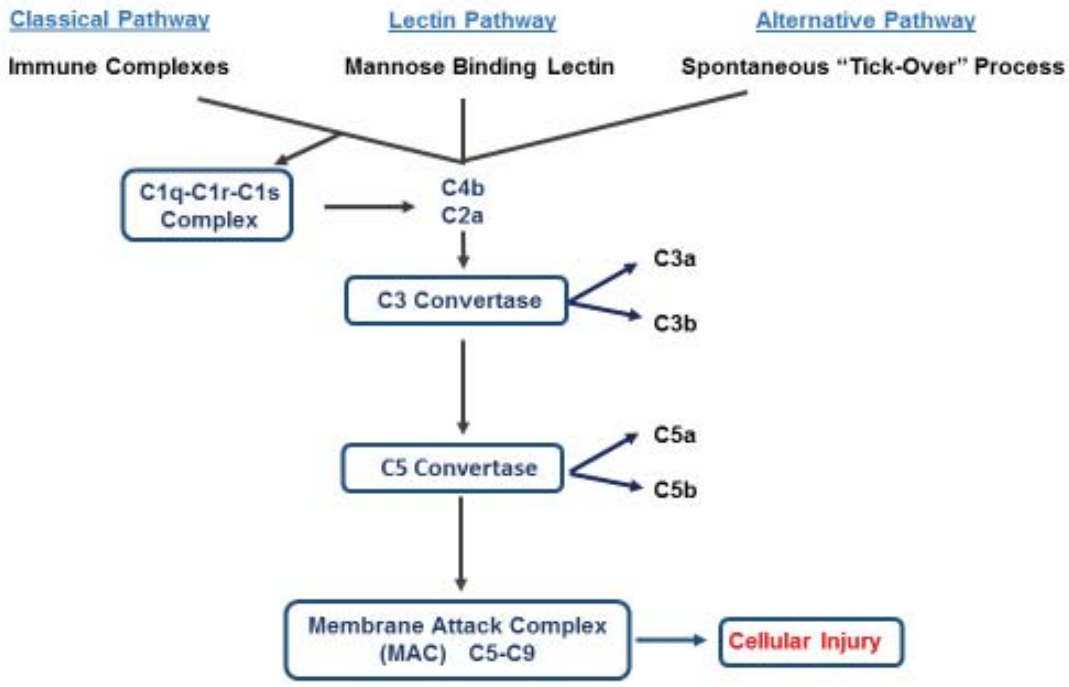

Figure 4. Complement Pathways. Complement activation can be initiated by three major pathways: classical, lectin, and alternative pathway. However, all three pathways converge to form C3 and C5 convertases, resulting in formation of MAC to mediate cell-injury or death. Adapted from Pandya and Wilkes; American Journal of Respiratory Cellular and Molecular Biology, 2014 (36).

Role of Complement in Innate and Adaptive Immunity

Recent advances in recognizing the functional properties of complement proteins, highlight their importance in bridging innate and adaptive immune responses. Previously, complement proteins' role was restricted to innate immunity. However, activated complement proteins with chemoattractant properties, such as C3a and C5a, can bind to their respective receptors and initiate downstream signaling pathways to regulate T cell differentiation and functions of APCs (42). Heeger's group reported that binding of C5a, to its receptor, C5aR, on T cells enhances survival and expansion of these effector T cells by inhibiting their apoptosis (43). Along with the expansion of T cells, signaling by complement proteins also affect T cell differentiation. Blocking the signaling by complement receptors, C3aR and C5aR, by gene therapy or pharmacological inhibitors on T cells, induces the differentiation of these alloreactive effector T cells to be more like pTregs, rather than the Th1 phenotype (44). It has also been suggested that C3a and C5a can induce distinct signaling pathways that lead to different T cell phenotypes. This has been observed in the murine allergic asthma model where C3a induced the formation of Th17 cells through IL-23 production from dendritic cells. However, in these studies C5a signaling inhibited production of Th17 cells (45), suggesting differential roles of C3a and C5a.

While direct binding of C3a and C5a to their receptors on T cells influences the subset of T effector cells being produced, C3a and C5a signaling on APCs can also contribute to T cell differentiation (42). It has been reported

that in certain conditions, C3a and C5a signaling on dendritic cells can induce cell surface expression of MHC II, cluster of differentiation 80 and 40 (CD80 and CD40, respectively), to initiateTh1 responses (46), (47), (48). Other APCs such as macrophages bearing the C3a and C5a receptors can mediate differentiation of T cells into Th1 and Th17 subsets (42), (49). However, the type of T effector cell being produced via complement signaling can greatly vary depending on the type of APC involved, the cytokine milieu in the environment, and the disease model that is being assessed (42), (49).

Complement proteins can also modulate B cell functions in adaptive immunity. The threshold for B cell activation can be lowered when the B cell receptor (BCR) colligates with a receptor complex consisting of complement receptor 2 (CR2) and cell surface proteins known as cluster of differentiation 19 and 81 (CD19 and CD81, respectively) (50), (Figure 5). The mechanism by which the threshold for B cell activation is decreased involves the antigen binding to the BCR, and the opsonized part of the antigen, that consists of C3b, binding to CR2 (50). Complement binding also protects B cells from apoptosis (51).

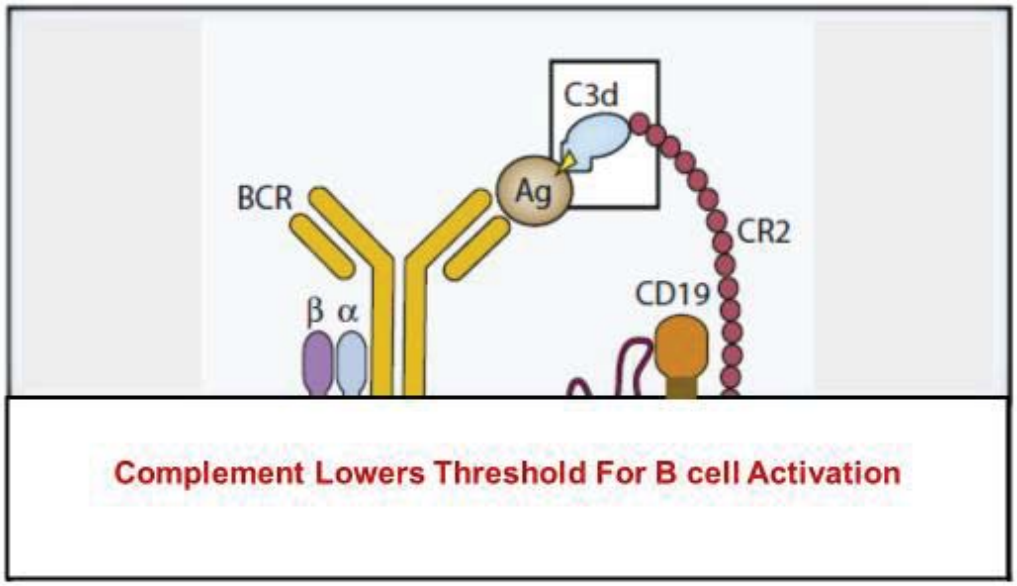

Figure 5. Complement Augments B Cell Activation. Co-ligation of co-receptor complex and BCR with complement bound antigen lowers the threshold for B cell activation. Adapted from Carroll and Isenman; Immunity Review, 2012 (50).

Complement Regulatory Proteins

While the complement system plays a pivotal role in immune-mediated protection against pathogens or other abnormal cells, excessive complement activation can lead to destruction and injury of normal cells or tissues (1), (52). Thus, to limit the activation of complement cascade to pathogenic surfaces or site of infected or abnormal cells, role of complement regulatory proteins (CRPs) becomes critical. While the well characterized role of CRPs in innate immunity is regulation of complement activity (53), they have been recently identified to also participate in regulating adaptive immunity (1). CRPs are present in soluble or membrane-bound forms on various cell types (Figure 6), (53), (54) to provide the precise balance of sufficient, but not excessive complement activation (1). In the complement pathways, CRPs function by regulating complement activation at the level of C3 convertase, C5 convertase, or MAC formation (53).

Soluble, or fluid-phase, CRPs that regulate formation of C3 convertases include C1 inhibitor (C1INH), C4 binding protein (C4BP), and Factor H (53), (55). C1INH regulates complement activity by preventing the autocatalytic activity of C1r and C1s, which inhibits their serine protease activity. In non-inflammatory conditions, C1INH, C1r, and C1s are reversibly bound to prevent complement cascade (53). However, when C1q binds to the Fc portion of the antibody in an immune complex, C1r and C1s can cleave and inactivate C1INH (Figure 6), (53). C1INH, similar to its role on C1q, can also act on mannose-binding protein-associated serine protease-2 (MASP-2) (27). C4BP dissociates C3 convertase (C4b2a), and acts as a cofactor for Factor I to cleave and inactivate C4b (Figure

6), (53), (56). Factor H regulates C3 convertase in the alternative pathway, and is characterized as a functional analogue of C4BP (53). It functions by competing with factor B to bind to C3b, as well as, displacing the already bound activated factor B (Bb) to the C3 convertase (Figure 6), (53), (57). Other soluble CRPs such as clusterin and S protein prevent MAC formation by inhibiting insertion of terminal complement proteins into the cellular membrane (Figure 6), (53).

The four membrane-bound CRPs include: complement receptor 1 (CR1), cluster of differentiation 46, 55, and 59 (CD46, CD55, and CD59, respectively). Similar to fluid-phase CRPs, membrane-bound CRPs also regulate complement activation at multiple phases of the pathways. CR1 expression is restricted to hematopoietic-derived cells such as lymphocytes, phagocytes, and erythrocytes (53). It is functionally characterized by dissociating preformed C3 and C5 convertases, and also mediates cofactor functions for Factor I which cleaves and inactivate C3b and C4b (Figure 6), (53). CD46, also known as membrane cofactor protein (MCP) is a type I transmembrane protein that has functions similar to CR1 (Figure 6). However, unlike CR1, expression of CD46 is distributed among epithelial cells, endothelial cells, and cells of the hematopoietic lineage except for erythrocytes (53). CD46 expression is restricted to reproductive organs in mice (58). However, the murine homologue of CD46, known as complement receptor related protein Y (CRRY) is distributed more widely in mice (Figure 6), (59), (60).

Both, CD55 and CD59, are structurally similar in that they are proteins that are anchored to the cellular membranes by glycosylphosphatidylinositol (GPI) (53). They are expressed on various types of cells ranging from cells in the hematopoietic-lineage, and non-hematopoietic lineage (61), (62), (63). While CD59 acts at the level of MAC to prevent its assembly into the cellular membrane, CD55, also known as decay accelerating factor (DAF), functions in a similar manner to CR1 and CD46 by dissociating and inhibiting formation of C3 or C5 convertases (Figure 6), (53), (55).

Notably some of these CRPs; such as CD46, CRRY, and CD55; also regulate adaptive immunity. CD46 and CRRY have costimulatory properties on human and mouse T cells, respectively (64), (65). It has been reported the complement activation through receptor binding on T cells, enhances proliferation and differentiation of effector T cells (44), (66). However, the presence of CD55 on these T cells, aids in regulating the T-cell mediated immunity associated with allograft rejection (67). CD55 is also involved in T cell regulation by forming a co-regulatory protein complex with cluster of differentiation 97 (CD97) (68). On monocytes, the short consensus repeat-1 (SCR-1) of CD55 can interact with epidermal growth factor (EGF) domains 1,2, and 5 of CD97 on T cells to enhance T cell proliferation and IFN-γ secretion (68).

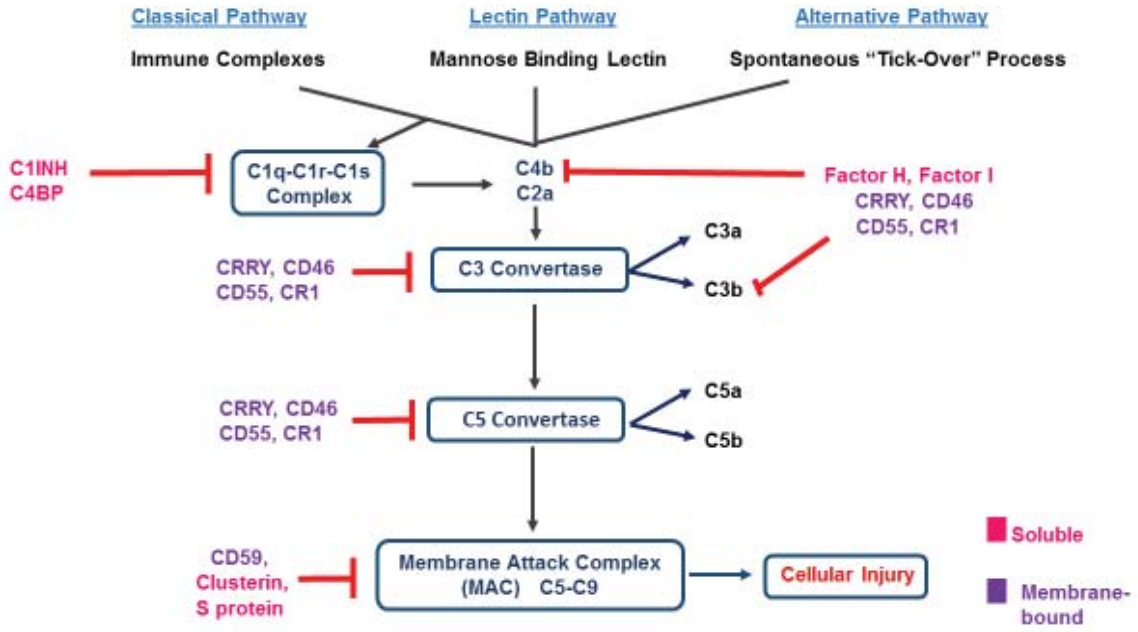

Figure 6. Complement Regulatory Proteins. Activation of complement can be regulated by soluble or membrane-bound CRPs. This regulation can occur in all three pathways. Adapted from Pandya and Wilkes; American Journal of Respiratory Cellular and Molecular Biology, 2014 (36).

C. CD55

CD55 Structure

CD55 (DAF), a GPI-anchored protein, consists of four SCRs that are linked together by two cysteine residues (Figure 7), (69). These SCRs contribute to the complement regulating characteristic of CD55 (70). In particular, SCR subunits 2, 3, and 4 are the primary sites at which regulation of complement activity from the classical and alternative pathways are mediated (70). Each one of the four SCR subunits are highly enriched with serine and threonine residues that serve to properly orient and position the CCPs (Figure 7), (69). This gives rise to variability in molecular weight of the protein (69). Other post-translational modifications such as O-glycosylation of CD55, which serves to stabilize the protein structure, also adds another factor of variability to CD55 expression. At the molecular level, alternative splicing results in two different CD55 transcripts (mRNA) to produce either the GPI-anchored form or the secreted form of the protein (71). The GPI-anchored form is expressed on membrane of several cell types including lymphocytes, erythrocytes, endothelial cells, and epithelial cells (Figure 7), (71), and, the soluble form is expressed in bodily fluids such as saliva, sweat, tears, plasma, and urine (69), (71). In the murine model two CD55 (DAF) genes, DAF 1 and DAF 2, are differentially expressed. DAF 1 is GPI-anchored protein that is more widely expressed, but DAF 2 is a transmembrane protein that is restricted to the testis (72). Despite the presence of various CD55 isoforms, the GPI-anchored CD55 is the predominant form in humans (73), (74).

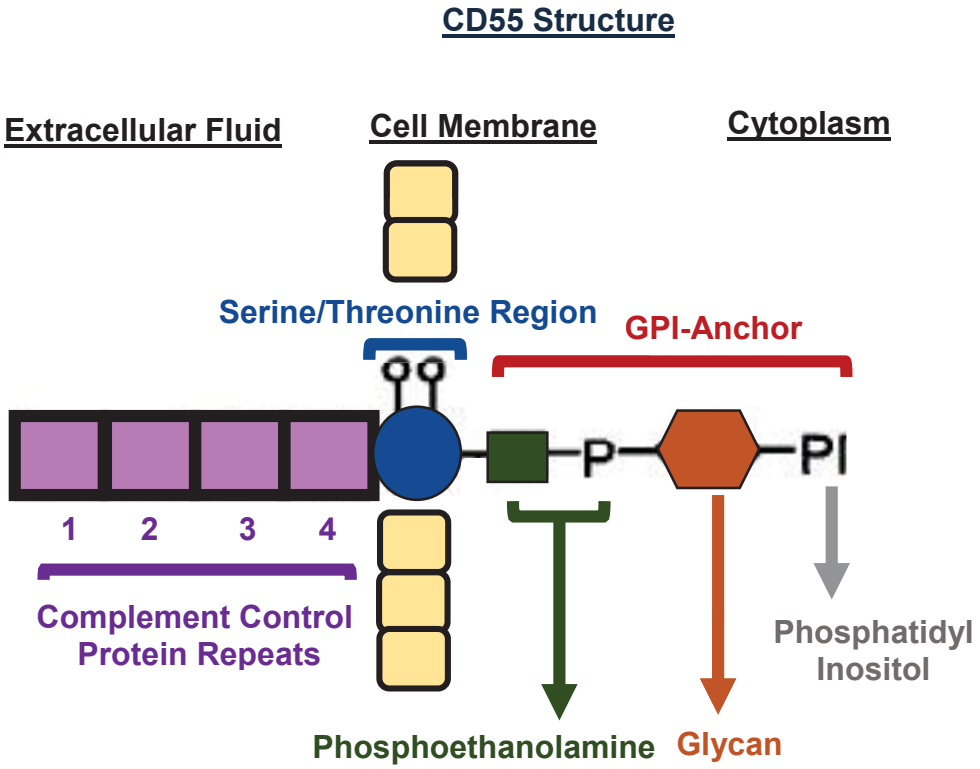

Figure 7. GPI Structure Of CD55. CD55 is a membrane-bound GPI anchored CRP. It regulates complement activity at the level of C3 and C5 convertases. Adapted from Mikesch et al; Cellular Oncology, 2006 (69).

Regulation of CD55

CD55 protein is constitutively expressed on cellular membranes (69). However, depending on the cell type and the environmental conditions that are present, such as the presence of certain cytokines or enzymes, CD55 expression can be induced or down-regulated. Down-regulation of CD55 is observed in pathological states involving infection or disease due to the necessity for complement activation to destroy the infectious pathogen or diseased cells/tissues. Certain pathogens, such as echovirus, utilize the GPI-anchored CD55 as means to enter the host cell (75). Thereby, down-regulating the surface expression of CD55 (75). Other mechanisms involve down-regulation of CD55 by mutation in gene that encodes for GPI-anchor, as observed in paroxysmal nocturnal hemoglobinuria (PNH) (27). CD55 expression was also decreased on airway epithelium in obliterative bronchiolitis (OB) (76). However, the process by which the down-regulation occurs remains unknown. Down-regulation of GPI-anchored CD55 can also be observed when it is cleaved by matrix metalloproteinase-7 (MMP-7) (77) or other enzymes such as phosphotidylinostyl phospholipase C (PIPLC) (78). On the contrary, pro-inflammatory mediators such as TNF-α, interleukin 1- beta (IL-1β), IFN-γ, and prostaglandin E_2 (PGE_2) can induce the expression of CD55 on cell surface under hypoxic tumorigenic conditions (77), (79), (80). However, a decrease in CD55 expression was observed in other forms of tumors such as aggressive breast tumors (81). This variability in CD55 protein expression in tumors or other disease models, can be attributed to the origin of tumor, presence of cytokine milieu in the environment,

and different cell types associated within the context of that particular disease model (81). Notably, complement activation has been observed in certain disease states where down-regulation of CD55 or other CRPs occur.

D. Complement Activation in Pulmonary Diseases

Obliterative Bronchiolitis (OB)

A form of chronic lung allograft dysfunction (CLAD) is known as OB, is a pulmonary disease characterized by irreversible progressive airway obstruction mediated by fibrosis (82). OB is the leading cause of death post lung transplantation. Onset of OB in the clinical setting or in murine orthotopic lung transplants show elevated complement activation in their bronchoalveolar lavage fluid (BALF) (76). Interestingly, studies by Suzuki and colleagues have also reported down-regulation of CRPs, such as CD55, observed on airway epithelium of clinical or murine OB, which may correlate with the local increase of complement seen in the BALF (76). Inhibiting complement activity through C5 neutralization, restored expression of CRPs such as CD55, and attenuated the onset of OB. Notably, neutralizing IL-17A in the OB murine model also resulted in recovery of CD55, along with other membrane-bound CRPs, resulting in down-regulation of C3a levels in the BALF (36), (76). Similarly, stimulation of allo or autoantigen reactive lymphocytes with C3a, induced IL-17A production, thereby, suggesting that the reciprocal regulation between IL-17A and complement

proteins contribute to OB pathogenesis by facilitating down-regulation of CD55 along with other CRPs (36), (76).

However, studies by Mohanakumar and colleagues suggest that obliterative airway disease (OAD), a model that reproduces some OB features, does not require complement activation (83), suggesting that the development of airway obliteration in OB may not be dependent on the pivotal role of complement proteins (83). The contrasting reports by Suzuki and Mohanakumar regarding the role for complement in OB can be attributed to the difference in the animal models being utilized, as well as, pathophysiological differences observed between OAD and OB (36), (83), (84).

a) Mice Strain Differences Impact OB Pathogenesis

It has been previously reported that lung transplant in mice with complete mismatch of major histocompatibility antigens resulted severe rejection and necrosis, but onset of OB was not observed (85). However, histopathological features of OB were observed in the murine lung transplant model where the C57BL/10 male mice are used as the lung donors and C57BL/6 male mice are the recipients in which the lung is transplanted. While both strains of mice possess the H-2b major histocompatibility antigen, they vary in their minor histocompatible antigens H-9, Igh-2, and Lv, but the specific roles for these particular minor antigens have not been elucidated (85). Interestingly however, naïve C57BL/6 and C57BL/10 mice lungs differentially express proteins involved

in the complement cascade. Figure 8 depicts that C57BL/10 mice express higher levels of CD55 in lung homogenates compared to the age and weight matched naïve C57BL/6 mice lung homogenates. On the contrary, CRRY expression remains similar between lung homogenates of naïve C57BL/6 and C57BL/10 mice (Figure 8). Similarly, while no difference was observed in C3aR expression between lung homogenates of naïve C57BL/6 and C57BL/10 mice strains (Figure 9), expression of C5aR was lower in naïve C57BL/10 mice compared to the age and weight matched naïve C57BL/6 mice lung homogenates (Figure 9). These results depict strain-specific differential expression of proteins in the complement pathways are obtained from male mice. Thus, it is also possible that gender of the mice model being utilized is equally as important as the strain of the mice. Notably, factors such as the microbiome and environmental factors can also result in different outcomes within the animal model for a particular disease. This is observed in the murine OB model due to vendor differences. As previously mentioned, the onset of OB occurs when C57BL/10 male mice donor lungs are transplanted into a C57BL/6 male mice (Figure 10) (85). However, this is only observed if the C57BL/10 male mice which are used at lung donors are obtained from Harlan Laboratories but not observed when the C57BL/10 mice are obtained from vendors like Jackson Laboratory (unpublished data). Due to higher CD55 expression in naïve C57BL/10 mice lungs, their down-regulation post-transplant could prevent the regulation of complement which may factor into the post-transplant rejection as previously mentioned (Figure 10) (76). As Wills-Karp and colleagues have

reported in the case of experimental allergic asthma C3a and C5a signaling can have differential roles and may either be protective or detrimental (45). Thus, it is possible that presence of increased C5aR in naïve C57BL/6 mice may result in C5a-mediated protection from lung injury (Figure 10) which may provide an explanation as to why C57BL/6 mice lungs are protected from the onset of OB when used the donor lungs. Collectively, these data suggests that factors like strain, gender, vendor, and microbiome of the animal model being used to recapitulate human diseases such as OB, can influence the outcome.

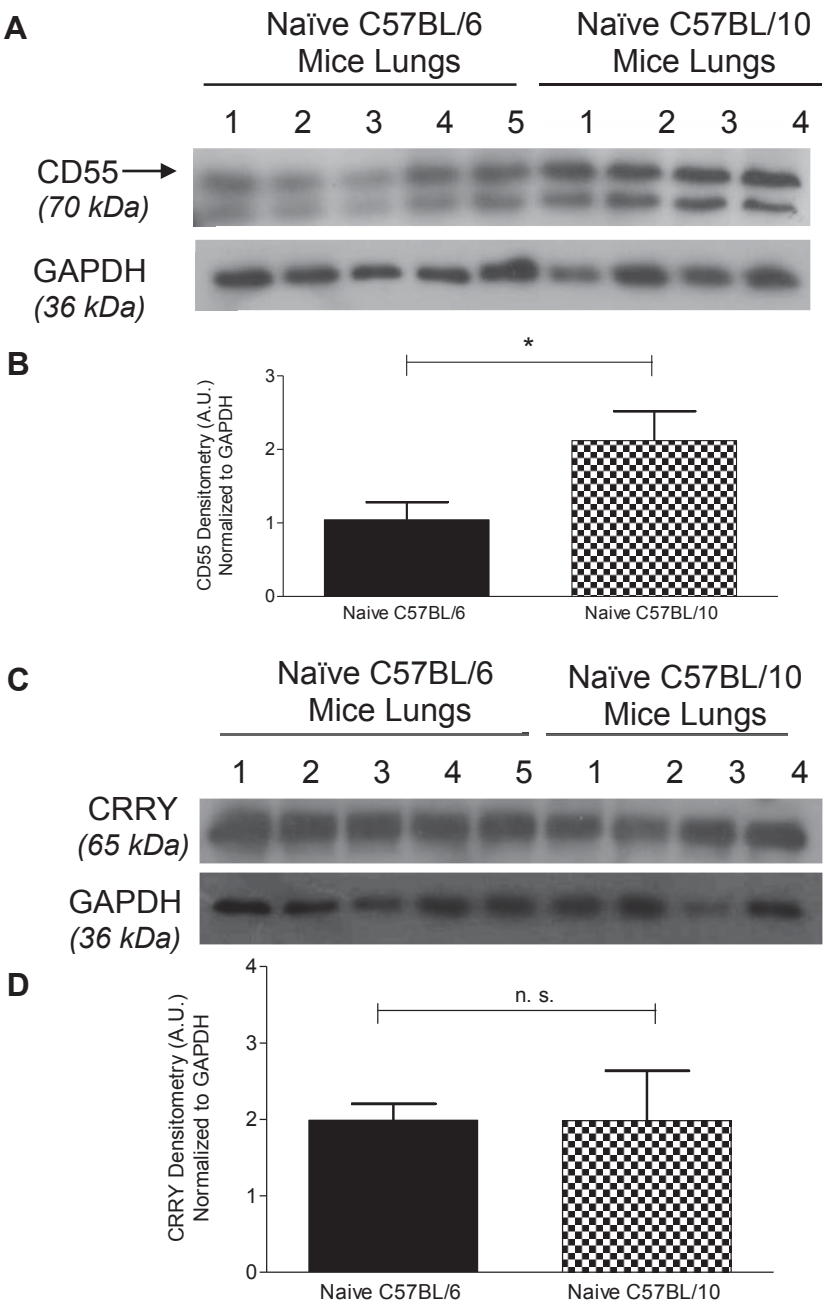

Figure 8. CD55 Expression Higher In Naïve C57BL/10 Lungs Than In C57BL/6 Lungs. **(A)** CD55 expression was probed in naïve C57BL/6 or C57BL/10 mice lungs. GAPDH was the internal loading control. **(B)** Densitometry analysis show elevated CD55 in naïve lungs from C57BL/10 vs C57BL/6. Values represent mean$\pm$ SEM, n$\geq$4, *p<0.05 vs C57BL/6; two-tailed unpaired t-test. **(C)** CRRY expression was probed in naïve C57BL/6 or C57BL/10 mice lungs. **(D)** Densitometry analysis show no statistical significance (n. s.) in CRRY expression in naïve lungs from C57BL/6 vs C57BL/10. Values represent mean$\pm$ SEM, n$\geq$4, n. s. two-tailed unpaired t-test.

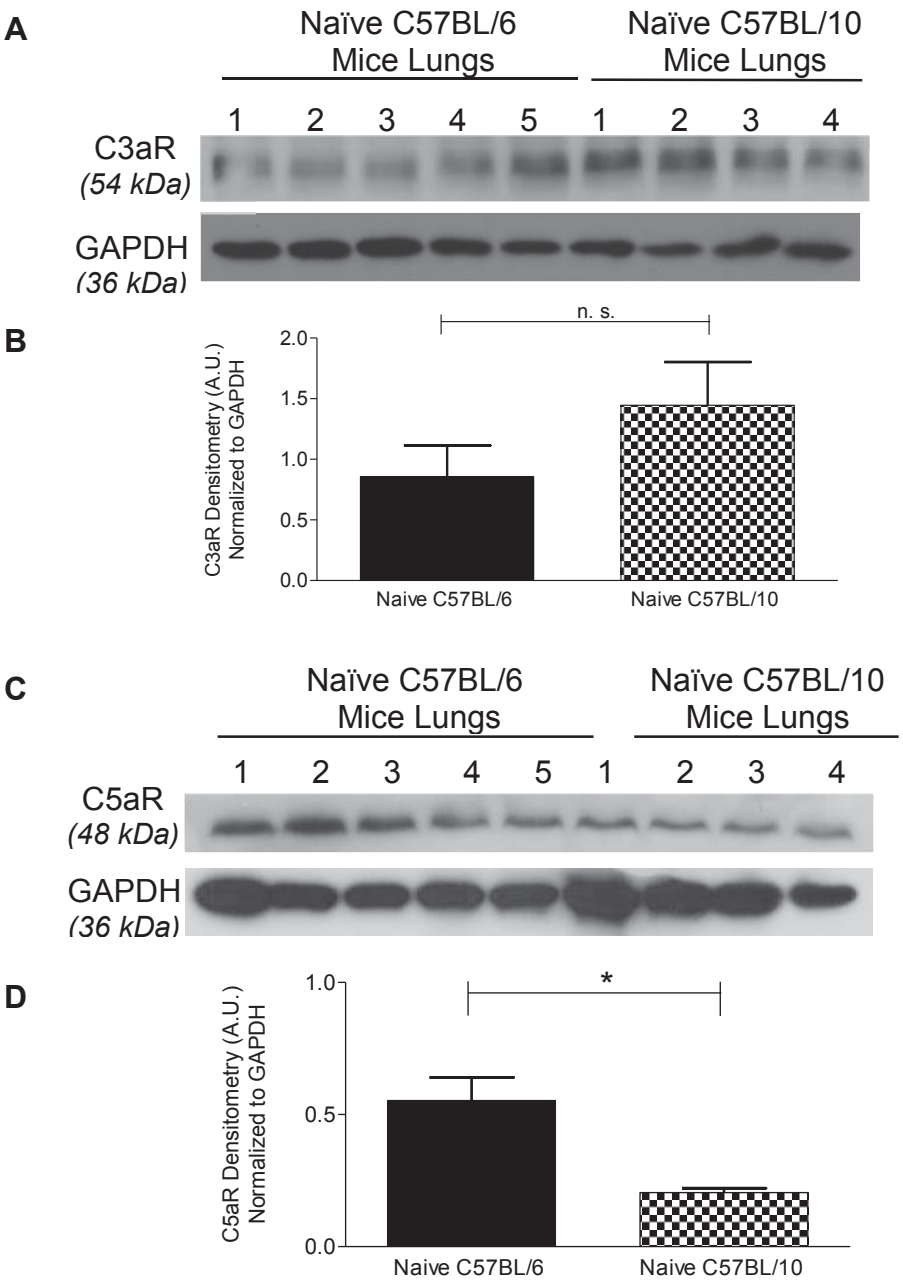

Figure 9. C5aR Expression Higher In Naïve C57BL/6 Lungs Than In C57BL/10 Lungs. **(A)** C3aR expression was probed in naïve C57BL/6 or C57BL/10 mice lungs. GAPDH was the internal loading control. **(B)** Densitometry analysis shows no statistical significance (n. s.) in CRRY expression in naïve lungs from C57BL/6 vs C57BL/10.Values represent mean$\pm$ SEM, n$\geq$4, n. s. two-tailed unpaired t-test. **(C)** CRRY expression was probed in naïve C57BL/6 or C57BL/10 mice lungs. **(D)** Densitometry analysis show elevated C5aR expression in naïve lungs from C57BL/6 vs C57BL/10. Values represent mean$\pm$ SEM, n$\geq$4, *p<0.05 vs C57BL/10; two-tailed unpaired t-test.

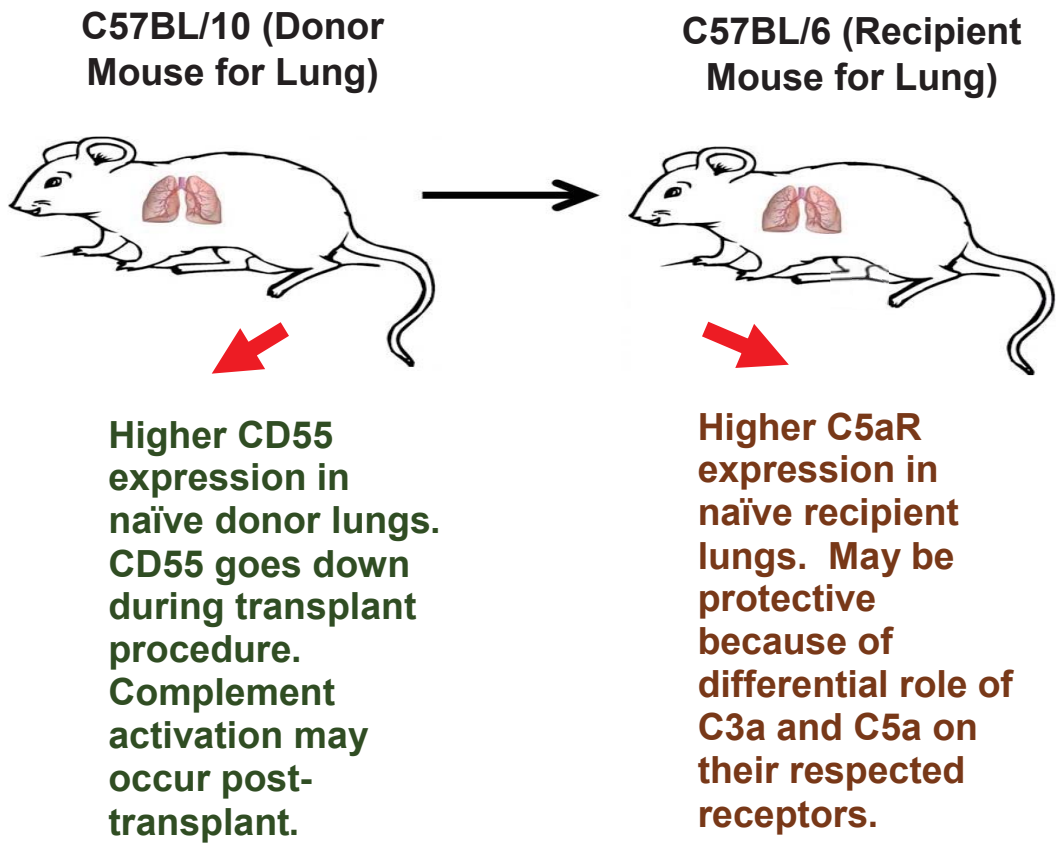

Figure 10. Naïve Lungs From C57BL/10 And C57BL/6 Mice Have Differential Complement Protein Expression. In the murine OB model, naïve C57BL/10 mice lungs are donor lungs and are transplanted into naïve C57BL/6 mice. Naïve C57BL/10 mice lungs have elevated CD55 expression compared to C57BL/6 mice lungs, whereas naïve C57BL/6 mice lungs have higher C5aR expression compared to naïve C57BL/10 mice lungs. This differential expression of complement proteins in these naïve mice, may predispose the donor lungs for getting OB.

Idiopathic Pulmonary Fibrosis (IPF)

The heterogeneity of IPF renders it a complex pulmonary disease to understand, often with poor prognosis (86), (87). Pathological manifestations of IPF include the presence of fibrotic foci in the lungs through the process known as epithelial to mesenchymal transition (EMT), which involves accumulation of extracellular matrix proteins such as collagen, as well as, increased activation and proliferation of fibroblasts (88). IPF is the most common and detrimental form of idiopathic interstitial pneumonias, due its irreversible nature and unknown etiology (88). While environmental factors such as smoking and genetic predisposition have been associated with the onset of IPF, mechanisms contributing to IPF remain elusive (89). However, a role for complement activation in IPF patients has been highlighted (36). Presence of activated complement proteins, and immune complexes that activate complement have been reported in the BALF of IPF patients (90), (91). IPF is observed in animal models involving bleomycin-induced lung fibrosis (92). Notably, complement depletion in these bleomycin injured animals attenuated lung fibrosis, mediated by EMT, which is the hallmark characteristic of IPF (36), (90), (93), (94), suggesting a possible correlation between lung fibrosis and complement activation (94). Mechanisms by which complement activation occurs in IPF have not been elucidated. However, indirect evidence for the role of classical pathway in complement activation in IPF has been suggested due to the presence of immune complexes and autoantibodies targeting the heat shock protein 70 (HSP-70) in IPF patients (95). Studies by Vittal's group indicate down-regulation

of CD55 on airway epithelial cells, which may contribute to the EMT process, and result in the observed complement activation in IPF or bleomycin-induced lung fibrosis (96).

Asthma

Asthma is a complex heterogeneous disease that develops due to variables such as genetics, and exposure to environmental factors (97), (98). The complex nature of this disease can be attributed to its phenotypic and pathogenic variability (97). The disparity in the symptoms associated with this multifaceted pulmonary disease, include variation in airway inflammation, mucus secretion, and bronchoconstriction (97).

At the core of the pathophysiological features of asthma, immune mechanisms include production of Th2 cytokines, IL-17A, and IgE (99), (100). Recent studies have indicated a role for complement activation in asthma as indicated by elevated C3a and C5a levels in BALF of asthmatic patients undergoing allergen challenge (101), (102). Due to structural and functional complexities associated with development of asthma in humans, it is difficult to obtain proper animal models for this disease (103). Among mammalian species, asthma is only developed in humans and in certain animals such as cats or horses (103). Therefore, a physiological characteristic of asthma known as airway hyperresponsiveness (AHR), which is initiated through acute exposures of antigens, such as ovalbumin, is utilized for animal studies involving rodents to

model asthma (103). Studies by Wills-Karp suggest that complement activation in asthma does not always correlate with exacerbation of lung injury (45), (104). Rather, differential roles of C3a and C5a in ovalbumin-induced allergic AHR can result in increase or decrease in AHR severity, respectively (45). Mechanisms that mediate the opposing effects of C3a and C5a in asthma or AHR have not been fully elucidated. However, it has been reported that C3a and C5a play reciprocal roles in IL-23 dependent Th17 production (45). C3a can induce IL-17A production from Th17 cells in AHR, as well as, recruit and activate Th2 cells (45), (105), (106). In contrast, the reverse is true for effects of C5a on TH17 cells in AHR (45). Notably, protective effects of C5a in AHR have also been reported in studies by Lewkowich, where it has been indicated that C5a mediated activation of plasmacytoid dendritic cells can drive T regulatory induced AHR tolerance (107). This observation of increased complement activation in ovalbumin-induced AHR model may be correlated to the observed decrease in bronchiolar expression of CRPs such as CD55 (J. Lott and D. S. Wilkes, unpublished observations).

Lung Ischemia Reperfusion Injury (IRI)

Ischemia reperfusion injury (IRI) consists of two critical components: the ischemic phase, and the reperfusion phase. Ischemia is characterized by the lack of blood flow to tissue or organ which results in inadequate oxygen levels, also known as hypoxia (108). Reperfusion involves restoring the blood flow to the ischemic organ or tissue, restoring oxygenation (108). Lung IRI occurs

during lung transplant (109). However, IRI can also ensue through assaults inflicted on the lung during hypotension, pneumonia, aspiration, and mechanical ventilation (110). One component of the immune response that contributes to inflammation in lung IRI within the context of lung transplantation, is activation of complement (111). Studies by Keshavjee and colleagues have utilized methods to inhibit activated complement proteins by administering soluble CR1 in a swine lung transplant model (112), as well as, in human lung allografts prior to reperfusion (113). These types of methods can improve oxygenation, reduce lung edema, and attenuate other inflammation that occur in the transplanted lung during IRI (112), (114), (115).

One mechanism by which detrimental effects of complement activation in lung IRI are achieved, involve the chemoattractant properties of activated complement proteins binding to their receptors on leukocytes such as neutrophils to initiate further inflammation (109), (116). Similar to other pulmonary diseases, complement activation is not the sole causative factor for pathogenesis of lung IRI (116). While inhibiting complement activation in lung IRI has abrogated some lung injury during transplant, the more suitable therapeutic intervention for lung IRI may involve targeting factors within lung IRI, such as hypoxia, that induce complement activation (108).

E. Lung Hypoxia

Hypoxia Occurring in Lung Pathologies

Hypoxia may be a consequence of lung injury occurring in IRI, pulmonary hypertension (PH), IPF, and the acute and chronic injury that may be observed in transplanted lungs (117), (118). The injury can occur in the airways which may induce hypoxia (Figure 11). Prolonged hypoxia is one of many multi-factorial injuries inflicted on the donor lung during the transplant procedure, which may predispose it for post-transplant rejection. Initiation of hypoxia occurs in the ischemic phase during the lung preservation process, and persists even after the lung is reperfused and transplanted (119), (120). This continuous hypoxic condition post-transplant can be attributed to the lack of revascularization of the bronchial artery, which provides the main supply of oxygenated blood to the lungs (Figure 12), (121), (122). While the pulmonary arteries and veins are reanastomosed during the transplant procedure, the bronchial artery remains unvascularized due to difficulties associated with this surgical technique (121), (122). The absence of a systemic arterial blood supply, delivered by the bronchial artery under normal conditions, leads to chronic airway hypoxia. Studies by Norgaard's group have assessed the clinical outcomes for restoring the bronchial artery during lung transplant (123). While the revascularization of bronchial artery increased survival in transplant patients, the restoration did not definitively attenuate rejection. Therefore, suggesting that other mechanisms along with hypoxia may be regulating lung rejection. It may also be plausible that the reanastomosis of bronchial artery does occur either through *de novo*

regrowth by angiogenesis or collateralization, but the microvasculature may still be rejected (122), (124), (125). This could result in hypoxia and activation of the immune responses which may affect and injure airway epithelial cells, leading to formation of fibrotic lesions which contributes to CLAD (126). Thus, suggesting that in addition to airway hypoxia other factors, such as hypoxia-mediated innate and adaptive immune-regulation, may also contribute to post-transplant lung rejection or onset of other pulmonary diseases (122), (124).

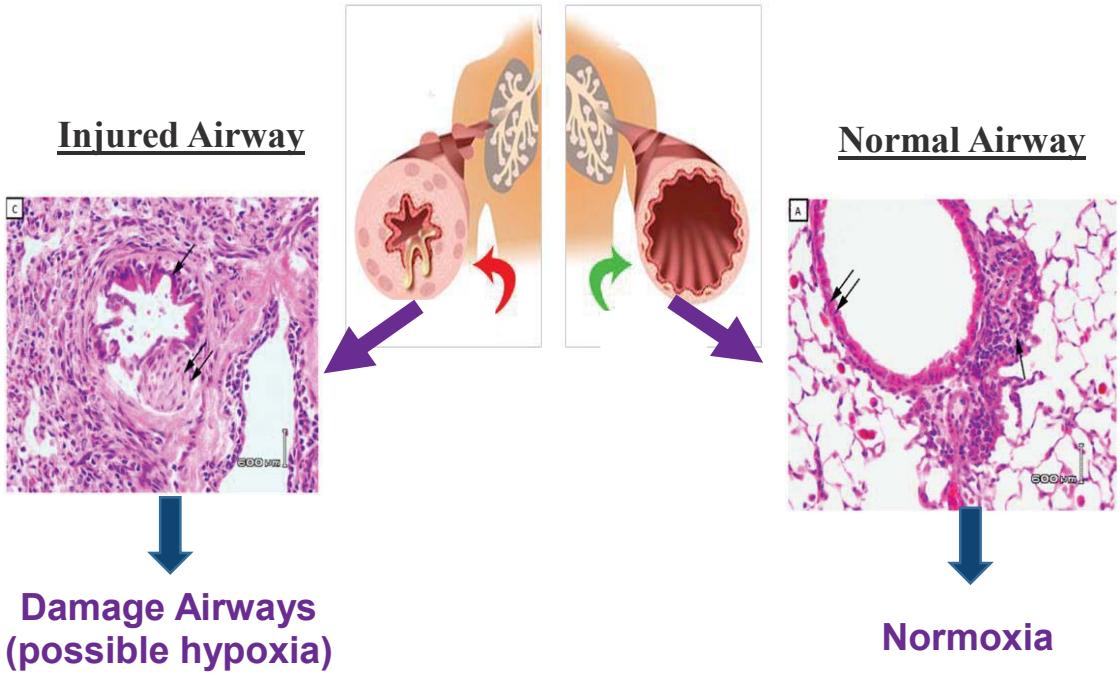

Figure 11. Structure Of The Airways. Normal airways allow for sufficient oxygenation to an individual. However, injury to airway epithelium may occlude the airways, resulting in inadequate oxygenation and hypoxia. Adapted from American Academy of Allergy, Asthma, and Immunology.

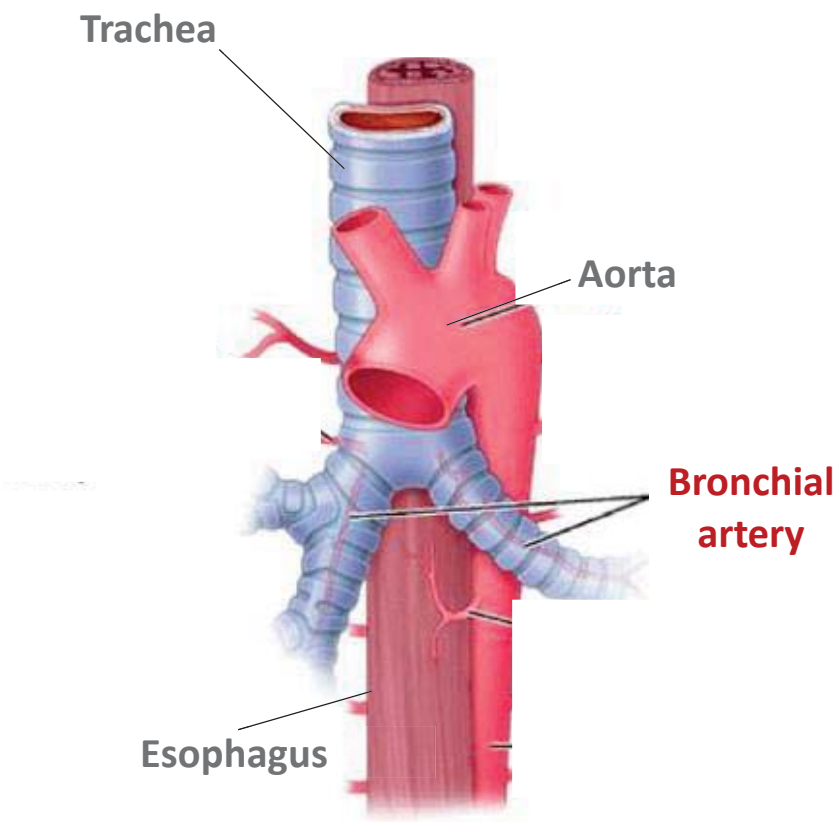

Figure 12. Bronchial Artery. Bronchial artery are the major source of oxygenated blood to the lung (122). Adapted from Medicine Anatomy Clinically oriented from Moore Lecture Notes; Quizlet: Anatomy of the Respiratory Tract, Neck, and Thyroid.

Effects of Hypoxia on Airway Epithelium

Injury to airway epithelium during lung transplant or other pulmonary conditions, play a key role in intiating pulmonary fibrotic lesions that characterize some of these lung diseases, such as IPF and OB (127). These fibroproliferative mechanisms may result from the hypoxic environment of the lung (125). Several studies have indicated a role for hypoxia in induction of fibrogenesis which occurs through a process known EMT (128). This involves transition of adherent epithelial cells to motile fibroblasts, resulting in fibrosis (129). Hypoxic conditions mediating EMT, have been vastly studied in cancer metastasis (130). However, recent studies have highlighted the role of hypoxia and hypoxia dependent transcription factors, such as hypoxia-inducible-factor-1 alpha (HIF-1α), in regulating molecular mechanisms associated with proteins or cell markers of mesenchymal or fibroblast cells (131). Other effects of hypoxia on airway epithelial cells involve the induction of apoptosis and inhibition of cellular proliferation (132). Lung hypoxia also generates the production of reactive oxygen species (ROS), which results in oxidative damage to airway epithelial cells (133).

F. Hypoxia-Inducible Factor-1 alpha (HIF-1α)

HIF-1α Structure

Being a member of the basic helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) protein family (134), HIF-1α structure is comprised of these bHLH and PAS motifs to enable interaction and binding to its constitutively expressed co-subunit known as hypoxia inducible subunit beta (HIF-1β). This interaction results in formation of heterodimeric complex (134). Other basic regions present in the HIF-1α structure mediate DNA binding (135). Downstream of bHLH and PAS motifs, are regulatory domains that facilitate HIF-1α stability and function in hypoxia (135). Stabilization of HIF-1α is regulated by oxygen-dependent degradation domain (ODDD) within the HIF-1α structure. The ODDD is comprised of proline residues critical for oxygen-mediated activity of enzymes that enable prolyl hydroxylation (135) which result in HIF-1α degradation. HIF-1α can also be regulated by two transactivation domains located in the carboxy-terminal end of HIF-1α protein (135). A slight overlap between ODDD and the amino transactivation domain (N-TAD) enables N-TAD to function in regulating HIF-1α protein stability by facilitating its binding to E3 ubiquitin ligase (134), (135). This interaction mediates ubiquitination of HIF-1α. Thus, targeting it for proteosomal degradation. However, the carboxy transactivation domain (C-TAD) plays a role in regulating the transcriptional activity of HIF-1α by enabling CH1 domain of the co-activators to interact with this region of HIF-1α in the heterodimeric complex (134). This interaction between the co-activator and the HIF-1α heterodimeric complex promotes the binding of this transcription factor

complex to hypoxia responsive elements (HREs) on the promotor regions of HIF-1α dependent genes (134), (136). N-TAD is also capable of transactivating HIF-1α dependent genes. However, it is done less effectively compared to C-TAD which is located in the carboxy termini of HIF-1α structure (136).

Regulation of HIF-1α

For physiological adaptations in hypoxic conditions, cells have evolved mechanisms to induce expression of genes associated with processes that can facilitate and promote survival where inadequate oxygen levels are maintained (134), (137). Up-regulation of such genes under hypoxia are predominantly regulated by the transcription factor HIF-1α. HIF-1α is constitutively transcribed, however, post-translational modifications such as hydroxylation, acetylation, and ubiquitination regulate its expression (134), (135). In normoxia where sufficient oxygen levels are maintained, prolyl hydroxylases (PHDs) mediate hydroxylation of proline residues 402 and 564 (P402 and P564) within the ODDD region of HIF-1α protein (Figure 13), (134). Other modifications that occur within the ODDD region of HIF-1α include acetylation of lysine residue 532 (K532) by an acetyl transferase known as arrest-defective-1 (ARD1) (Figure 13), (134). Collectively, both of these post-translational modifications facilitate the interaction of HIF-1α with an E3-ubiquitin ligase known as von-Hippel Lindau protein (pVHL) (Figure 11). This E3 ubiquitin ligase functions by ubiquitinating the HIF-1α protein (Figure 14). Thus, promoting the proteosomal degradation of HIF-1α by 26S proteosome as previously described (Figures 13, 14), (134). However, under

hypoxic stimuli, lack of sufficient oxygen levels result in inactivation of PHDs, and HIF-1α residues P402 and P564 are not hydroxylated (Figures 13, 14). This prevents E3-ubiquitin ligase mediated degradation, and results in stable HIF-1α protein expression (134), (135). Subsequently, HIF-1α translocates to the nucleus to form a heterodimeric complex when bound to its constitutively expressed subunit HIF-1β, also known as aryl hydrocarbon nuclear translocator, (ARNT) (134), (135). Transcription of HIF-1α dependent genes are mediated by binding of the heterodimeric complex interacting with coactivators, such as creb binding protein (CBP)/p300 to HREs, on promoter regions of HIF-1α dependent genes (134), (136).

Activity of PHDs in normoxia are dependent on presence of oxygen, and iron (138). They belong to 2-oxoglutartarte (2-OG)-dependent dioxygenase family and consists of three isoforms: PHD1, PHD2, and PHD3. While PHD1 and PHD3 preferably regulate HIF-2α, PHD2 is the predominant hydroxylase that regulates HIF-1α protein stability in normoxia (139), (140). On the contrary, activity of the acetyl transferase, ARD1, is independent of oxygen levels, but the protein levels are decreased in hypoxia which may explain why increased acetylation of K532 is observed in normoxia compared to hypoxia (134).

Along with the post-translational modifications of HIF-1α that occur in the ODDD region, HIF-1α activity can also be regulated by changes occurring within C-TAD (134). Asparagine residue 803 (N803) is hydroxylated by factor inhibiting HIF-1α (FIH-1) which serves to be an oxygen sensor, but does not influence HIF-1α protein (Figure 13), (134). FIH-1 regulates the transcriptional activity of HIF-

1α by inhibiting the C-TAD interaction with the CH1 domain of coactivators CBP/p300, and preventing the transcription of HIF-1α dependent genes (134).

Stabilization of HIF-1α protein can also occur in normoxic conditions. This can be achieved by mammalian target of rapamycin (mTOR), or phosphatidyl-inositol 3 kinase (PI3K) signaling pathways associated with certain stimuli such as growth factors and cytokines (135). Direct phosphorylation of HIF-1α through mitogen-activated protein kinase (MAPK)-dependent pathways can also regulate HIF-1α dependent mechanisms by increasing its transcriptional activity (134).

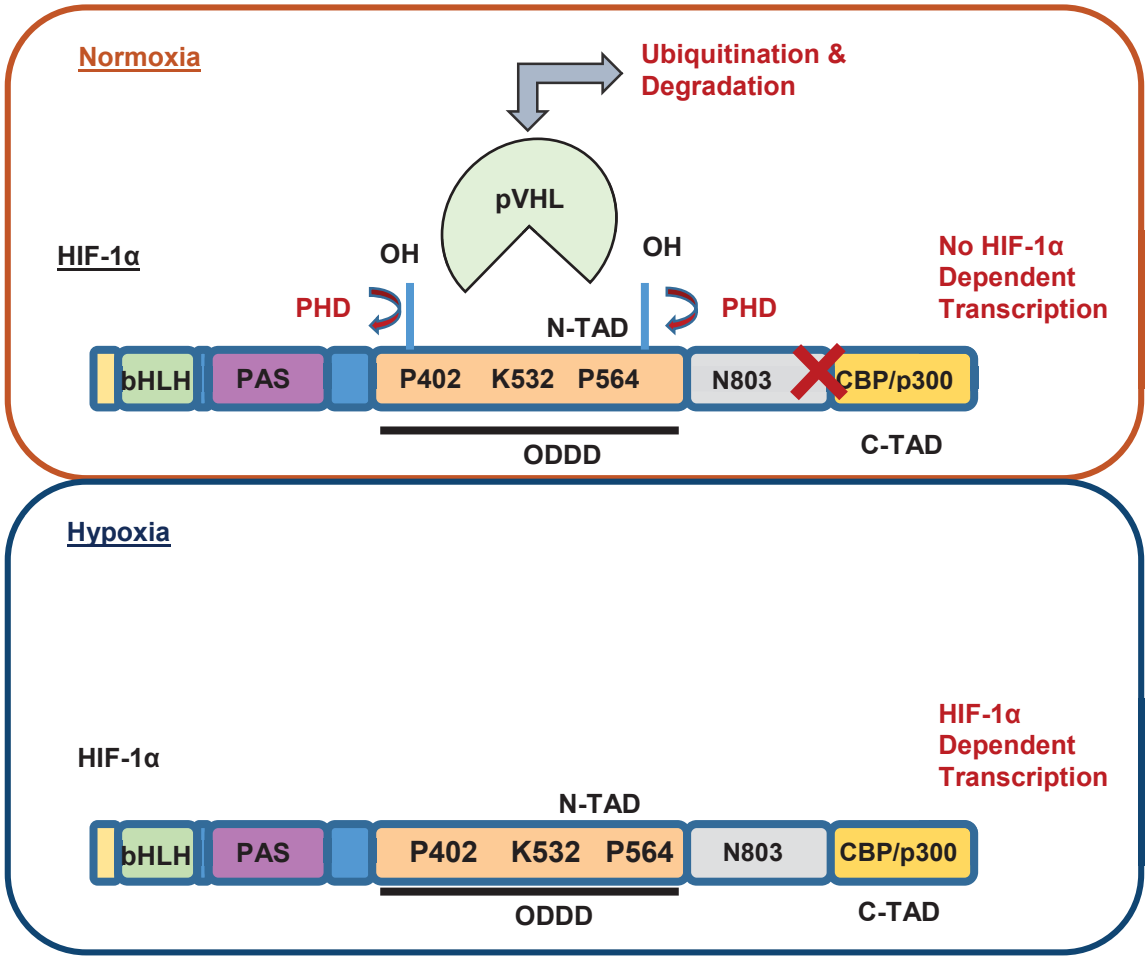

Figure 13. Structure And Regulation Of HIF-1α. In normoxic conditions, HIF- 1α becomes hydroxylated at proline residues 402 and 564 by PHDs. FIH-1 also hydroxylates asparagine 803. Collectively, all these hydroxylations result in degradation of HIF-1α. In hypoxia, these enzymes responsible for hydroxylation are not activated. Therefore, HIF-1α expression is preserved. Adapted from Yeom et al; Cancers, 2011 (141).

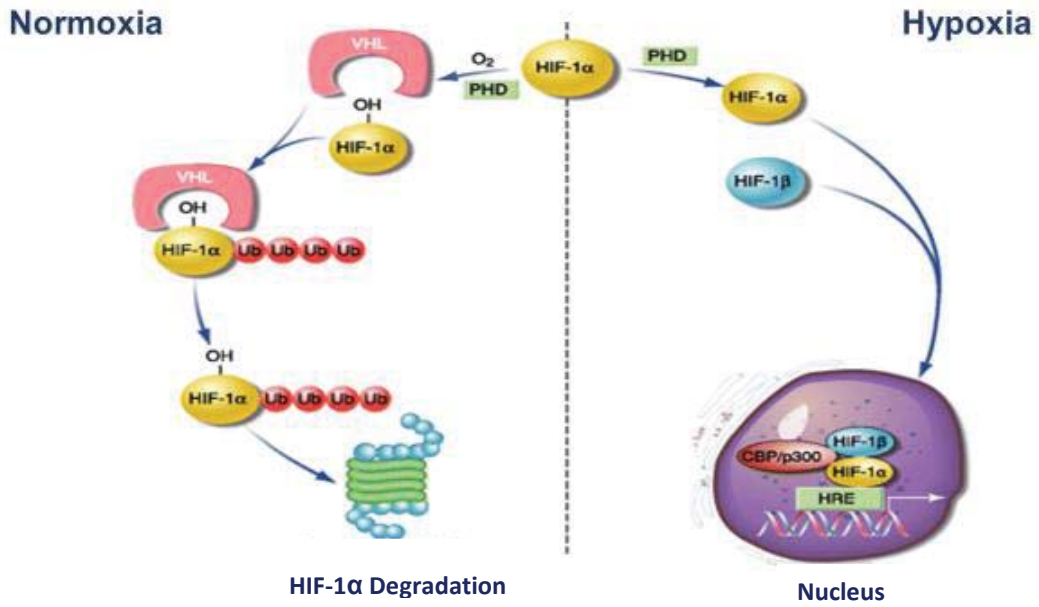

Figure 14. HIF-1α Pathway. In normoxia, HIF-1α is degraded by 26S proteosome due to PHD-mediated hydroxylation. However, in hypoxic environment, expression of HIF-1α is stabilized, because the hydroxylating enzymes are inactivated (135). Adapted from Shimoda and Semenza; American Journal of Respiratory Critical Care Medicine, 2011 (142).

Carbonic Anhydrase IX, CA9, An Indicator of HIF-1α Transcriptional Activity

Hypoxia, through HIF-1α dependent transcriptional mechanisms, is responsible for induction of genes critical for cell survival in hypoxic conditions. HIF-1α dependent genes play critical role in metabolism, cell survival, cell proliferation, angiogenesis, erythropoiesis, and pH regulation during hypoxia (143), (144). Although it has been reported that carbonic anhydrase IX *(CA9)* can be regulated by other transcription factors such as SP1 and SP3 (145), it is also known to be the most uniformly induced genes by HIF-1α which makes it a robust indicator of HIF-1α dependent transcriptional activity (Figure 15), (146).

CA9 is a membrane-bound metalloenzyme that catalyzes reversible hydration of carbon dioxide (CO_2) to bicarbonate and protons (143), (144), which helps regulate pH under hypoxia (Figure 13), (146). Role of CA9 in regulating pH under hypoxia has been studied in tumorigenic conditions (144), where tumors adapt to inadequate oxygen levels by reducing their pH (144). Due to bicarbonate being a factor in synthesis of pyrimidine nucleotides, CA9 can also mediate cellular growth in hypoxic conditions through bicarbonate formation for pH regulation (144), (146).

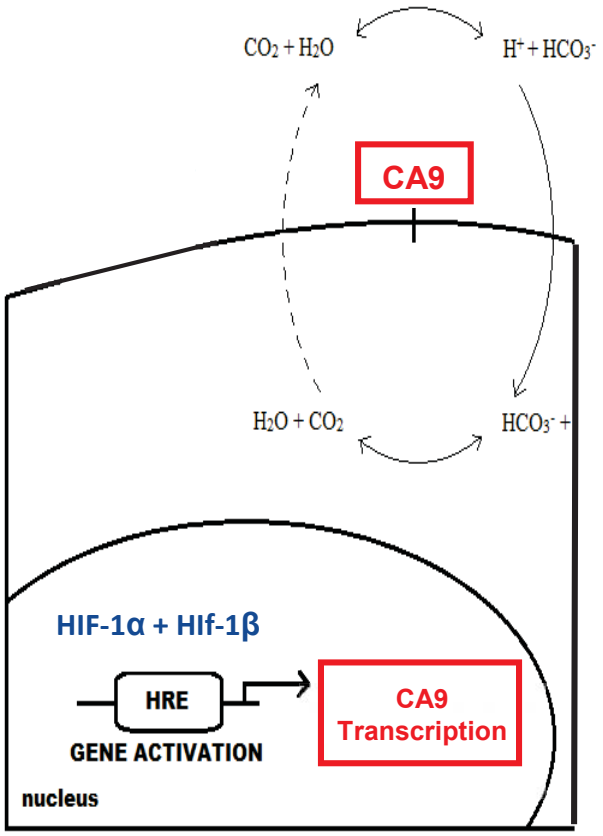

Figure 15. Carbonic Anhydrase IX *(CA9)* Is An Indicator Of HIF-1α Transcriptional Activity. It functions in regulating pH in hypoxic environment by catalyzing reversible hydration of carbon dioxide (CO_2) to bicarbonate and protons (144), (146). It serves to be a good indicator for HIF-1α dependent transcriptional activity. Adapted from Nordfors et al; Oncology, 2013.

Stabilizers and Inhibitors of HIF-1α

Expression of HIF-1α can be pharmacologically stabilized by hypoxia-mimetic agents such as cobalt chloride ($CoCl_2$) (147), desferrioxamine (DFO), (148), and dimethyloxaloylglycine (DMOG) (149) (Table 2 and Figure 16). One mechanism implemented by hypoxia-mimetics to stabilize HIF-1α expression, is to inhibit the activity of PHDs (Figure 16), (150), (151). Inhibition of PHDs by iron chelators, such as $CoCl_2$ and DFO, remove the iron in the active site of PHD critical for its function (Figure 16), (152), (153). As previously mentioned, in normoxic conditions PHD1 and PHD3 preferably regulate HIF-2α, whereas, PHD2 is known to regulate HIF-1α activity (139). Since all three isoforms of PHDs require iron to function (139), these iron chelators can inhibit the function of all three PHDs, thus, resulting in activation of HIF-1α, HIF-2α, and HIF-3α. Another hypoxia-mimetic, DMOG, is an analogue of PHD co-substrate known as 2-oxoglutatarate (154). DMOG competes with all three PHDs for regulation of HIF isoforms (Figure 16). Collectively, these hypoxia-mimetics result in the absence of hydroxylated proline residues in ODDD region of HIF-1α or HIF-2α, thus, preventing their ubiquitination-mediated proteosomal degradation. For mechanisms by which HIF-1α specific expression can be induced, techniques associated with molecular biology are implemented, such as the use of adenoviral vectors encoding HIF-1α gene. Semenza and colleagues have designed a constitutively active HIF-1α, AdCA5, by substituting two critical proline residues with threonine and glutamine, as well as, deletion of certain

amino acids in the HIF-1α ODDD (155). These vectors can be utilized to induce HIF-1α expression in absence of hypoxia.

HIF-1α Stabilizers	Mechanism of Action	Specificity
$CoCl_2$	Inhibits PHDs by chelation of iron	Possible targets: HIF-1α and HIF-2α
DFO	Inhibits PHDs by chelation of iron	Possible targets: HIF-1α and HIF-2α
DMOG	Inhibits PHDs by competition	Possible targets: HIF-1α and HIF-2α
AdCA5	Constitutively active HIF-1α vector	HIF-1α

Table 2. HIF-1α Stabilizers

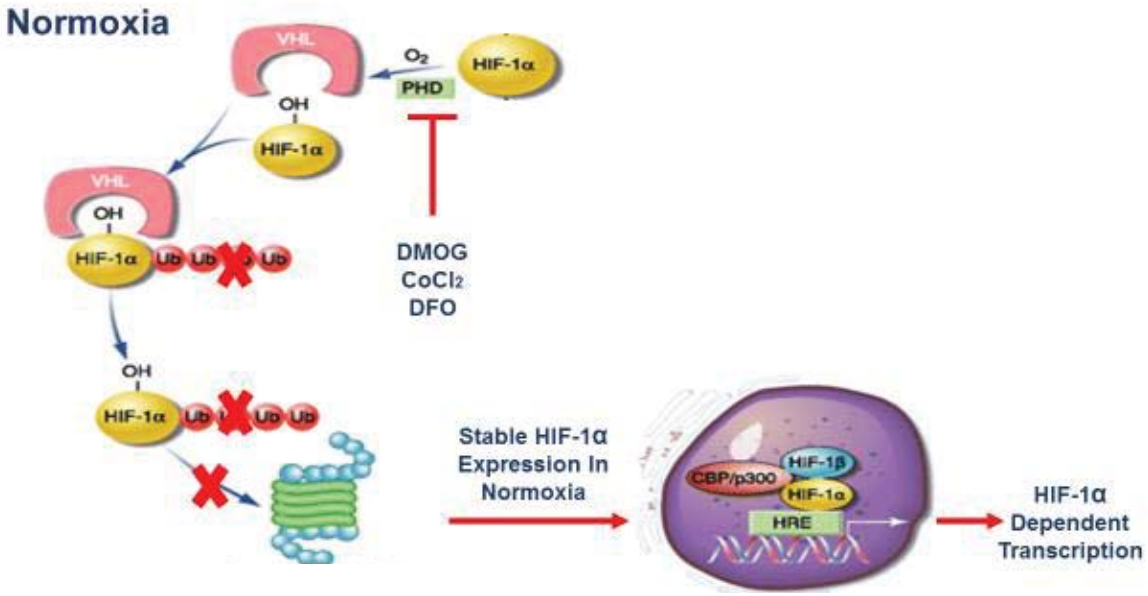

Figure 16. Pharmacological Stabilizers Of HIF-1α. In normoxic environment, HIF-1α protein can be stabilized by inhibiting the action of PHDs. This can be achieved by DMOG which competes with PHDs or by $CoCl_2$ and DFO that chelate irons in the PHD active site (147), (148), (149). Adapted from Shimoda and Semenza; American Journal of Respiratory Critical Care Medicine, 2011 (142).

Pharmacological inhibitors of HIF-1α include chetomin, or digoxin (Table 3). Chetomin is a small molecule, and a natural metabolite of the fungal species *Chaetomium*. It can inhibit HIF-1α dependent transcriptional activity by inhibiting the interaction of C-TAD of HIF-1α or HIF-2α with the CH1 domain of coactivators (CBP/p300), (156), (157), (Figure 17). While chetomin also has the capability to inhibit transcriptional activity of HIF-2α, the effects of chetomin on HIF-1α are more widely studied. Digoxin and other cardiac glycosides have been reported to inhibit HIF-1α expression independent of the well-known PHD mediated ubiquitination-proteosomal degradation pathway (158). The mechanism of action for this may involve digoxin-dependent inhibition of *de novo* HIF-1α synthesis and translation (Table 3), (158). Due to non-specific effects of these pharmacological treatments for HIF-1α inhibition specific mechanisms, such as silencing of HIF-1α through small interfering ribonucleic acid (siRNA) or short-hairpin ribonucleic acid (shRNA) can also be utilized (Table 3).

HIF-1α Inhibitors	Mechanism of Action	Specificity
Chetomin	Inhibits HIF-1α interaction with coactivators	Targets: HIF-1α, and HIF-2α
Digoxin	May inhibit de novo HIF-1α synthesis and translation	Targets: HIF-1α
HIF-1α siRNA	Transient silencing of HIF-1α gene	Targets: HIF-1α
HIF-1α shRNA	Short-hairpin mediated long-term HIF-1a gene silencing	Targets: HIF-1α

Table 3. HIF-1α Inhibitors

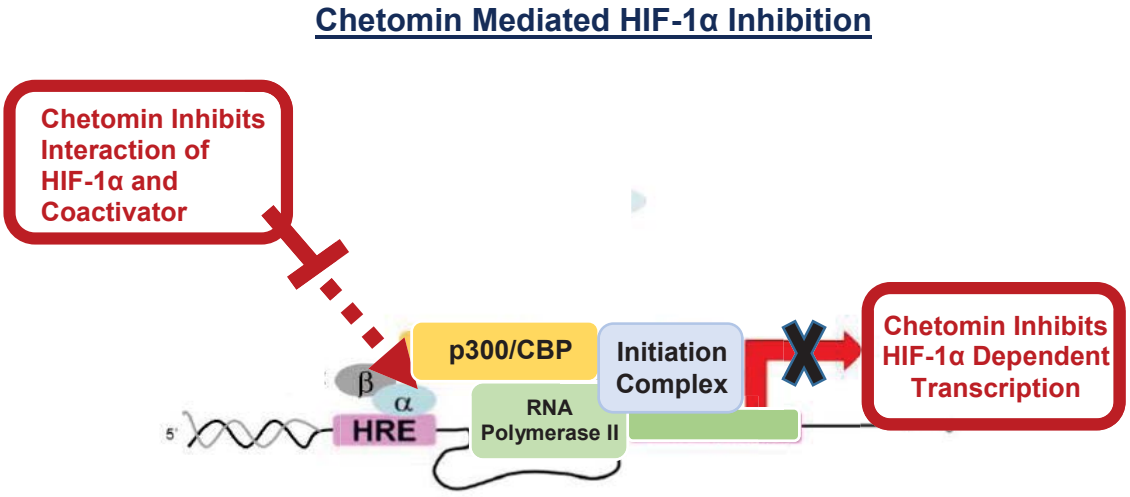

Figure 17. Chetomin, A Pharmacological Inhibitor Of HIF-1α. Chetomin inhibits HIF-1α dependent transcriptional activation of genes by disrupting the HIF-1α or HIF-2α interaction with the coactivator complex (156), (157). Adapted from Yeom et al; Cancers, 2011 (141).

Role of HIF-1α in Airway Epithelium

The role for HIF-1α has been described in several pulmonary diseases due to presence of hypoxia resulting from obstruction or constriction of airways (142), (155). While vast amount of studies focus on role of HIF-1α on alveolar epithelial cells, limited studies in the literature highlight HIF-1α-mediated effects on airway epithelium, particularly in airway epithelial cells that are not immortalized or transformed. However, it has been noted that fibrosis contributes to obstruction of airways by mediating EMT on airway epithelial cells (142). Studies in other models, suggest that HIF-1α may contribute to the EMT process involved in pulmonary fibrosis due to HIF-1α dependent induction of EMT regulating transcription factors such as Snail and TWIST (Figure 18), (159), (160), (161). The EMT regulators can repress epithelial cell markers and induce mesenchymal markers of fibroblast-like phenotypes (162). HIF-1α can also protect the airway epithelium from barrier dysfunction associated with oxidative damage by induction of antioxidant protein, Sestrin-2, which reduces oxidative stress by and preserves tight junction proteins (163). It has also been reported that protection of airway epithelial cells can also be regulated by HIF-1α mediated anti-apoptosis (164).

Notably, HIF-1α has several other roles in the lung which are not restricted to the airway epithelium (142). Due to the hypoxic nature during embryonic development, HIF-1α mediates development of the lung by airway branching (142), (165). HIF-1α mediated gene regulation in the lung also involves

angiogenesis and metastasis of lung-specific cancers (142), or contraction of smooth muscle cells and vascular remodeling as observed in PH.

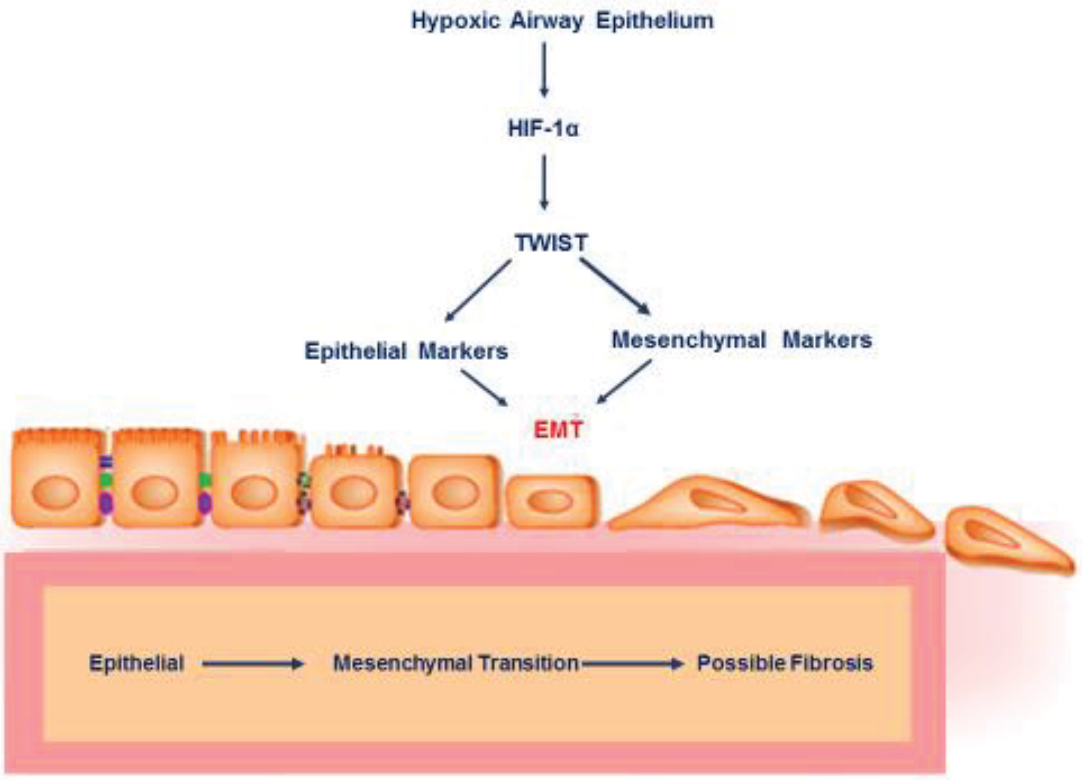

Figure 18. HIF-1α Mediates EMT On Airway Epithelium. Hypoxic conditions induce airway epithelial expression of HIF-1α and, subsequently, induce the expression of transcription factors, such as TWIST, which play a role in EMT (159), (160), (161). Adapted from Aroeira et al; Journal of The American Society of Nephrology. 2007 (166).

Role of HIF-1α in Innate and Adaptive Immunity

Hypoxia initiates injury and inflammatory cascades by production of ROS after reoxygenation (167). However, hypoxic stimuli can also mediate innate and adaptive immune responses through mechanisms involving HIF-1α. Colgan's group reported a role for HIF-1α in regulating innate immunity by showing that HIF-1α dependent induction of epithelial CRP, CD55, can remove neutrophils following their transmigration (80). Notably, HIF-1α also regulates transcription factors, such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), that play a part in innate immunity (168). PHDs, enzymes that regulate HIF-1α degradation, also regulate NFκB activity by inhibiting IκB kinase-beta (IKKβ). This IKKβ enzyme is responsible for phosphorylating and degrading I kappa B alpha (IκBα), resulting in NFκB release and activation (169). Thus, when PHDs are not active in hypoxia due to insufficient oxygen levels, HIF-1α and NFκB become activated (169).

HIF-1α has also been linked to adaptive immunity because of its role in regulating Th17 and Tregs (170), (171). A transcriptional complex forms when HIF-1α binds to RAR-related orphan receptor gamma t (RORγt), and coactivator, p300. This complex can then bind to the IL-17 promoter region and initiate transcription this and other Th17-specific genes (171). Transcriptional activation of RORγt by HIF-1α can also regulate generation of Th17 cells (171). In contrast, HIF-1α negatively regulates Treg cells by binding to FOXP3, transcription factor involved in Treg development, and targeting it for degradation (171). It has also been reported that HIF-1α also mediates activation and

maturation of dendritic cells which enables them to activate allogeneic T cells (172). These studies suggest that the presence of lung hypoxia not only affects airway epithelium, but may also affect lymphocytes or other leukocytes via induction of HIF-1 α.

Other HIF Isoforms

Contrary to the ubiquitous and well-studied hypoxia regulated transcription factor HIF-1α, the role of other members of the bHLH-PAS family, such as hypoxia inducible transcription factors hypoxia-inducible factor 2 alpha (HIF-2α) and hypoxia-inducible factor 3 alpha (HIF-3α), have not previously been well-recognized (135). However, recent studies highlight some of their functional characteristics. While all isoforms of HIF proteins share structural and functional similarities, their expression varies in organ or cell-specific manner (173). Expression of HIF-1α is ubiquitous, however, it has been reported to be predominantly expressed in epithelial cells (135). Whereas, expression of HIF-2α and HIF-3α are more cell specific, and restricted lung endothelium, heart, or the brain (134), (140). Notably, it has also been reported that HIF-2α and HIF-3α can also be induced in epithelial cells of the lung (174), (175).

Cooperative interaction between HIF-1α and HIF-2α in hypoxia has been observed. While HIF-2α can mediate some HIF-1α dependent functions in hypoxia, such as erythropoiesis (176), non-redundant functions between the two isoforms have also been noted. These distinct differences can be attributed to

factors such as the variability in amount of time required for hypoxic exposure, the cell type involved, or temporal differences associated with induction of HIF-1α and HIF-2α (135). It is also possible that due to the short half-life of HIF-1α, expression of HIF-2α may be induced following HIF-1α protein degradation (135), (177). In contrast, while HIF-3α can mediate gene transcription by binding to HREs on promoter regions, a splice variant of HIF-3α known as inhibitory PAS (IPAS) can serve to be a negative regulator of HIF-1α (134). This negative regulation of HIF-1α by HIF-3α is mediated through the interaction of IPAS to the N-TAD of HIF-1α (134).

G. Research Goal

Prolonged hypoxia occurs in the airways post-lung transplant, and in pulmonary diseases associated with obstruction of the airways by fibrosis or bronchiolar constriction (122), (178). Interestingly, these particular pulmonary diseases, such as IPF and OB, have also shown local complement activation which correlates with the observed airway epithelial down-regulation of membrane-bound CRPs such as CD55 (Figure 19), (76), (96). Wilkes and colleagues have reported down-regulation of CD55 transcripts occurring as early as 24 hours post-transplant prior to initiation of any alloimmune responses (76). Down-regulation of CD55 on airway epithelium of lung isografts (data not shown), further confirmed that its suppression was independent of an alloimmune response. These data suggest that injury to the donor lung during the transplant procedure, like hypoxia from IRI, may regulate CD55 expression. Studies by Vittal's group have also reported down-regulation of airway epithelial CD55 expression, and local complement activation in IPF patients (96). Due to this rapid CD55 down-regulation observed in pulmonary diseases associated with epithelial injury and hypoxia, we sought to determine if hypoxia regulated airway epithelial CD55 expression. Specifically, our goal was to assess if HIF-1α regulated CD55 expression on airway epithelium.

H. Hypothesis

HIF-1α regulates CD55 expression on airway epithelium.

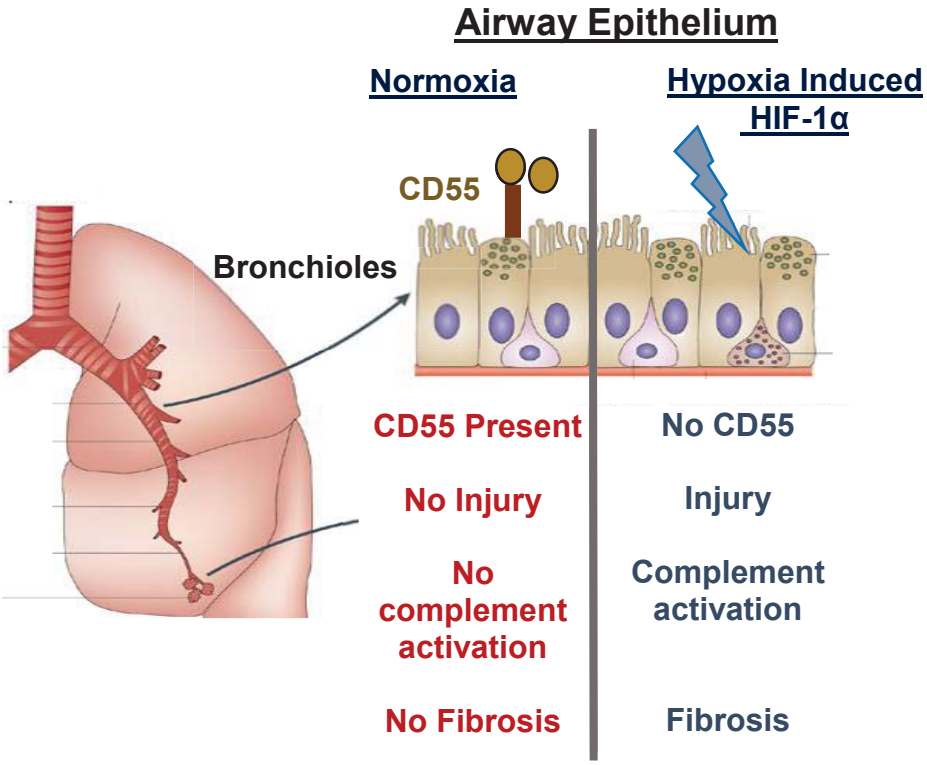

Figure 19. Down-regulation Of CD55 On Airway Epithelial Cells. CD55 expression is present on normal airway epithelium which protects against complement-mediated lung injury. Hypoxia induced HIF-1α mediates the loss of CD55 expression on airway epithelium. Thus, resulting in complement related lung injury and induction of the fibrotic cascade. Adapted from Mouse Lung Anatomy; Revival Corp.

II. MATERIALS AND METHODS

A. Human Small Airway Epithelial Cells (SAECs)

Normal primary human SAECs, from different donors, were purchased from (Lonza Clonetics, Walkersville, MD, USA), and cultured in sterile conditions (96).

B. Animals (Mice)

C57BL/10 or C57BL/6 male mice 6-8 weeks old (25–30g) were purchased from (Harlan, Indianapolis, IN, USA). Animals were maintained in pathogen-free environment, and housed in the Laboratory Animal Resource Center at Indiana University School of Medicine, according to institutional guidelines. All studies were approved by the Indiana University School of Medicine Institutional Care and Use Committee (76). For all *in vivo* hypoxia studies C57BL/10 male mice were utilized.

C. Formulations of Buffers and Media

SAECs Growth Medium (SAGM)

To prepare a 100 ml of SAGM*,* 100 ml of small airway basal medium (SABM) (Lonza Clonetics, Walkersville, MD, USA) was added into a 1 L sterile beaker. To this, the following components (Lonza Clonetics, Walkersville, MD, USA) were added: 100 µl of epinephrine, 100 µl transferrin, 100 µl retinoic acid, 100 µl insulin, 100 µl gentamicin, 100 µl human epidermal growth factor (hEGF),

100 µl hydrocortisone, 100 µl triiodothyronine, 400 µl bovine pituitary extract (BPE), and 1 ml bovine serum albumin (BSA). The media was mixed by pipetting the basal medium containing the growth supplements, and stored at 4^{o}C until ready for use.

Washing Buffer (Western Blot)

In a 1 L bottle, 100 ml sterile 10X phosphate buffered saline (PBS) was added to 900 ml Milli-Q H_2O. To give the wash buffer its detergent-like characteristics, 500 µl of Tween-20 (Sigma Aldrich, St. Louis, MO, USA) was also added to the mixture.

RNA Lysis Buffer

In a 15 ml canonical tube, 10 µl of beta-mercaptoethanol (β-ME) (Sigma Aldrich, St. Louis, MO, USA) was added to 990 µl of RLT lysis buffer (Qiagen Inc., Valencia, CA, USA) to obtain 1:100 dilution.

D. Culturing of SAECs

Cryopreserved normal human SAECs obtained from (Lonza Clonetics, Walkersville, MD, USA), were thawed in 37^{o}C water bath, and collected into a 50 ml tube. Cells were washed with 1 ml SAGM, and spun down at 1500 revolutions per minutes (rpm) for 5 minutes. Supernatant was aspirated off, and

cell pellet was resuspended in 10 ml SAGM for culturing in one T75 tissue culture flask. This was termed passage 0. Once SAECs reached 70-80% confluency, the cells were sub-cultured and passaged further. SAECs were rinsed with HEPES-buffer saline solution (Lonza Clonetics, Walkersville, MD, USA), and 6 ml of trypsin (Lonza Clonetics, Walkersville, MD, USA), was added to the cells for 2-4 minutes to disrupt the adherent cell monolayers. Following the disruption of cell adherence, 12 ml of trypsin-neutralizing solution (Lonza Clonetics, Walkersville, MD, USA), was added to inhibit the activity of trypsin. SAECs were collected and spun down at 1500 rpm for 5 min. Supernatant was aspirated off, and cell pellet was resuspended in 20 ml of SAGM to culture into two T75 tissue-culture flasks (10 ml cell suspension/ flask). This served to be passage 1. Similarly, cells were sub-cultured again to passage 2. However, four T75 tissue-culture flasks were used with 10 ml cell suspension/flask). At this point, SAECs were either sub-cultured to passage 3 for experiments or harvested for cryopreservation until further use.

For cryopreservation of SAECs, cells were collected by tryspin/EDTA and trypsin-neutralizing solution, as indicated above. Cells were spun down for 5 minutes at 1500 rpm and media was aspirated off. The remaining cell pellet was resuspended in 1ml total volume containing 80% SAGM, 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA), and 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Rockford, IL, USA). This 1 ml containing SAECs was transferred into a cryovial and a slow freeze process of cells was implemented by placing them in Mr. Frosty^{TM} freezing container (Thermo Fisher Scientific,

Rockford, IL, USA) for 12 hours. For long-term storage of SAECs, the cryovials containing the cells were placed in liquid nitrogen until ready for use. All SAEC studies were performed at cell passage 3.

E. Preparation of RNA Lysates

Preparation of Cellular RNA

To obtain RNA lysates for studies involving SAECs, 150 µl of the prepared RLT lysis buffer (Qiagen, Valencia, CA, USA) (as indicated above) was added to the cells in tissue culture plates. The cells were then scraped off, collected, and transferred to an RNAse-free tube (Qiagen, Valencia, CA, USA). RNA from these cellular lysates were extracted using the mini RNeasy RNA extraction kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions.

Preparation of RNA from Mice Lungs

To extract RNA from mice lungs, a hand held homogenizer was used to ensure that the lungs with 700 µl of Tri-reagent (Molecular Research Center Incorporation) were properly homogenized. Following this, the lung homogenates were mixed with 7 µl of polyacryl carrier (Molecular Research Center, Inc., Cincinnati, OH, USA). For phase separation, 100 µl of 1–bromo–3–chloropropane (BCP) (Molecular Research Center Incorporation) was added to the lung homogenate following the addition of polyacryl carrier. This lung

homogenate mixture was spun down at 14000 rpm for 15min. The clear supernatant was then transferred to the RNAse free columns (Qiagen, Valencia, CA, USA). RNA was collected, and extracted using the RNeasy RNA extraction kit (Qiagen, Valencia, CA, USA) as suggested by manufacturer's instructions.

F. Preparation of Protein Lysates

Preparation of Protein Lysates from Cells and Mice Lungs

Cytoplasmic/nuclear extraction kit (Thermo Fisher Scientific, Rockford, IL, USA) was used to collect cytoplasmic and nuclear fractions from SAECs or mice lung homogenates. This was used to detect CD55 and HIF-1α, respectively, as indicated in the protocol provided by the manufacturer. Nanospectrophotometer (Nanodrop 1000; Fisher Scientific, Wilmington, DE, USA) was used to measure protein concentration. For detection of cytoplasmic proteins in SAECs and in mouse lung homogenates, primary antibodies specific to CD55 (Santa Cruz Biotechnology sc-9156 (H-319) Santa Cruz, CA, USA), beta-actin (β-actin) (Abcam ab8224, Cambridge, MA, USA), CD46 (Santa Cruz Biotechnology (M177) sc-52647), CRRY (Santa Cruz Biotechnology (M180) sc-30214), GAPDH (Meridian Life Sciences H86504M), C3aR (NBP2-15649, Novus Biologicals, Littleton, CO, USA), C5aR (NBP1-61567, Novus Biologicals, Littleton, CO, USA), and vinculin (Abcam ab18058, Cambridge, MA, USA) were used as indicated by their respective manufacturer's instructions. Membrane cytoskeletal proteins vinculin and β-actin were used as the loading controls depending on their

availability at the time of the protein assessment. Nuclear proteins were detected in SAECs or mouse lung homogenates by incubation with antibodies specific to HIF-1α (R&D Systems AF1935, Minneapolis, ME, USA), and the nuclear loading control lamin β1 (Abcam ab16048, Cambridge, MA, USA) according to the manufacturer's protocol. Following the incubation with their specific primary antibodies, polyvinylidene fluoride (PVDF) membranes were washed three times with tris-buffered saline with Tween-20 (TBS-T). Subsequently, the blots were then incubated with their corresponding horseradish peroxidase (HRP) - secondary antibody: goat, rabbit, or mouse (Jackson ImmunoResearch, West Grove, PA, USA). Supersignal West Pico or Femto (Thermo Fisher Scientific, Rockford, IL, USA) were used for detection of protein by chemiluminescence. Quantification of proteins were performed by densitometric analyses as previously described (96).

G. Intratracheal Instillation

C57BL/10 male mice (Harlan, Indianapolis, IN, USA) were anesthesized with isoflurane until no signs of reflex were observed. Due to midline region of the neck being the site for the procedure, mice fur was shaved from this region, and Betadine (Purdue Products, Stamford, CT, USA) solution was applied to the area. In the neck region of these mice, skin incision of approximately 1 cm was made to separate the muscles and expose the trachea for instillation. To instill 50 µl of instillate directly into the trachea, a 30 gauge needle (BD precision glide needles, Franklin Lakes, NJ, USA), that is bent at a 90 degree angle, was used.

Vet bond skin glue (3M Animal Care Products, St. Paul, MN, USA) was used to patch up the incision. Following the instillation and sealing of the injured site, mice were monitored for normal breathing, and full recovery following the procedure (179).

H. Exposure to Hypoxia

In Vitro Studies (SAECs)

To recapitulate hypoxic conditions *in vitro*, SAECs were placed in hypoxic chamber (The Baker Company, Ruskinn Technology Ltd., Sanford, ME, USA), and exposed to 1% O_2 for various time-points. SAECs exposed to ambient normoxic O_2 concentration of 21%, served as our controls (180), (181).

In Vivo Studies (Mice)

For hypoxic animal studies, C57BL/10 male mice (Harlan, Indianapolis, IN, USA), were placed in a custom-made hypobaric hypoxia chamber, and exposed to 10% O_2 for 24 hours. The chamber mediates slow, gradient decrease in oxygen levels from the normal (control) 21% O_2 to the hypoxic 10% O_2. The normoxic control group consisted of age-matched C57BL/10 male mice exposed to the ambient 21% O_2 that is present in the atmospheric air. Animals were allowed *ad libitum* access to food and water (180).

I. Preparation of Chetomin

Stimulation of SAECs with Chetomin

For pharmacological inhibition of HIF-1α in cells, SAECs were treated with 100nM of chetomin (Sigma-Aldrich, St. Louis, MO, USA) 2 hours prior to the hypoxic exposure. The stock 1mg of chetomin was reconstituted in 1.2 ml DMSO to obtain 0.8 mg/ml chetomin. To prepare 100nM of chetomin, 1.3 µl of the 0.8 mg/ml chetomin was diluted in 14.998 ml SAGM. The 100nM chetomin was mixed by vortexing, and 300 µl/well were added to the cells. DMSO was used as the appropriate vehicle controls. For RNA analysis, SAECs were treated with 100 nM of chetomin for 2 hours in normoxic conditions, followed by 6 hours of hypoxic (1% O_2) exposure. To obtain protein data, SAECs were treated with 100 nM chetomin for 2 hours in normoxic conditions, followed by 24 hours of hypoxic exposure (1% O_2). This longer hypoxic exposure for protein data was performed due to lag phase between transcript and protein expression.

Intratracheal Instillation of Chetomin in Mice

For *in vivo* pharmacological inhibition of HIF-1α, 1 mg/ml of chetomin was intratracheally instilled in C57BL/10 male mice (Harlan, Indianapolis, IN, USA) prior to 24 hour hypoxic (10% O_2) exposure. For chetomin preparation, the stock 1 mg chetomin was reconstituted in 100 µl DMSO to give 10mg/ml. To obtain 1 mg/ml chetomin in total volume of 50 µl for intratracheal instillation in mice, 5 µl of the 10 mg/ml chetomin was diluted in 45 µl of 1X PBS. Intratracheal instillation

was performed as stated above (179). Due to the slow gradient decrease in oxygen levels mediated by the animal hypoxic chamber, assessment of RNA and protein analysis in hypoxic animals could not be performed prior to 24 hours of hypoxia. Thus, all *in vivo* chetomin RNA and protein analysis were performed at 24 hours of hypoxia following intratracheal instillation of 1 mg/ml chetomin.

J. HIF-1α siRNA Preparation

Transfection of SAECs with HIF-1α siRNA or Control siRNA

In vitro silencing of HIF-1α in hypoxic SAECs was achieved by lipofectamine- mediated transfection with HIF-1α small interference RNA (siRNA) sequences that target human HIF-1α (M-004018-05-0005; Dharmacon Technologies, Lafayette, CO, USA). The transfection process involved formulating siRNA-lipofectamine complexes to mediate delivery into the cells. The formation of these complexes were achieved by diluting 50 nM of HIF-1α siRNA or the control siRNA known as RNA Induced Silencing Complex (RISC) siRNA (D-001600-01-05; Dharmacon Technologies, Lafayette, CO, USA) with Opti-MEM I reduced serum medium (Gibco, Life Technologies, Grand Island, NY, USA), and dilution of 5 µl lipofectamine (Invitrogen, Life Technologies, Grand Island, NY, USA) with Opti-MEM as indicated by the manufacturer's protocol. Transfection of these SAECs with HIF-1α siRNA or the control siRNA was mediated by the direct addition of these complexes to the cells for 6 hours. Subsequently, the transfection medium was replaced with growth medium, and

the transfected cells were cultured in hypoxic conditions for additional 6 hours or 24 hours for CD55 RNA and protein analysis, respectively.

Intratracheal Instillation of HIF-1α siRNA or Control siRNA in Mice

For *in vivo* knock-down of HIF-1α, HIF-1α siRNA was administered into mice lungs via intratracheal instillation. To complex the siRNA and lipofectamine together for delivery into the mice lungs, 50 μg HIF-1α siRNA targeting mouse HIF-1α (L-040638-00-0005; Dharmacon Technologies, Lafayette, CO, USA), or 50μg control RISC siRNA (D-001600-01-05; Dharmacon Technologies, Lafayette, CO, USA), as well as, 5 μl lipofectamine (Invitrogen, Life Technologies, Grand Island, NY, USA) were diluted in Opti-MEM I reduced serum medium (Gibco, Life technologies, Grand Island, NY, USA) as indicated by the manufacturer's instructions. Intratracheal instillation of HIF-1α or control siRNA in mice lungs occurred after the siRNA- lipofectamine were complexed together at room temperature for 20 minutes (179). Following the 72 hours post-intratracheal instillation of HIF-1α or control siRNA, the mice were placed in the hypoxia chamber for 24 hours.

K. Preparation of DMOG

Stimulation of SAECs with DMOG

For pharmacological stabilization of HIF-1α in cells, SAECs were treated with 1 μM of DMOG (Sigma-Aldrich, St. Louis, MO, USA). DMOG was prepared by reconstituting the 10 mg stock with 200μl of 1X PBS to obtain 50 mg/ml DMOG. To dilute down to 1 μM, 3.5 μl of the 50mg/ml DMOG was diluted in 996.5 μl of 1X PBS to get 1000 μM of DMOG. This was followed by diluting 10 μl of 1000μM DMOG with 9990 μl of SAGM to get 10 ml of 1 μM DMOG. The 1 μM DMOG was mixed by vortexing, and 300 μl/well were added to the cells. 1X PBS was used as the appropriate vehicle control. For RNA analysis, SAECs were treated with 1 μM of DMOG 6 hours in normoxic conditions (21% O_2). For protein analysis, SAECs were stimulated with 1 μM of DMOG for 24 hours in normoxic conditions (21% O_2).

Intratracheal Instillation of DMOG in Mice

In vivo, HIF-1α expression was stabilized in normoxic conditions by intratracheal instillation of C57BL/10 male mice (Harlan, Indianapolis, IN, USA) 1 mg/ml DMOG. To prepare 1 mg/ml DMOG, 10 mg stock of DMOG was resuspended in 200 μl of 1X PBS to get 50 mg/ml DMOG. DMOG was then diluted by taking 1 μl of 50 mg/ml DMOG, and adding it to 49 μl of 1X PBS to obtain 50 μl of 1 mg/ml DMOG. Intratracheal instillation of the 1 mg/ml DMOG in 50 μl was performed as stated above (179). Due to the specific nature of DMOG

activity on HIF-1α stabilization, RNA and protein analysis of these mice were done as early as 6 hours post-DMOG instillation.

L. Stimulation of SAECs with AdCA5

Constitutive HIF-1α expression in normoxic SAECs was mediated by gene therapy involving adenoviral vector, AdCA5. Modifications within the ODDD region of HIF-1α, such as amino acid deletions (residues 392-520) and two point mutations (Pro567Thr and Pro658Gln) (182), led to stabilization and activation of HIF-1α even in the absence of hypoxia (Vincent and Kelly). AdCA5 and the control vector for transduction efficiency, AdLacZ, (encoding Beta-galactosidase), were generously given to us by Dr. Gregg Semenza from John Hopkins University. SAECs were transduced for 24 hours with 50 plaque forming units (PFUs)/cell of AdCA5 or AdLacZ under normoxic conditions (182).

M. Quantitative Real-Time PCR of Genes

For mRNA quantification within *in vitro* and *in vivo* studies, total RNA from SAECs and mice lungs were isolated using mini RNeasy RNA extraction kit (Qiagen, Valencia, CA, USA) (76). Analysis of the RNA purity and concentration was assessed using the Nano Drop ND-1000 (Thermo Scientific, Wilmington, DE, USA) (76). qScript cDNA SuperMix (Quanta Biosciences Inc., Foster City, CA, USA) was used to synthesize cDNA using 25 ng of total RNA, as indicated by the manufacturer's protocol. Reverse transcription and real-time PCR was

performed on Applied Biosystems 7500, as suggested by manufacturer's instructions, to assess mRNA expression levels with taqman gene expression mastermix (Applied Biosystems Life Technologies, Grand Island, NY, USA). The process of cDNA amplification occurred using 2 µL of cDNA which was amplified for 40 cycles at 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 30 seconds. Primers for *CD55, CA9, β-actin* were purchased as follows: mouse *CD55*, Mm00438377_m1 taqman primer (Applied Biosystems Life Technologies, Grand Island, NY, USA); mouse *CA9*, Mm00519870_m1 Taqman primer (Applied Biosystems Life Technologies, Grand Island, NY, USA); mouse *β-actin*, Mm00607939_s1 taqman primer (Applied Biosystems Life Technologies, Grand Island, NY, USA); human *CD55*, Hs00892618_m1 taqman primer (Applied Biosystems Life Technologies, Grand Island, NY, USA); human *CA9*, Hs00154208_m1 taqman primer (Applied Biosystems Life Technologies, Grand Island, NY, USA); human *β-actin*, Hs01060665_g1 taqman primer (Applied Biosystems Life Technologies, Grand Island, NY, USA), and eukaryotic *18S ribosomal RNA,* Hs99999901_s1 (Applied Biosystems Life Technologies, Grand Island, NY, USA) for endogenous control. The type of endogenous control chosen depended on its availability at the time of mRNA analysis. For analysis of the relative expression of target genes, each sample was ran in triplicates. *β-actin* served as the endogenous control, and normalization of the cycle threshold (Ct) values were obtained using the $2^{-\Delta\Delta Ct}$ method (76). Transcripts and mRNA are used interchangeably in the results section.

N. Immunohistochemistry (IHC) Analysis of Mice Lungs

Mice lungs were perfused, inflated, and fixed with neutral buffered formalin. These formalin-fixed lungs were paraffin-embedded and sectioned into 4μM tissue sections (prepared at Indiana University School of Medicine). For CD55 staining of mice lungs, the tissue sections underwent process of de-waxing and rehydration. Antigen retrieval was obtained by incubating in rodent decloaker (Biocare Medical, Concord, CA, USA) at 95°C for 40 minutes in a pressure cooker (Biocare Medical, Concord, CA, USA). This revealed epitopes on the lungs for antibody binding during the staining procedure. To prevent non-specific antibody binding during staining, the sections were blocked with 3% hydrogen peroxide followed by blocking with rodent block M (Biocare Medical, Concord, CA, USA) for 10 minutes and 30 minutes, respectively. Staining of CD55 was achieved by incubating the sections with 1:100 dilution of rabbit anti-human CD55 (sc9156), (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour at room temperature. Following the primary antibody incubation, tissue sections were washed and, subsequently, incubated with the rabbit on rodent HRP secondary antibody (Biocare Medical, Concord, CA, USA) for 20 minutes. CD55 staining was developed by incubating the sections with immPACT DAB peroxidase substrate (Vector Laboratories, Burlingame, CA, USA) for 1 minute and 30 seconds, followed by counter-staining with Gills hematoxylin (Vector Laboratories, Burlingame, CA, USA). Similar procedure was used for CRRY staining but the antibody used was the following: CRRY (Santa Cruz Biotechnology, Dallas, TX, USA (M180) (sc-30214).

For HIF-1α staining, 4-μm sections of formalin-fixed paraffin-embedded mouse lung sections were de-paraffinized and rehydrated. The sections were stained with HIF-1α primary antibody at 1:100 dilution for 30 minutes (NB.100-479, Novus Biologicals, Littleton, CO, USA). Antigen retrieval was achieved by Dako's 'PT Module' (Dako North America Inc., Carpinteria, CA, USA) with their high pH-Target Retrieval Solution using Dako's Flex+Mouse avidin–biotin system (Dako North America Inc., Carpinteria, CA, USA). This process involved incubation with Flex+M linker (Dako North America Inc., Carpinteria, CA, USA), and Flex-HRP (Dako North America Inc., Carpinteria, CA, USA) for 30 minutes each. Three wash steps in Dako TBS wash buffer were performed between each step. A 10 minute incubation with HRP-conjugated to the final reagent was used to develop the brown diaminobenzidine chromogen (Dako North America Inc., Carpinteria, CA, USA). The sections were counter-stained using hematoxylin (Thermo Fisher Scientific, Rockford, IL, USA). HIF-1α staining was performed at Indiana University department of pathology and laboratory medicine.

Images of the stained mice lung sections were visualized and captured using whole slide digital imaging system (Aperio Scan Scope CS Systems, Vista, CA, USA). The system imaged all slides at 20x or 10x.

IHC Quantification

IHC staining was quantified using Aperio Scanscope (ImageScope) 11.2.0.780 imaging software (Leica Biosystems, Buffalo Grove, IL, USA). The

positive pixel count algorithim for image quantification were selected on whole lung or airways, and analyzed as previously described (76).

O. C3a and C5a Enzyme-Linked Immunosorbant Assay (ELISA)

Complement activation was assessed by measuring C3a (MBS703819 MyBioSource Inc., San Diego, CA, USA), and C5a (MBS700538 MyBioSource Inc., San Diego, CA, USA) levels in mice BALF by ELISA, as indicated by manufacturer's instructions. For detection of activated complement proteins, these assays utilized the quantitative sandwich enzyme immunoassay approach. The ELISA plate was read at 450 nm on a Spectra Max Plus (Molecular Devices, Sunnyvale, CA, USA). Analysis of the results were performed on Softmax Pro 3.1.2 software (Molecular Devices, Sunnyvale, CA, USA).

P. Statistical Analysis

Statistical analysis of all the data were performed using GraphPad Prism 4 Software (GraphPad Prism Software, San Diego, CA, USA). One-way ANOVA with Bonferroni post-test, or two-tailed paired t-test, and unpaired t-test were used for statistical analysis. Data are expressed as mean $\pm$ standard error mean (SEM). Statistical significance was observed when $p<0.05$.

III. RESULTS

A. Part I: Hypoxia Induces Expression of HIF-1α and *CA9,* An Indicator of HIF-1α Transcriptional Activity

Expression of HIF-1α is Induced in Hypoxic Human SAECs

The onset of hypoxia in several cells and tissues initiates the induction of HIF-1α to adapt to conditions with insufficient oxygen levels (134). While up-regulation of HIF-1α in hypoxic environment is widely studied in several pulmonary cells (183), the induction of this key hypoxic regulator has not been reported *in vitro* in human SAECs. Thus, to determine whether hypoxic SAECs stimulate HIF-1α expression, western blot analysis was performed, and antibodies against HIF-1α were used to probe for its expression. A time-dependent induction of HIF-1α expression was observed in hypoxic SAECs, with significantly higher expression achieved within 6 hours of hypoxia (1% O_2) (Figure 20A, B, $p<0.05$).

Increased Expression of CA9, An indicator of HIF-1α Transcriptional Activity in Hypoxic SAECs

While figure 20A, B showed induction of HIF-1α in hypoxic SAECs, its functional activity as a transcription factor was not assessed. Therefore, to confirm that the presence of HIF-1α in hypoxic SAECs correlated with its functional characteristic as a transcription factor, mRNA quantification of *CA9* was assessed (144), (146). Transcript levels for *CA9* in these specific SAEC

donors were elevated by 16 hours of hypoxia, with statistical significance being observed at 16-72 hours of hypoxia (Figure 20C, $p<0.05$, $p<0.01$). This interval between induction of HIF-1α and increase in *CA9* transcripts, may correspond to the time it takes for the stabilized HIF-1α to translocate into the nucleus, to direct transcriptional activation of *CA9* (144), (146).

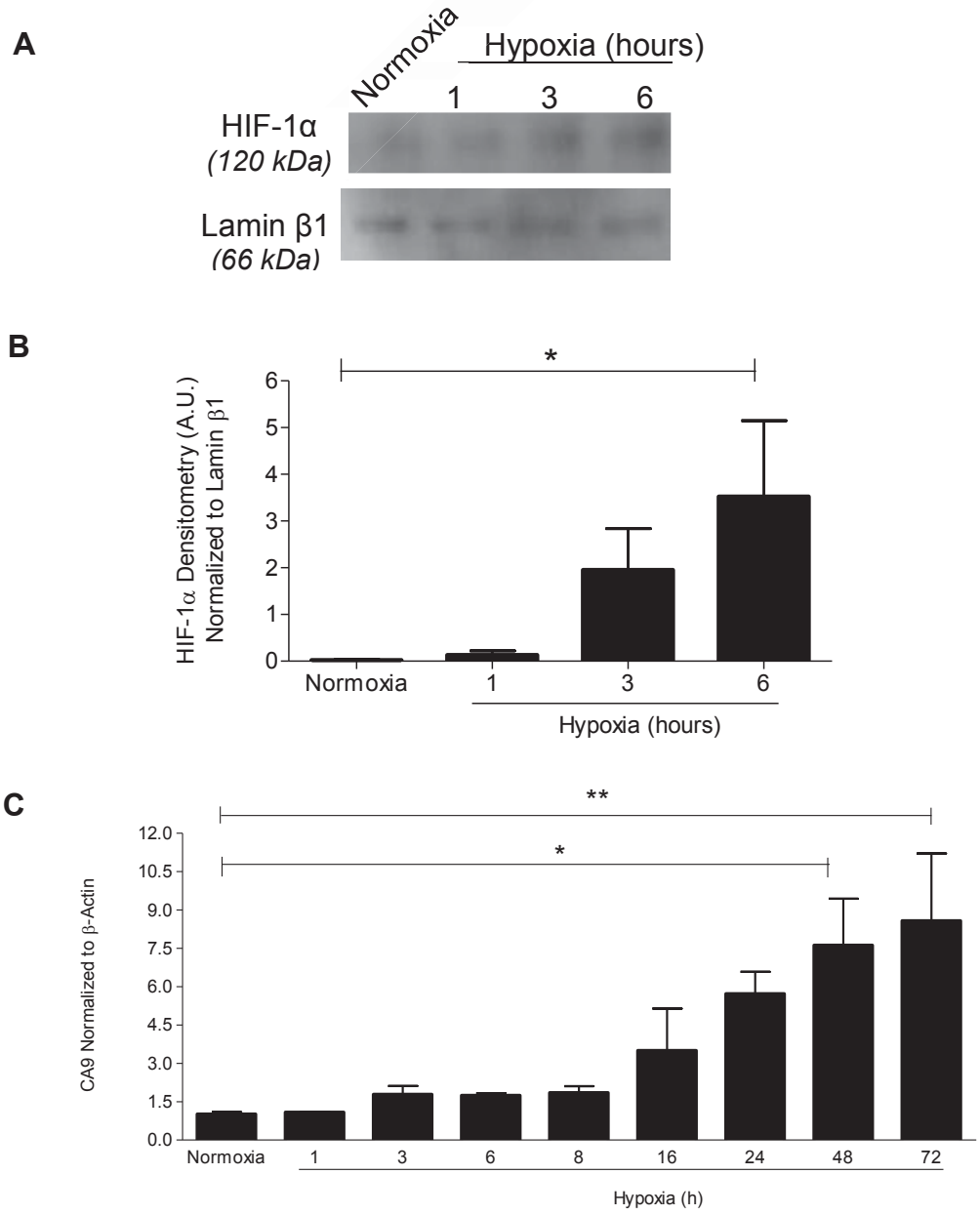

Figure 20. Hypoxic SAECs Induce HIF-1α Expression. SAECs were stimulated with hypoxic conditions by exposure to 1% O_2 for various time-points. Cells exposed to normoxia (21% O_2) were controls. **(A)** Protein lysates of hypoxic SAECs were probed for expression of HIF-1α. Lamin β1 as an internal loading control. **(B)** Densitometry analysis indicated significant up-regulation of HIF-1α by 6h of hypoxia vs normoxic (0h) conditions. Data are representative of means±SEM, n=5, *p<0.05 vs normoxia; One-Way ANOVA with Bonferroni post-test. **(C)** Quantification of *CA9* mRNA showed an induction with increasing hypoxic exposure compared to normoxia. β-actin was used as the loading control. Data represent mean±SEM, n=3, *p<0.05 or **p<0.01 vs normoxia; One-Way ANOVA with Bonferroni post-test.

Hypoxia Induces CA9 Gene Expression in Mice Lungs

Induction of HIF-1α within hypoxic lungs was assessed *in vivo* by exposing C57BL/10 male mice to 10% O_2 for 24 hours. Animal studies required a gradual decrease in oxygen levels over a 24 hour period to 10% O_2 to protect against lethal side effects mediated by the rapid decline in oxygen (184), (185). Due to the short-half-life of HIF-1α protein, and the gradual gradient change in the oxygen levels, expression of HIF-1α may be readily degraded, thus, making its expression difficult to assess in our *in vivo* studies. Therefore, expression of *CA9*, an indicator of HIF-1α transcriptional activity, was analyzed in whole lung homogenates of hypoxic mice. *CA9* transcripts increased in 24 hour hypoxic mice lungs, compared to lungs of mice in normoxic control conditions (Figure 21, $p<0.05$).

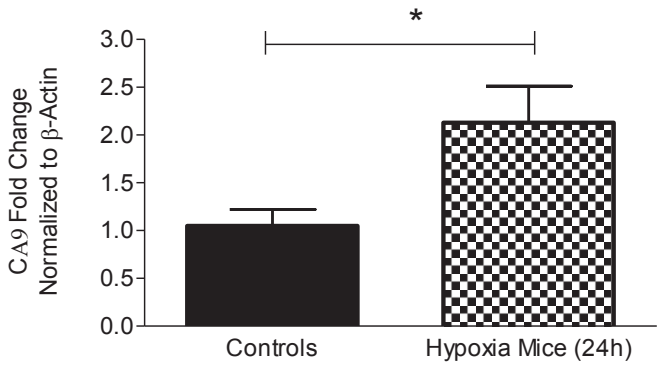

Figure 21. *In Vivo* Hypoxia Results In Induction Of *CA9* In Mice Lungs. **(A)** *CA9* was induced at the mRNA level in lungs of 24h hypoxic mice compared to normoxic control mice. β-actin was used as the loading control. Values represent mean±SEM; n≥4; *p<0.05 vs controls; two-tailed unpaired t-test.

B. Part II: HIF-1α Dependent Down-regulation of CD55 in Airway Epithelium

CD55 Expression is Down-regulated in Hypoxic Human SAECs

To determine whether hypoxic conditions correlate with CD55 down-regulation in these SAECs, *CD55* transcript and protein expression were assessed in hypoxic SAECs (1% O_2) using reverse transcription-polymerase chain reaction (RT-PCR), and western blot analysis, respectively. *CD55* transcripts were down-regulated within 6 hours of hypoxia in the SAECs, compared to the control normoxic SAECs (Figure 22A, $p<0.05$). Notably, the transcriptional repression of *CD55*, and the induction of HIF-1α occurred at the same time point in these hypoxic SAECs (Figure 20A and Figure 22A). These data suggest that hypoxia-induced HIF-1α may correlate with CD55 down-regulation in SAECs. While *CD55* transcript down-regulation occurred as early as 6 hours post-hypoxia, down-regulation of CD55 protein expression was not observed until 72 hours of hypoxia for these particular donors (Figure 22B, C, $p<0.05$).

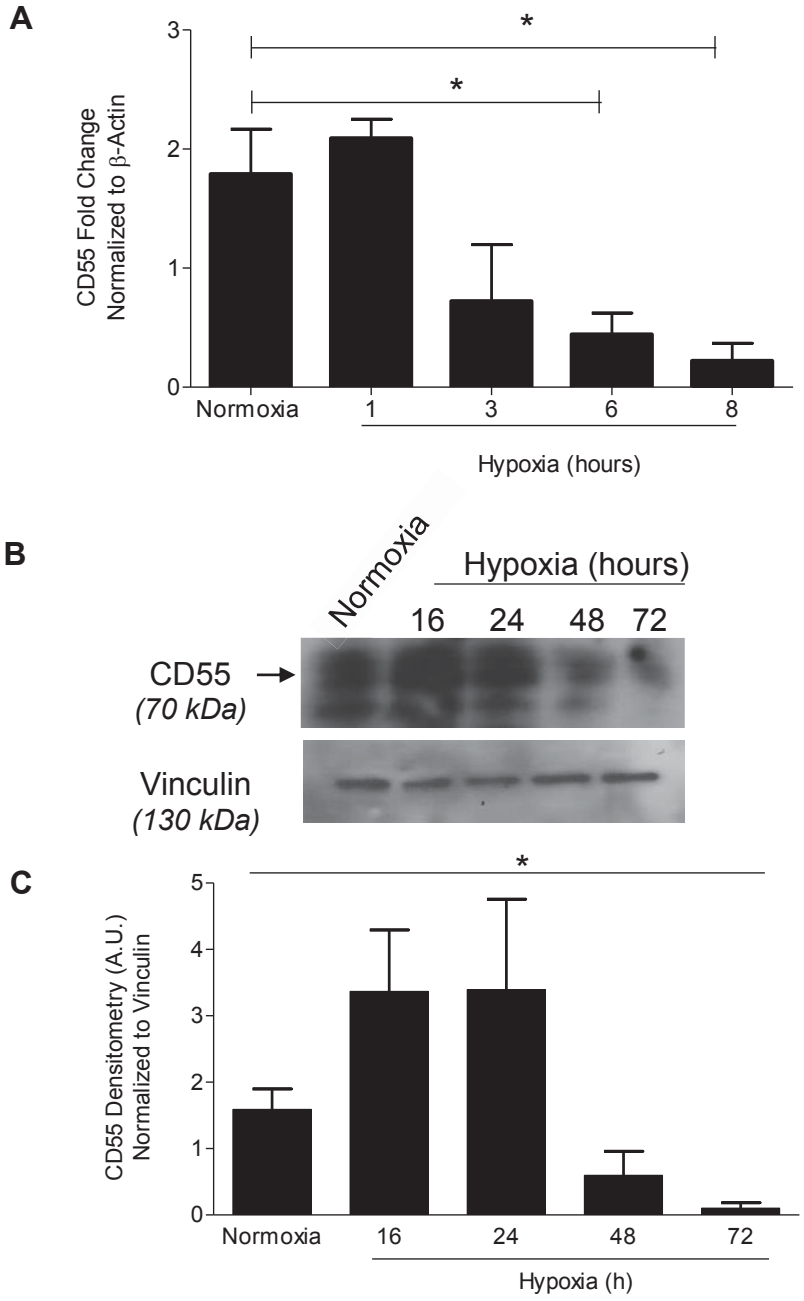

Figure 22. Hypoxic SAECs Down-regulate CD55 Expression. **(A)** *CD55* transcript levels were significantly down-regulated by 6h hypoxia compared to the controlled normoxic conditions. β-actin was used as the loading control. Values represent means±SEM; n=3; *p<0.05 vs normoxia; One-Way ANOVA with Bonferroni post-test. **(B)** Protein lysates of hypoxic SAECs were probed for expression of CD55 in hypoxic SAECs. Vinculin was used as internal loading control. **(C)** Densitometry analysis indicated significant down-regulation of CD55 protein by 72h of hypoxia vs normoxic conditions. Data represent mean±SEM, n=3, *p<0.05 vs normoxia; One-Way ANOVA with Bonferroni post-test.

Differential Regulation of CD46 mRNA and Protein in Hypoxic Human SAECs

To assess if hypoxia mediated down-regulation of membrane-bound CRP was only specific to CD55 or if this phenomenon was also observed on other membrane-bound CRPs that target C3 and C5 convertases, we assessed the expression of CD46 in hypoxic human SAECs. *CD46* mRNA levels are down-regulated by 8 hours of hypoxia on SAECs compared to normoxic controls (Figure 23A, $p<0.01$). On the contrary, CD46 protein expression was not down-regulated even at longer hypoxic exposures (Figure 23B). Thus was further confirmed by densitometry analysis of these western blots which showed no statistical significant difference in CD46 expression between normoxic controls and various time-points for hypoxic exposures (Figure 23C, n. s.)

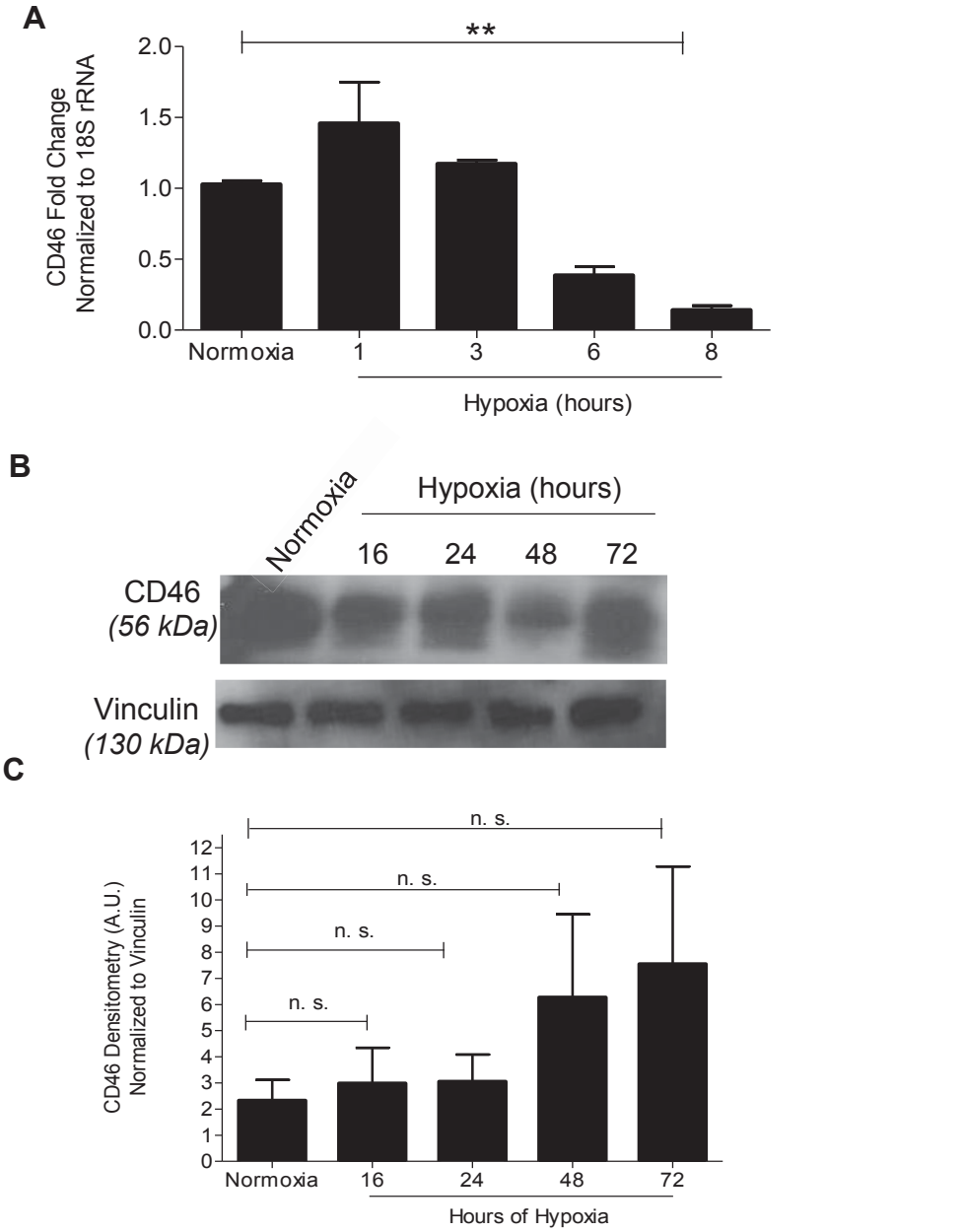

Figure 23. Hypoxic SAECs Down-regulate *CD46* mRNA Expression.
(A) *CD46* mRNA levels are down-regulated in hypoxic SAECs.
Values represent means±SEM; n=3; ***p*<0.01 vs normoxia; One-Way ANOVA with Bonferroni post-test. **(B)** CD46 expression was probed in hypoxic SAECs. Vinculin was used as the loading control. **(C)** Densitometry analysis show no statistical significant difference between any groups when compared to CD46 expression in normoxic SAECs. Data represents mean±SEM; n=3; n. s. One-Way ANOVA with Bonferroni post-test.

Down-regulation of CD55 is Observed in Hypoxic Mice Lungs

To correlate the down-regulated CD55 expression observed in hypoxic SAECs with our *in vivo* model, we analyzed CD55 expression in C57BL/10 mice exposed to hypoxic environment for 24 hours. Significant down-regulation of *CD55* transcripts (Figure 24A, $p<0.01$) and protein expression (Figures 24B, C, $p<0.01$) were observed in whole lung homogenates of mice exposed to 24 hours of hypoxia compared to lung homogenates of normoxic mice. IHC analysis was performed to further confirm that the observed CD55 down-regulation was occurring within the hypoxic airway epithelium of these mice, compared to the CD55 expression present in the airway epithelium of normoxic mice (Figure 24D). Quantification of the IHC analysis show CD55 down-regulation on C57BL/10 hypoxic mice airways compared to the normal control mice airways (Figure 24E, $p<0.05$)

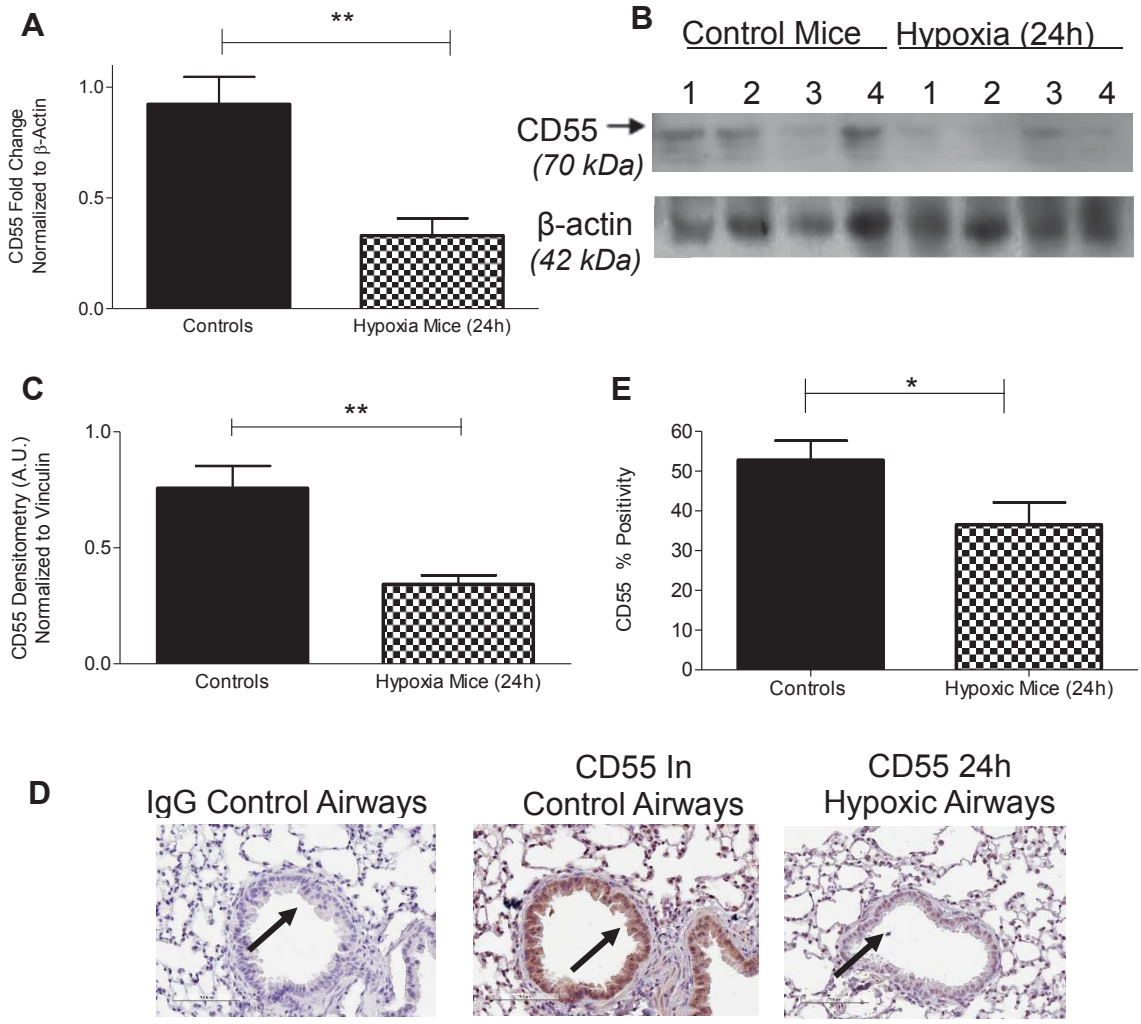

Figure 24. *In Vivo* Hypoxia Results In CD55 Down-regulation In Mice Lungs. **(A)** *CD55* transcript levels were significantly down-regulated in 24h hypoxic mice lungs compared to lungs of normoxic control mice. β-actin was used as internal loading control. Values represent means±SEM; n≥6; ***p*<0.01 vs controls; two-tailed unpaired t-test. **(B)** CD55 expression was probed for CD55 in lung homogenates of 24h control and hypoxic mice. β-actin was used as the loading control. **(C)** Densitometry analysis depict statistically significant down-regulation of CD55 in 24h hypoxic mice lungs compared to controls. Data represents mean±SEM; n=4; ***p*<0.01 vs control mice; two-tailed unpaired t-test. **(D)** IHC analysis show CD55 down-regulation in 24h hypoxic mice airways compared to control mice. **(E)** Quantification of IHC shows CD55 down-regulation on hypoxic mice airway epithelium. Values represent mean±SEM; n=8; **p*<0.05 vs control mice; two-tailed unpaired t-test.

Differential Regulation of CRRY mRNA and Protein Observed in Hypoxic Mice Lungs

Similar to *in vitro* CD46 data, human orthologue of CRRY, C57BL/10 mice lungs that are hypoxic for 24 hours show *CRRY* mRNA down-regulation (Figure 25A, $p<0.05$). However, protein expression of CRRY remains unchanged compared to CRRY expression in normal control C57BL/10 lungs (Figure 25B). This was further confirmed by IHC quantification, which showed no statistical significance in CRRY expression between 24 hours hypoxic C57BL/10 mice lungs and normal control lungs (Figure 25C, p= n. s.).

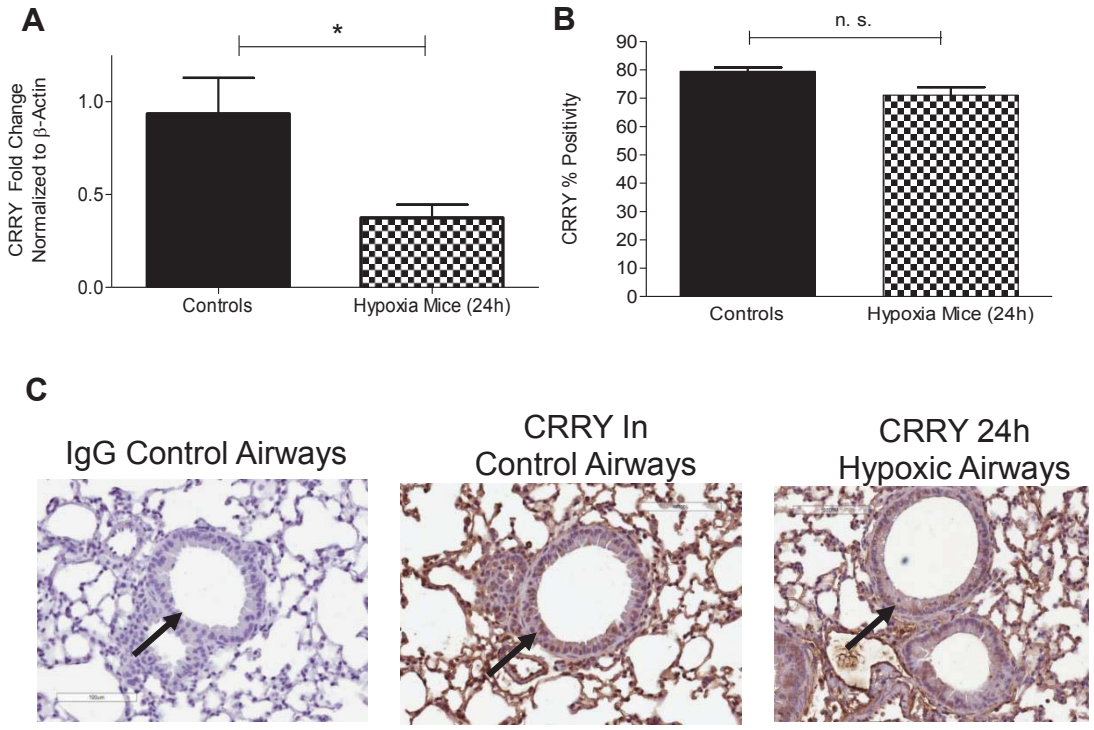

Figure 25. Hypoxic Mice Lungs Show Down-regulation Of *CRRY* Transcripts. **(A)** *CRRY* transcripts are down-regulated in lungs of hypoxic (24h) C57BL/10 mice vs controls (normoxic mice). Data represents mean±SEM; n≥6; ***p*<0.05 vs control mice; two-tailed unpaired t-test. **(B)** IHC quantification shows that CRRY protein expression in hypoxic (24h) C57BL/10 mice airways are not altered compared to control airways (normoxic mice). Data represents mean±SEM; n=3; n. s. two-tailed unpaired t-test.

Hypoxia Does Not Result in CD55 and CRRY Down-regulation in C57BL/6 Mice Lungs

To assess if hypoxia resulted in CD55 or CRRY down-regulation in C57BL/6 mice lungs like it did in C57BL/10 mice lungs, expression of CD55 and CRRY were assessed by IHC in C57BL/6 mice lungs exposed to 24 hours of hypoxia (Figure 26). Compared to normal control C57BL/6 mice lungs expression of CD55 and CRRY did not change in 24 hour hypoxic C57BL/6 mice lungs (Figures 26A, B). IHC quantification of CD55 and CRRY further confirmed that there was no statistical significance between normal control C57BL/6 mice lungs or 24 hour hypoxic mice lungs of the same strain (Figures 26C, D). Transcript levels for *CD55* and *CRRY* were not assessed in these mice strains.

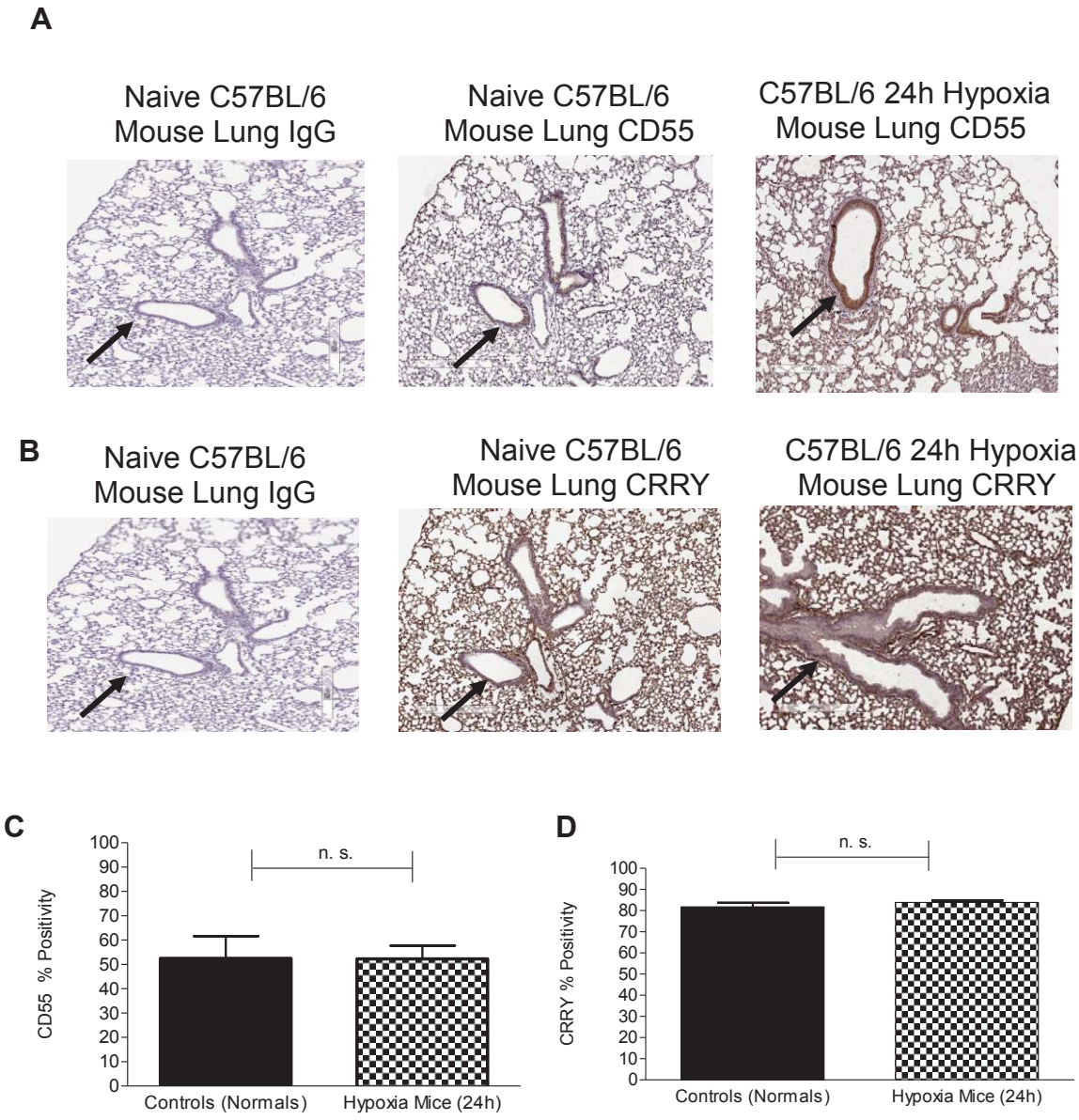

Figure 26. Hypoxia In C57BL/6 Mice Has No Effect On CD55 Or CRRY In The Lungs. **(A)** IHC analysis of C57BL/6 hypoxic (24h) lungs show no change in CRRY expression compared to normal (naïve) controls. **(B)** IHC analysis of C57BL/6 hypoxic (24h) lungs show no change in CD55 expression compared to normal (naïve) controls. **(C)** Quantification of CRRY IHC in hypoxic (24h) C57BL/6 mice airways shows no change. Data represents mean±SEM; n=4; n.s. two-tailed unpaired t-test. **(D)** IHC quantification shows that CD55 expression in hypoxic (24h) C57BL/6 mice airways are not altered compared to control airways (normoxic mice). Data represents mean±SEM; n=4; n. s. two-tailed unpaired t-test.

In Vivo, CD55 Down-regulation Results in Local Complement Activation

To determine if the observed down-regulation of CD55 in hypoxic mice lungs led to complement activation, we assessed local complement levels in hypoxic mice versus normoxic control mice. Down-regulation of CD55 on airway epithelium of 24 hour hypoxic mice, resulted in complement activation as observed by increased C3a levels in BALF of hypoxic mice compared to control mice (Figure 27, $p<0.05$). Thus, suggesting a correlation between airway epithelial CD55 down-regulation and local complement activation as indicated by C3a levels in BALF.

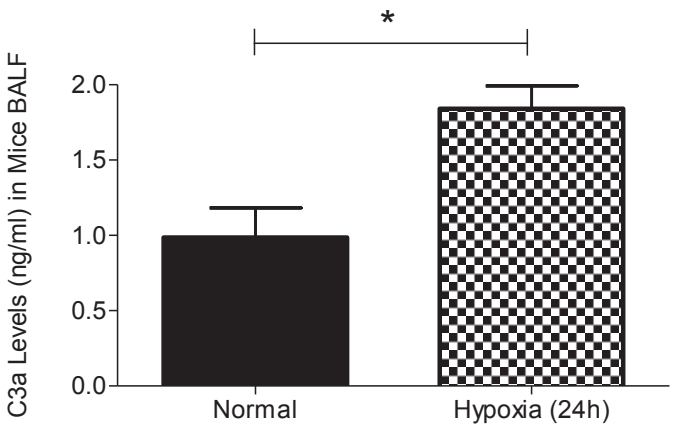

Figure 27. CD55 Down-regulation In Hypoxic Mice Results In Local Complement Activation. C3a levels in BALF of control or 24h hypoxic mice Values represent mean±SEM; n=3; *p<0.05 vs controls; two-tailed unpaired t-test.

C. Part III: Inhibition of HIF-1α in Hypoxic Conditions Restores CD55 Expression

Chetomin Mediated HIF-1α Inhibition Restores CD55 Expression in Hypoxic Human SAECs

Due to evidence for HIF-1α regulating CD55 expression in hypoxic *in vitro* and *in vivo* studies being only a correlation, we investigated the effects of pharmacological HIF-1α inhibition on CD55 expression during hypoxia. Following a 2 hour pre-treatment of SAECs with the HIF-1α inhibitor, chetomin, the cells were exposed to hypoxic conditions (1% O_2) for either 6 or 24 hours for RNA or protein analysis of CD55, respectively. Quantification of *CD55* mRNA in hypoxic conditions with vehicle control showed down-regulation within 6 hours, compared to the normoxic controls (Figure 28A, $p<0.05$). Optimal dosing for chetomin mediated effects on hypoxic SAECs was confirmed in preliminary studies (data not shown). Compared to untreated cells, chetomin (100nM) recovered *CD55* transcripts in SAECs exposed to 6 hours of hypoxia (Figure 28A, $p<0.001$). Contrary to the CD55 protein down-regulation observed at 72 hours of hypoxia (Figures 22B, C), a decrease in CD55 protein expression was observed at 24 hours of hypoxia in these and succeeding experiments, which may be attributed to donor-dependent variability of the SAECs. However, chetomin treatment restored expression of CD55 in 24 hour hypoxic SAECs compared to hypoxic SAECs in absence of chetomin treatment (Figures 28B, C, $p<0.05$).

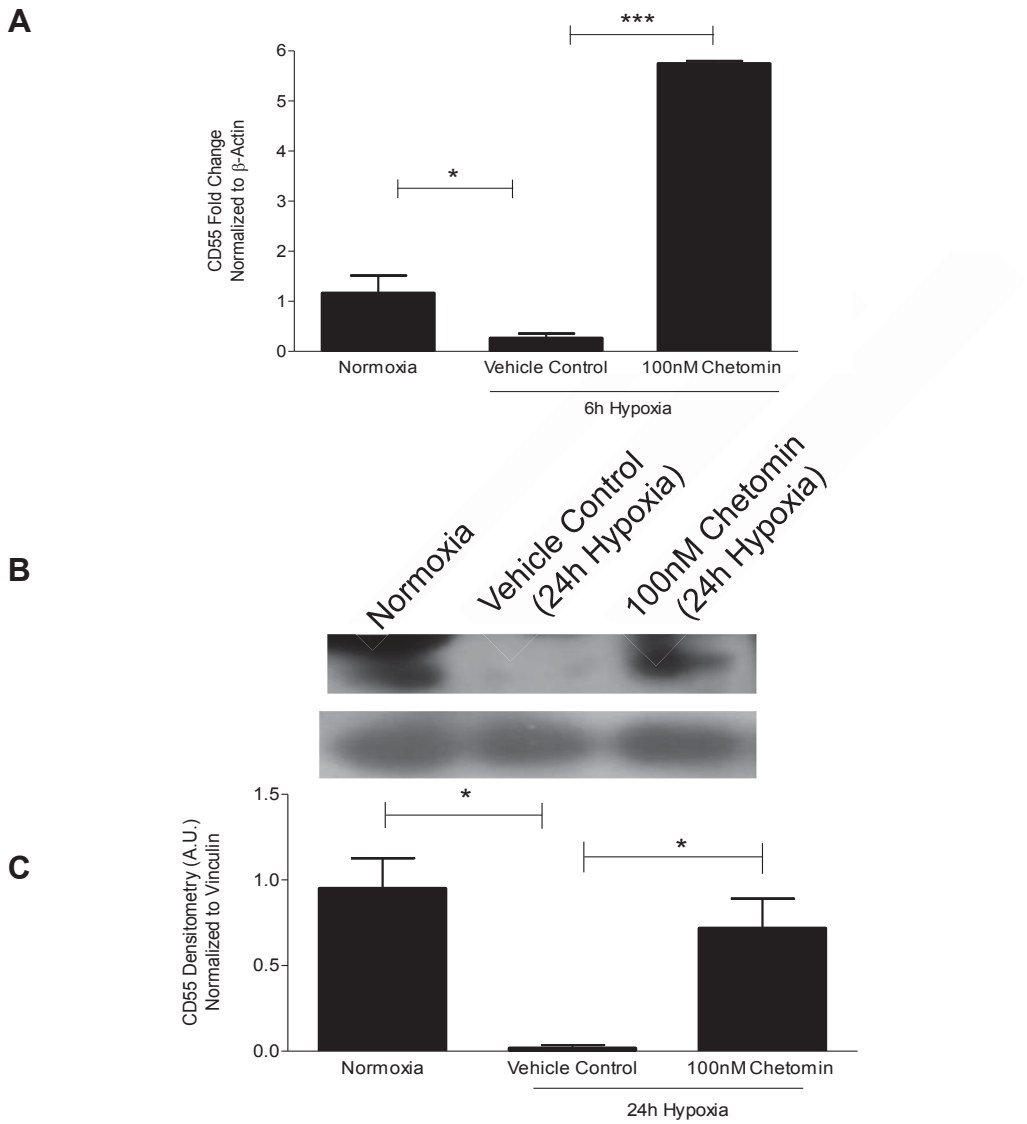

Figure 28. Chetomin Mediated HIF-1α Inhibition Restores CD55 Expression In Hypoxic SAECs. **(A)** *CD55* transcripts were restored in chetomin (100nM) treated cells exposed to 6h hypoxia compared to 6h hypoxic cells in absence of chetomin or in normoxia. Values represent mean±SEM; n=3; **p*<0.05 vs nomoxia, or ****p*<0.001 vs 6h vehicle control in hypoxia; One-Way ANOVA with Bonferroni post-test. **(B)** Protein lysates were probed for expression of CD55 in normoxic SAECs and in the presence or absence of 100nM chetomin in 24h hypoxic SAECs. Vinculin was used as internal loading control. **(C)** Densitometry analysis indicated significant down-regulation of CD55 in 24h hypoxia compared to normoxic levels. CD55 expression on SAECs was recovered in 24h hypoxic SAECs treated with chetomin (100nM) compared to hypoxic SAECs in absence of chetomin stimulation. Data represent mean±SEM, n=3, **p*<0.05 vs normoxia or 24h hypoxia; One-Way ANOVA with Bonferroni post-test.

CA9, An Indicator of HIF-1α Transcriptional Activity, is Down-regulated in Chetomin Treated Hypoxic Human SAECs.

Mechanistically, chetomin functions by inhibiting HIF-1α mediated transcriptional activation, while having no affect on the stabilization of HIF-1α protein. This pharmacological inhibition by chetomin involves disruption of the interaction between HIF-1α and the co-activator complex with the HREs located in the promoter regions of HIF-1α dependent genes (186), resulting in chetomin-mediated transcriptional repression of HIF-1α dependent genes (186). Thus, to confirm the HIF-1α specificity of chetomin, we analyzed the transcript levels of *CA9* an indicator of HIF-1α transcriptional activity (187), (188), which is an indicator of HIF-1α transcriptional activity. Treatment with 100 nM chetomin in 6 hour hypoxic SAECs resulted in down-regulation of *CA9* transcripts compared to 6h hypoxic SAECs in the absence of chetomin (Figure 29, $p<0.05$), confirming that effects of chetomin are HIF-1α specific.

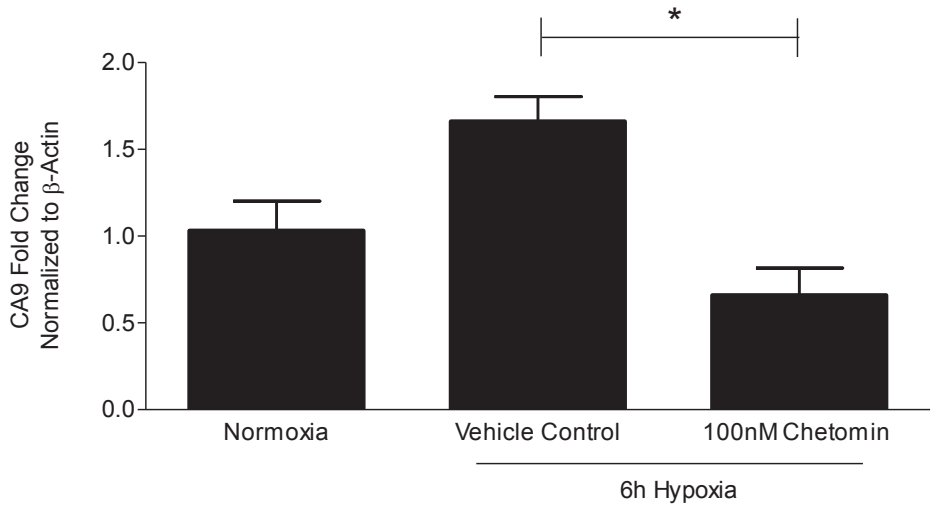

Figure 29. Transcripts Of *CA9* Are Down-regulated In Chetomin Treated Hypoxic SAECs. Transcript levels for *CA9* were increased in 6h hypoxic SAECs compared to those in normoxia, but were down-regulated in chetomin (100nM) treated cells compared to those in 6h hypoxic SAECs. β-actin was used as internal loading control. Values represent mean±SEM; n=3; *p<0.05 vs 6h hypoxia with vehicle control; One-Way ANOVA with Bonferroni post-test.

Chetomin Mediated HIF-1α Inhibition Restores CD55 Expression in Hypoxic Mice Lungs

Intrapulmonary HIF-1α inhibition, *in vivo,* was achieved by intratracheal instillation of 1mg/ml chetomin or vehicle control in 24 hour hypoxic mice. Compared to CD55 down-regulation in control or vehicle control mice lungs, CD55 expression was restored in chetomin treated hypoxic mouse lung homogenates (Figures 30A, B, $p<0.01$). Because CD55 is being down-regulated on the airway epithelium of these hypoxic mice (Figure 24D), we utilized IHC analysis to further confirm that chetomin mediated recovery of CD55 occurred on airway epithelium during *in vivo* hypoxic conditions. Airway epithelial CD55 recovery was observed in chetomin treated hypoxic mice compared to 24 hour hypoxic mice treated with the appropriate vehicle control (Figure 30C).

CA9 is Down-regulated in Lungs of Chetomin Instilled Hypoxic Mice

To confirm the HIF-1α specific effects of chetomin *in vivo*, mRNA quantification of *CA9* was performed in 24 hour hypoxic mice lungs instilled with chetomin or the appropriate vehicle control. Interestingly, these studies indicate that *CA9* transcripts were elevated in 24 hour hypoxic mice lungs compared to the control lungs of normoxic mice (Figure 30D, $p<0.01$). However, in presence of chetomin, *CA9* transcripts were reduced in lung homogenates of these 24 hour hypoxic mice compared to control mice lungs or lungs of hypoxic mice instilled

with vehicle control (Figure 30D, $p<0.01$). Thus, confirming the role of chetomin in regulating the transcriptional activity of HIF-1α.

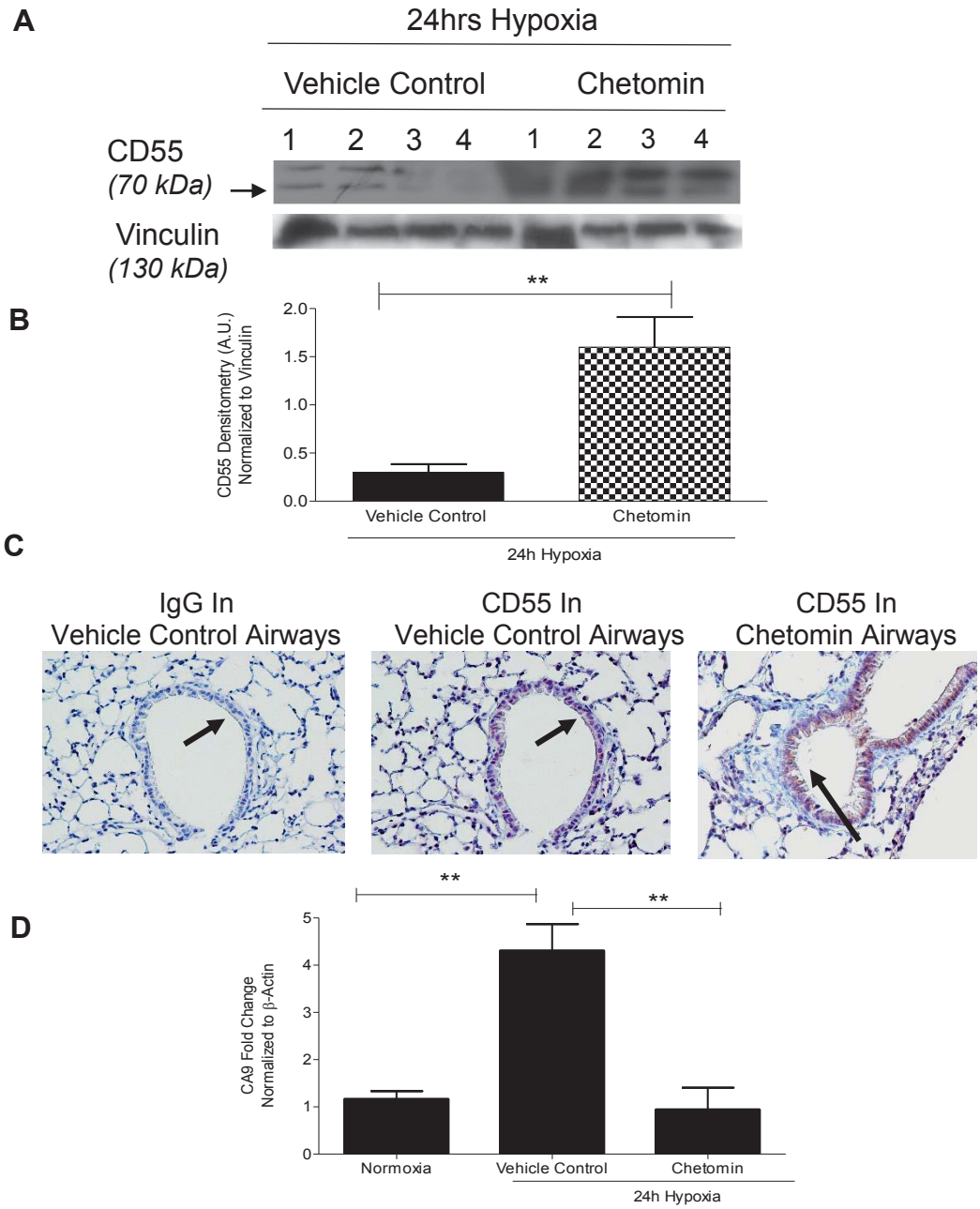

Figure 30. Chetomin Recovers CD55 Expression In Hypoxic Mice Lungs. **(A)** CD55 expression was probed 24h hypoxic mice lungs intratracheally instilled with chetomin (1mg/ml) or with vehicle control. Vinculin was the internal loading control. **(B)** Densitometry analysis show CD55 down-regulation in 24h hypoxic mice lungs instilled with chetomin (1mg/ml) vs vehicle controls. Values represent mean$\pm$ SEM, n=4, $^{**}p<0.01$ vs vehicle control; two-tailed unpaired t-test. **(C)** IHC data show CD55 down-regulation in chetomin instilled 24h hypoxic mice lungs vs vehicle control hypoxic mice lungs. **(D)** *CA9* transcripts were down-regulated in chetomin instilled 24h hypoxic mice lungs compared to vehicle control. Values represent mean$\pm$SEM; n$\geq$3; $^{**}p<0.01$ vs control or vehicle control; One-Way ANOVA with Bonferroni post-test.

HIF-1α Expression is Silenced in HIF-1α siRNA Transfected Hypoxic Human SAECs

Chetomin provided indirect evidence of HIF-1α regulating CD55 on airway epithelium. Therefore, inhibition of HIF-1α via specific mechanisms such as the use silencing RNA (siRNA) were exploited. Prior to the assessment of CD55 expression, control experiments were performed to determine the efficacy of HIF-1α siRNA. Following transfection of SAECs with 50nM HIF-1α siRNA or 50nM control siRNA, the cells were exposed to 6 hours of hypoxia. HIF-1α expression was induced in 6 hour hypoxic SAECs transfected with control siRNA compared to its expression in normoxic SAECs (Figures 31A, B, $p<0.05$). However, SAECs transfected with HIF-1α siRNA showed a significant decrease in HIF-1α expression when compared to normoxic SAECs or to control siRNA transfected hypoxic SAECs (Figure 31B, $p<0.05$).

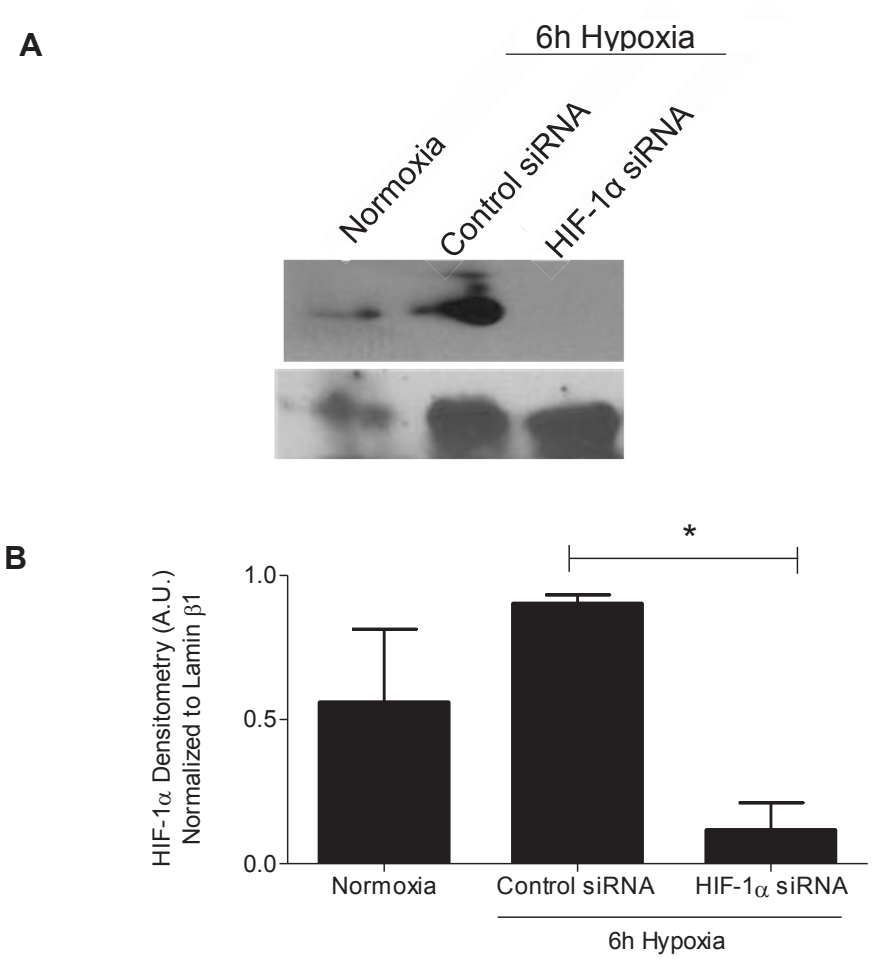

Figure 31. Silencing HIF-1α In Hypoxic SAECs. **(A)** HIF-1α protein expression is silenced in 6h hypoxic SAECs transfected with 50nM HIF-1α siRNA compared to the induced HIF-1α expression in 6h hypoxic SAECs with control siRNA (50nM) and HIF-1α expression present in normoxic basal hypoxia. Lamin β1 was used as internal loading control. **(B)** Densitometry analysis indicated significant down-regulation of HIF-1α in SAECs transfected with HIF-1α siRNA (50nM) under 6h hypoxia compared to normoxic basal levels in SAECs or control siRNA (50nM) in hypoxic SAECs. Data represent mean±SEM, n=3, *p<0.05 vs normoxia or control siRNA in 24h hypoxia; One-Way ANOVA with Bonferroni post-test.

CA9 Expression is Repressed in HIF-1α siRNA Transfected Hypoxic Human SAECs

Silencing of HIF-1α via siRNA in 6 hour hypoxic SAECs was further confirmed by quantifying *CA9* mRNA. *CA9* transcripts were reduced in 6 hour hypoxic SAECs transfected with HIF-1α siRNA compared to normoxic SAECs or 6 hour hypoxic SAECs transfected with or without the control siRNA (Figure 32, $p<0.01$). Due to induction of HIF-1α occurring at 6 hours of hypoxia in SAECS, longer hypoxic exposure may be necessary for *CA9* transcription in SAECs (Figure 20C). Thus, *CA9* transcript levels remain at basal levels in normoxic and 6 hour hypoxic SAECs with or without control-siRNA. The specificity of the HIF-1α siRNA was confirmed by data showing that silencing HIF-1α in 6 hour hypoxic SAECs down-regulated *CA9* transcript levels (Figure 32, $p<0.01$) compared to normoxic SAECs or 6h hypoxic SAECs transfected with or without control siRNA.

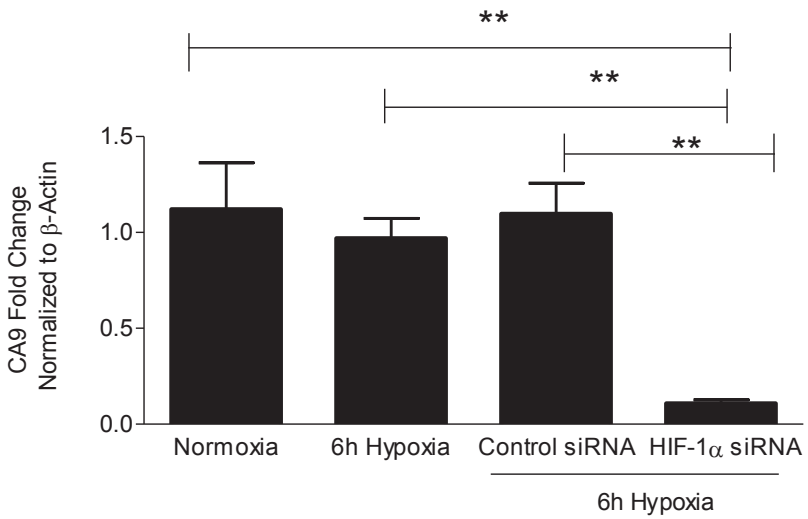

Figure 32. Silencing HIF-1α In Hypoxic SAECs Results In CA9 Down-regulation. *CA9* transcript levels were down-regulated in HIF-1α siRNA transfected 6h hypoxic SAECs compared to 6h hypoxic SAECs with or without control siRNA transfection. β-actin was used as internal loading control. Values represent mean±SEM; n=3; ** $p<0.01$ vs nomoxia, 6h hypoxia, or control siRNA; One-Way ANOVA with Bonferroni post-test.

Silencing HIF-1α in Hypoxic SAECs Restores CD55 Expression

To assess effects of silencing HIF-1α on CD55 expression in hypoxic SAECs, cells were exposed to 6 hours of hypoxia following transfection with 50nM HIF-1α siRNA or control siRNA. Compared to *CD55* transcript levels in normoxic cells or hypoxic cells transfected with the control siRNA, an increase in *CD55* transcripts were observed in SAECs where HIF-1α is silenced within 6 hours of hypoxia (Figure 33A, $p<0.05$). Interestingly, CD55 protein expression was also restored in hypoxic SAECs where HIF-1α was silenced compared to normoxic control cells or hypoxic cells transfected with control siRNA (Figures 33B, C, $p<0.05$).

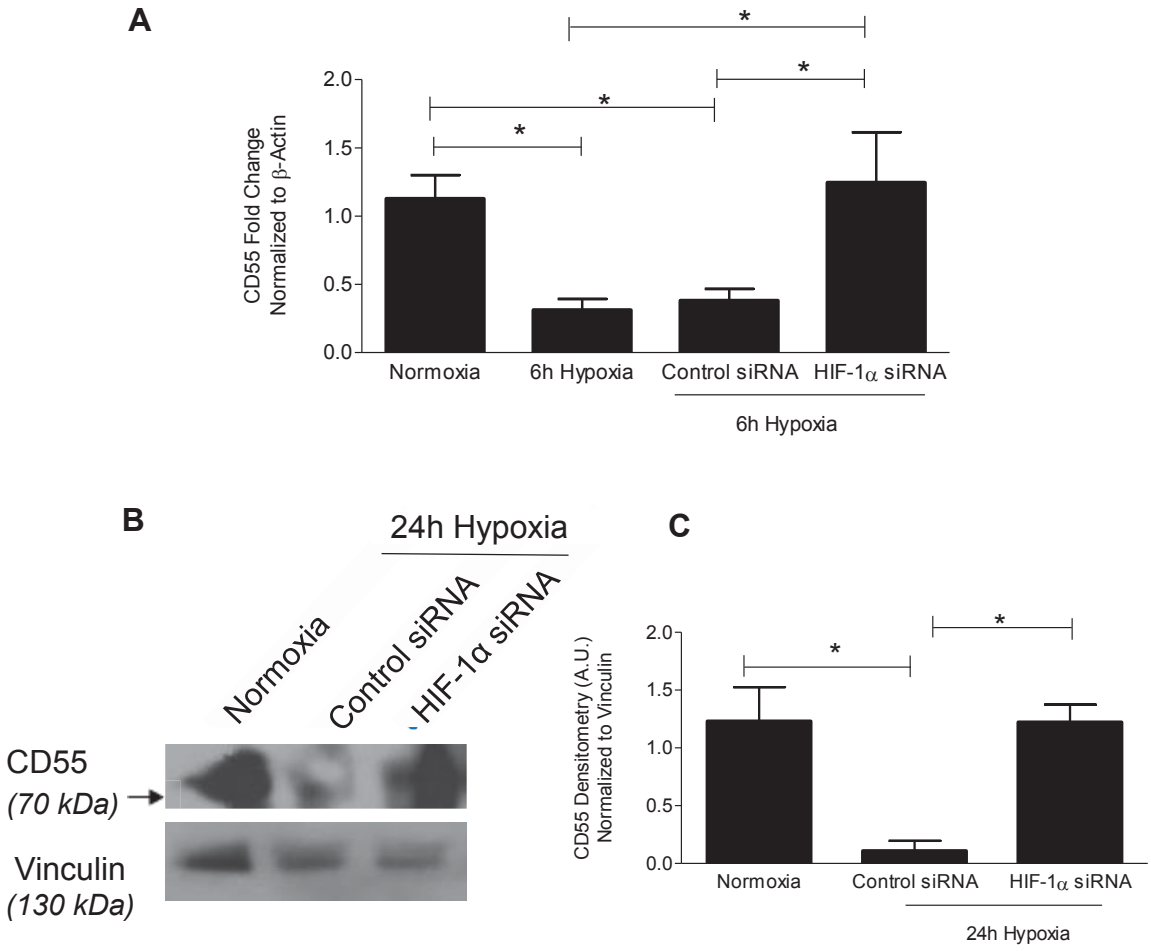

Figure 33. Silencing HIF-1α In Hypoxic SAECs Restores CD55 Expression. **(A)** *CD55* transcript levels were recovered in HIF-1α siRNA (50nM) transfected 6h hypoxic SAECs compared to 6h hypoxic SAECs in the presence or absence of control siRNA (50nM). β-actin was used as internal loading control. Values represent mean±SEM; n=3; *p<0.05 vs nomoxia, 6h hypoxia, or controls siRNA; One-Way ANOVA with Bonferroni post-test. **(B)** CD55 protein expression was probed for in normoxic, or 24h hypoxic HIF-1α siRNA (50nM) or control siRNA (50nM) transfected SAECs. Vinculin was used as internal loading control. **(C)** Densitometry analysis depict recovery of CD55 protein in 24h HIF-1α siRNA (50nM) transfected SAECs compared to control siRNA (50nM) or normoxic basal levels. Values represent mean±SEM; n=3; *p<0.05 vs nomoxia, 24h hypoxia, or controls siRNA; One-Way ANOVA with Bonferroni post-test.

HIF-1α Expression is Knocked-down in Mice Lungs After Intratracheal Instillation of HIF-1α siRNA

We also silenced HIF-1α in our hypoxic *in vivo* model to investigate its effect on CD55 expression within mice lungs. To knock-down HIF-1α expression specifically in the lungs, intratracheal instillation of HIF-1α siRNA or control siRNA was performed 72 hours prior to their 24 hour exposure to hypoxia (10% O_2). Efficacy of HIF-1α siRNA was confirmed by western blot analysis of hypoxic mice lungs (Figure 34A). Protein quantification of HIF-1α through densitometric analysis show HIF-1α silencing in 24 hour hypoxic mice lungs instilled with HIF-1α siRNA compared to the lungs instilled with control siRNA. (Figures 34A, B, $p<0.01$). IHC analyses was performed to confirm the down-regulation of HIF-1α in the airway epithelium of these 24 hour hypoxic mice instilled with HIF-1α siRNA (Figure 34C). Modest down-regulation of HIF-1α is observed in airways of hypoxic mice instilled with HIF-1α siRNA compared to controls (Figure 34C) but IHC readout is not reliable due to the patchy and location-dependent down-regulation of HIF-1α observed in these mice lung sections. Therefore, a more reliable readout to confirm HIF-1α silencing *in vivo* is needed. This patchy nature of HIF-1α induction and down-regulation observed within the hypoxic mice lungs, may be attributed to the temporal variability in stabilization of HIF-1α, and other HIF isoforms present among various cell types in the lung (189), (174).

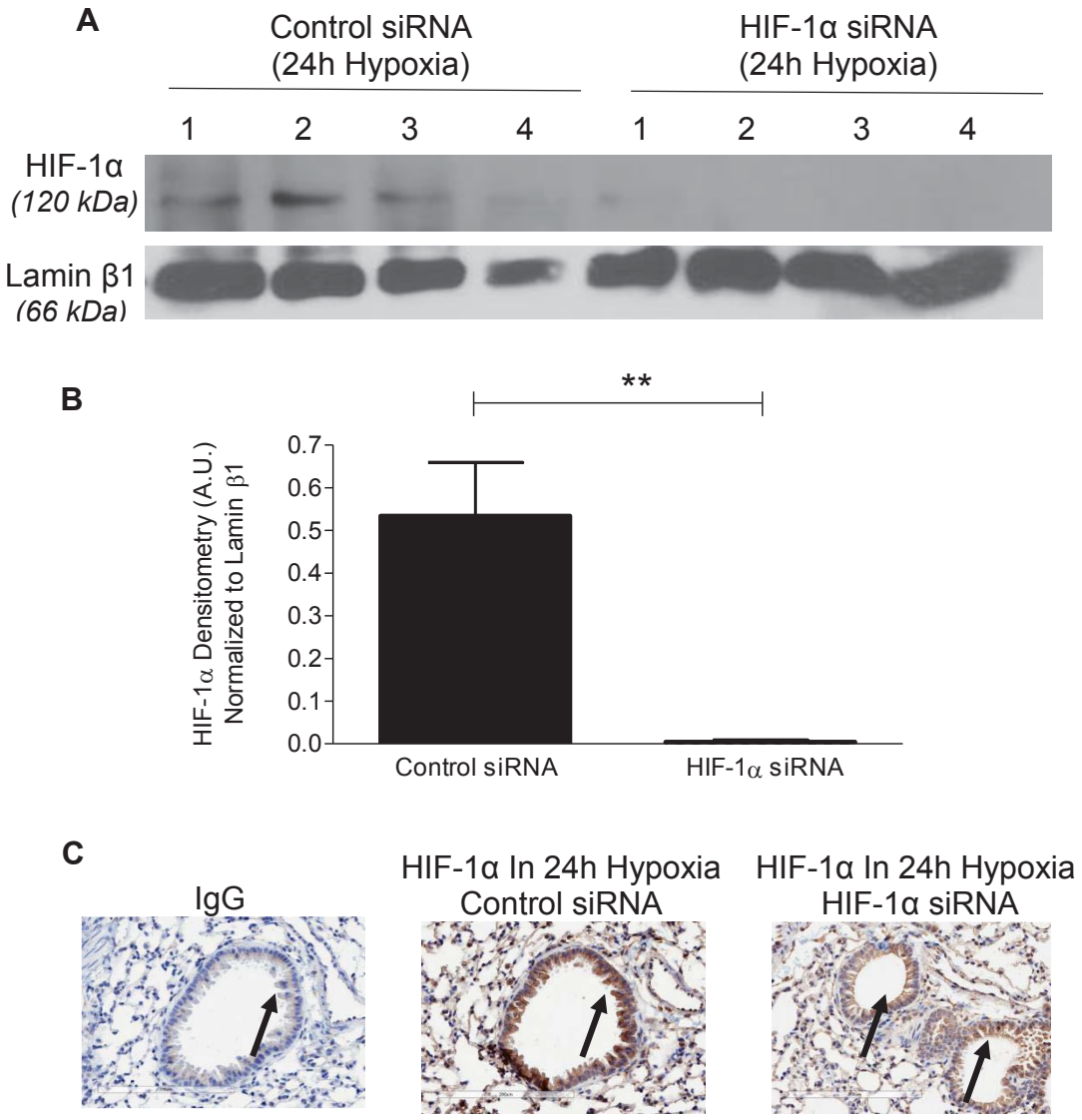

Figure 34. Intratracheal Silencing Of HIF-1α In Hypoxic Mice Lungs. **(A)** HIF-1α expression was probed for in lung homogenates of 24h hypoxic mice intratracheally instilled with HIF-1α siRNA (50μg) or control siRNA (50μg). Lamin β1 was used as internal loading control. **(B)** Densitometry analysis indicated significant down-regulation of HIF-1α in lung homogenates of 24h hypoxic mice intratracheally instilled with HIF-1α siRNA (50μg) compared to control siRNA (50μg). Values represent mean±SEM, n=4, $^{**}p<0.01$ vs control siRNA; two-tailed unpaired t-test. **(C)** IHC analysis show HIF-1α down-regulation in hypoxic mice lungs where HIF-1α is silenced compared to control hypoxic mice lungs.

CA9 is Down-regulated in HIF-1α Silenced Hypoxic Mice Lungs

While these *in vivo* HIF-1α siRNA studies show HIF-1α knock-down via western blot analysis, the inconsistent HIF-1α expression observed in IHC data implicate the need for a more reliable readout to verify HIF-1α silencing in hypoxic condition, *in vivo*. Thus, to confirm HIF-1α knockdown in these studies, mRNA quantification of *CA9*, and indicator of HIF-1α transcriptional activity, was implemented. Increase in *CA9* transcripts were detected in 24 hour hypoxic mice lungs compared to controls (Figure 35, $p<0.001$), but were down-regulated in 24 hour hypoxic mice lungs with HIF-1α siRNA compared to control siRNA (Figure 35, $p<0.05$).

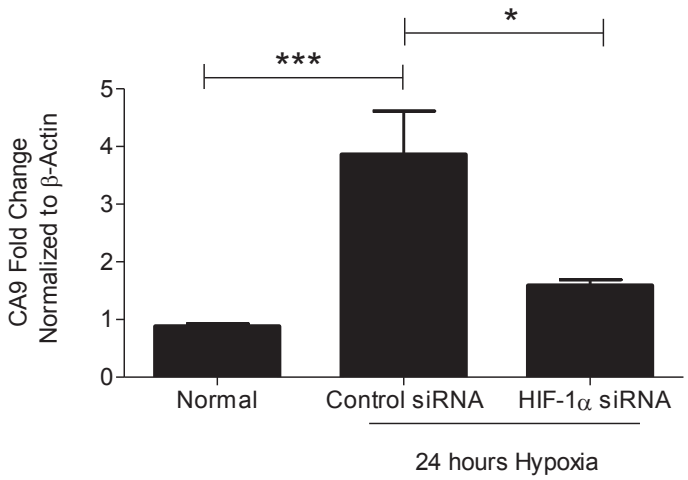

Figure 35. Silencing HIF-1α Via Intratracheal Instillation Down-regulates *CA9* Transcripts In Hypoxic Mice Lungs. Transcript levels for *CA9* are induced in hypoxic mice lungs instilled with control siRNA, but down-regulated in hypoxic mice lungs where HIF-1α expression is silenced. β-actin was used as internal loading control. Data represents mean$\pm$SEM, n$\geq$3; ***p<0.001 vs controls, *p<0.05 vs control siRNA; One-Way ANOVA with Bonferroni post-test.

Silencing HIF-1α in Hypoxic Mice Lungs Restores CD55 Expression

CD55 expression was assessed by western blot analysis on lungs extracts of mice intratracheally instilled with either HIF-1α siRNA or control siRNA (Figure 36A). CD55 protein expression was significantly restored in hypoxic mice lungs instilled with HIF-1α siRNA compared to mice lung homogenates with control siRNA (Figure 36B, $p<0.05$). Similarly, IHC analysis depict recovery of CD55 expression occurring, specifically, in the airway epithelium of 24 hour hypoxic mice when HIF-1α is silenced (Figure 36C). Therefore, suggesting an inverse regulation between induction of HIF-1α and down-regulation of CD55 in lungs, specifically, in the airway epithelium of the lungs, during hypoxia. Despite the recovery of CD55 in HIF-1α silenced mice airway epithelium, complement activation still occurred (Figure 37). C3a levels in BALF were comparable between HIF-1α silenced hypoxic mice and hypoxic mice instilled with control siRNA (Figure 37).

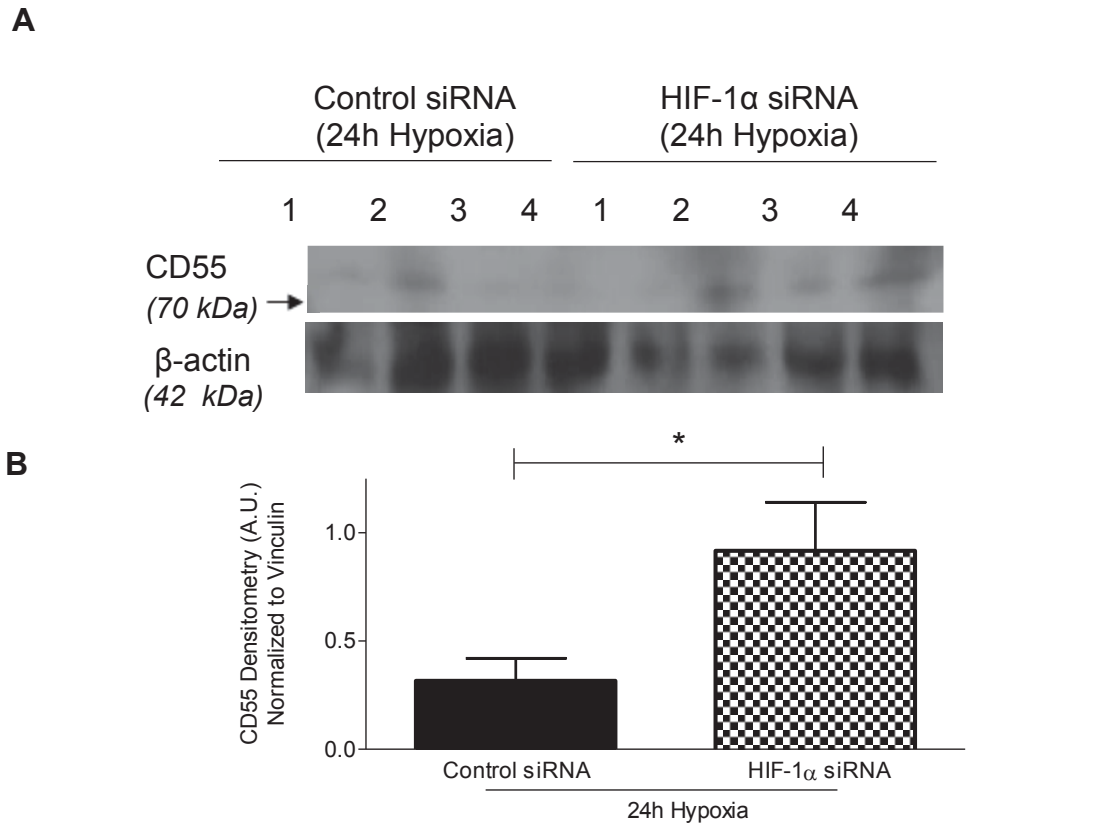

Figure 36. Intratracheal Silencing Of HIF-1α In Hypoxic Mice Lungs Recovers CD55 Expression. **(A)** CD55 expression was probed for in lung homogenates of 24h hypoxic mice intratracheally instilled with HIF-1α siRNA (50μg) or control siRNA (50μg). β-actin was used as internal loading control. **(B)** Densitometry analysis indicated CD55 was restored significantly in lung homogenates of 24h hypoxic mice intratracheally instilled with HIF-1α siRNA (50μg) compared to control siRNA (50μg). Data represents mean±SEM, n=4, **p*<0.05 vs control siRNA; two-tailed unpaired t-test. **(C)** IHC analysis show CD55 down-regulation in hypoxic mice lungs where HIF-1α is silenced compared to hypoxic mice lungs with control siRNA.

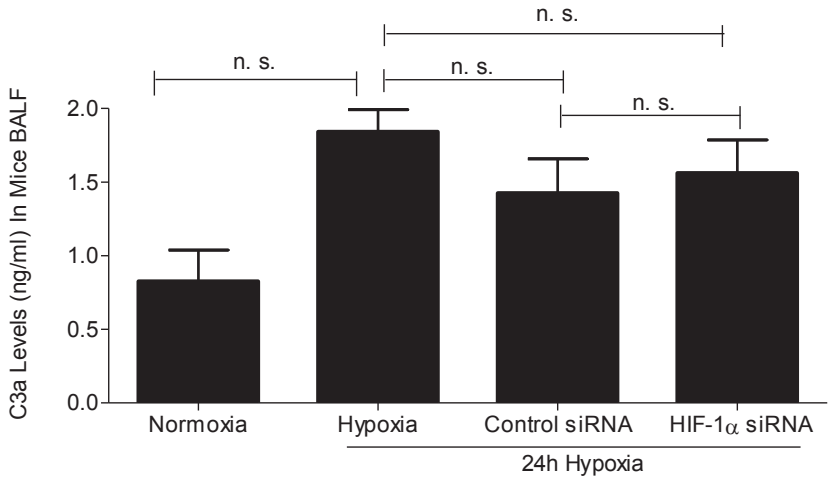

Figure 37. CD55 Recovery In HIF-1α Silenced Mice Lungs, Resulted In No Change In Local Complement Activation. C3a levels in BALF of control or 24h hypoxic mice Values represent mean±SEM; n≥3; no statistical significance (n. s.) vs normoxia, hypoxia, or control siRNA; two-tailed unpaired t-test.

D. Part IV: Induction of HIF-1α in Normoxic Conditions Down-regulates CD55 Expression

DMOG Treatment Induces HIF-1α Expression in Normoxic SAECs

To determine if HIF-1α expression was sufficient for CD55 down-regulation in the absence hypoxic stimuli, we investigated CD55 expression in normoxic SAECs where HIF-1α expression was stabilized with DMOG treatment to inhibit the PHD enzymes. To confirm the HIF-1α specificity of DMOG, control experiments were performed to demonstrate that HIF-1α expression was stabilized post-DMOG treatment in normoxic SAECs. Western blot analysis confirmed significant induction of HIF-1α in normoxic SAECs treated with 1μM of DMOG for 6-24 hours (Figures 38A, B, $p<0.05$). Optimal dosing for DMOG mediated effects on SAECs for HIF-1α and CD55 expression were verified in preliminary studies (data not shown).

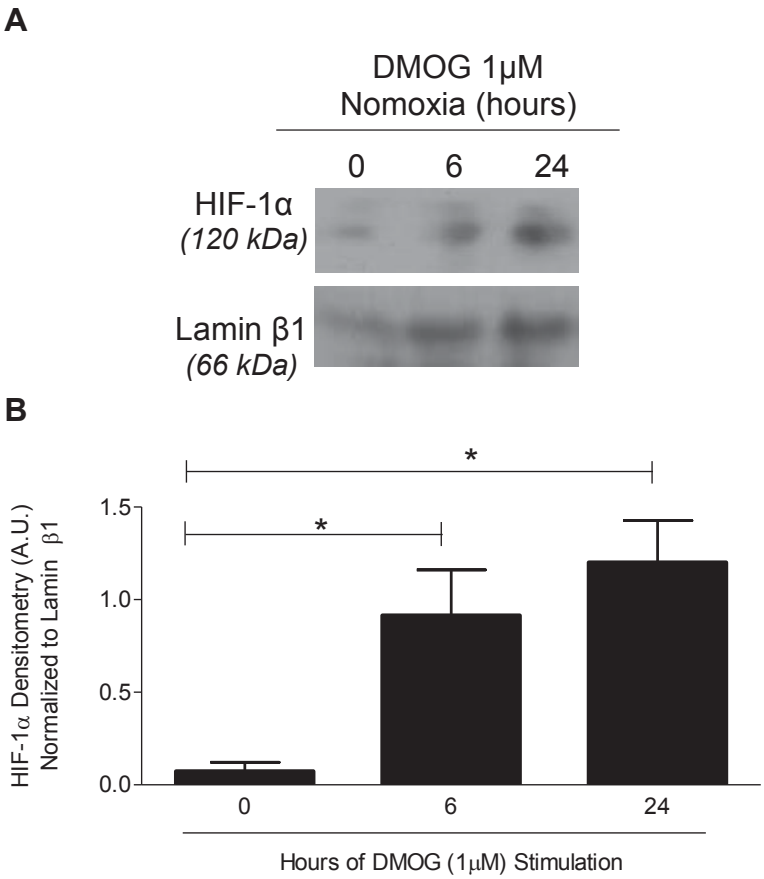

Figure 38. Stabilization Of HIF-1α In Normoxic SAECs By DMOG. **(A)** DMOG (1μM) treated SAECs were probed for HIF-1α protein expression. Lamin β1 was used as internal loading control. **(B)** Densitometry analysis indicated significant induction of HIF-1α within 6-24h DMOG (1μM) treated SAECs compared to cells without DMOG treatment. Values represent mean±SEM, n=3, *p<0.05 vs non-treated cells (0hr controls); One-Way ANOVA with Bonferroni post-test.

CA9 Expression is Induced in DMOG Stimulated Normoxic Human SAECs

To verify the HIF-1α activation by DMOG treatment on normoxic SAECs, we also quantified *CA9* mRNA in these DMOG treated SAECs for 6 hours post-treatment (Figure 39, $p<0.05$), and for 24 hours post-treatment (Figure 39, $p<0.01$).

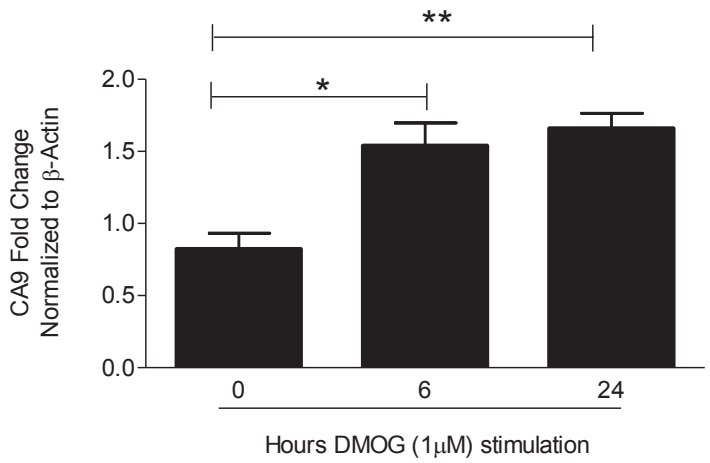

Figure 39. DMOG Mediated HIF-1α Stabilization In Normoxic SAECs Induces *CA9* Transcription. Transcription levels for *CA9* were up-regulated in DMOG (1μM) treated SAECs compared to non-treated cells. β-actin was used as internal loading control. Data represents mean±SEM; n=3; *p<0.05, or **p<0.01 vs non-treated cells (0hr controls); One-Way ANOVA with Bonferroni post-test.

DMOG Mediated HIF-1α Stabilization Under Normoxia Down-regulates CD55 Expression in SAECs

To assess if stabilizing HIF-1α in normoxic SAECs is adequate for CD55 down-regulation in absence of hypoxic conditions, CD55 expression was analyzed in 1μM DMOG-treated normoxic SAECs. Transcriptional down-regulation of *CD55* was observed following 6 hours of DMOG treatment in SAECs compared to the controls (Figure 40A, $p<0.01$). However, consistent with the longer time-frame required for CD55 protein down-regulation, as seen in our *in vitro* studies, western blot analysis shows down-regulation of CD55 occurring at 24 hours post-1μM DMOG treatment in normoxic SAECs (Figures 40B, C, $p<0.01$). Therefore, these *in vitro* DMOG studies suggest that expression of HIF-1α is sufficient for mediating CD55 suppression in airway epithelial cells, even in the absence of an hypoxic environment.

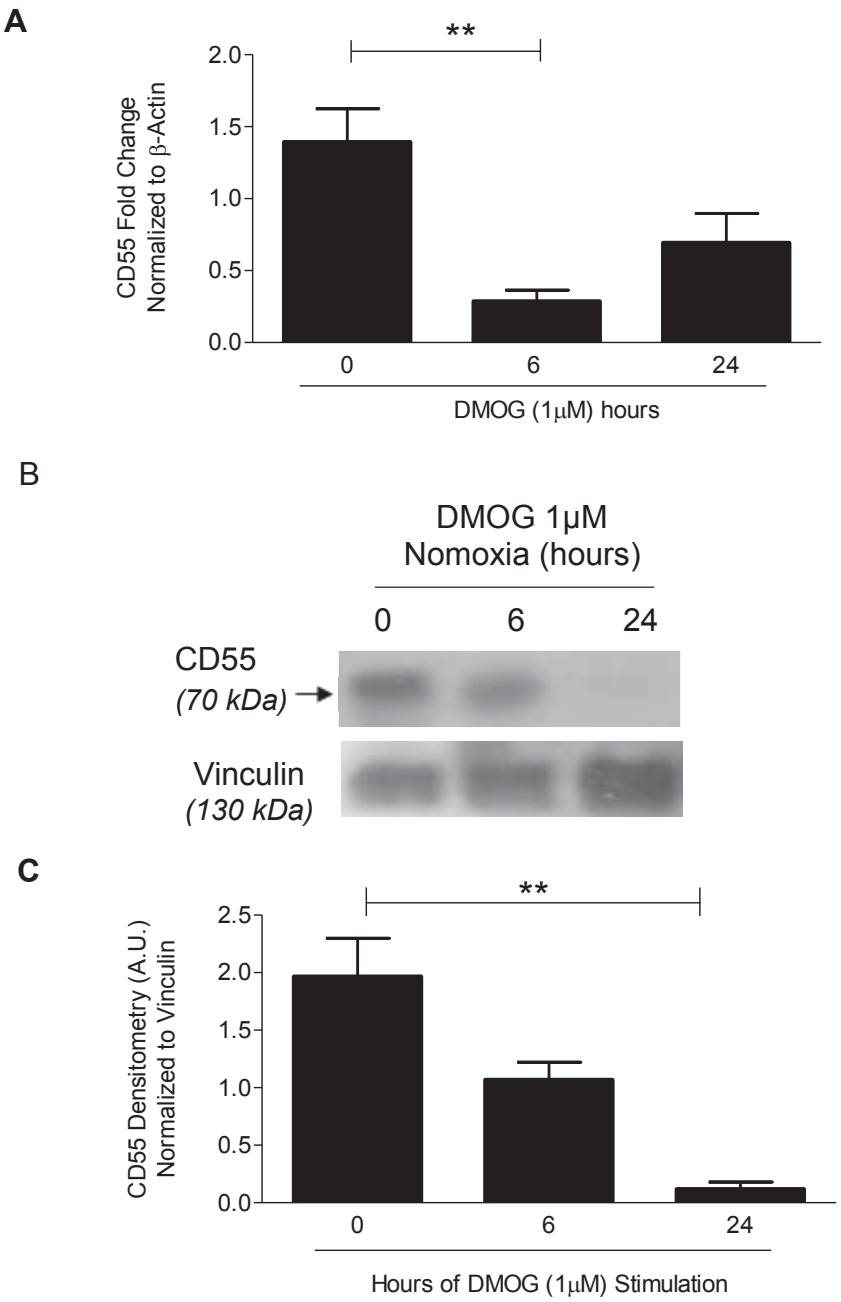

Figure 40. Induction Of HIF-1α In Normoxic SAECs Results In CD55 Down-regulation. **(A)** *CD55* transcript levels were down-regulated in 6h DMOG (1μM) treated SAECs compared to the non-treated cells (0hr controls). Data represents mean±SEM; n≥3; ***p*<0.01 vs 0h (non-treated cells); One-Way ANOVA with Bonferroni post-test. **(B)** CD55 protein expression was probed for in DMOG (1μM) treated SAECs. Lamin β1 was used as internal loading control. **(C)** Densitometry analysis show statically significant CD55 down-regulation by 24h of in DMOG (1μM) treated SAECs compared to the non-treated cells (0hr controls). Values represent mean±SEM; n=3; ***p*<0.01 vs non-treated cells (0hr controls); One-Way ANOVA with Bonferroni post-test.

Intratracheal Instillation of DMOG Stabilizes Lung HIF-1α During Normoxia

To address whether presence of HIF-1α in non-hypoxic conditions is also sufficient enough to regulate CD55 expression *in vivo*, intratracheal instillation of 1mg/ml of DMOG was implemented in normoxic C57BL/10 mice. Similar to the *in vitro* DMOG studies, HIF-1α specificity of DMOG was confirmed in our *in vivo* model. Significant stabilization of HIF-1α was observed as early as 6 hours in lungs of normoxic mice intratracheally instilled with 1mg/ml DMOG compared to normoxic mice instilled with the appropriate vehicle control (Figures 41A, B, $p<0.05$). To confirm that DMOG mediated-induction of HIF-1α occurred in airway epithelium, expression of HIF-1α was assessed by IHC. Staining data from these IHC studies show HIF-1α stabilization in airway epithelium 6 hours post-1mg/ml DMOG intratracheal instillation in normoxic mice, compared to its expression in airway epithelium of vehicle control mice (Figure 41C).

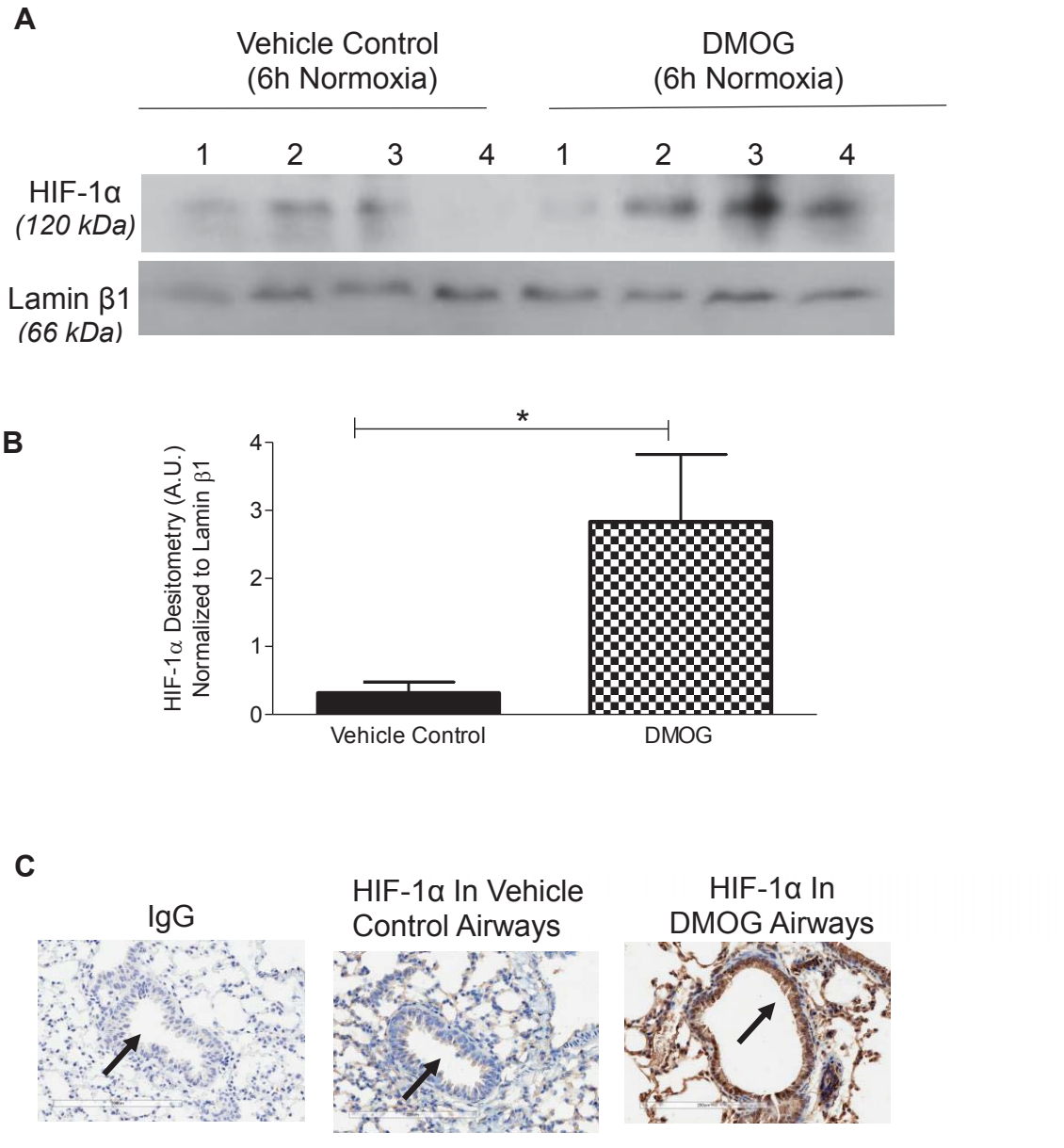

Figure 41. Intratracheal Induction Of HIF-1α In Normoxic Mice Lungs Down-regulates CD55 Expression. **(A)** HIF-1α expression was probed in lung homogenates 6h post mice intratracheal instillation with DMOG (1mg/ml) or vehicle control. Lamin β1 was used as internal loading control. **(B)** Densitometry analysis indicated significant induction of HIF-1α in lung homogenates of intratracheally instilled with DMOG (1mg/ml) or vehicle control. Values represent mean±SEM, n=4, *p<0.05 vs vehicle controls; two-tailed unpaired t-test. **(C)** IHC analysis indicate induction of HIF-1α in mice airways within 6h post-DMOG instillation compared to vehicle control airways.

DMOG Induces CA9 In Vivo

Assessment of *CA9*, an indicator of HIF-1α transcriptional activity, was performed to further confirm HIF-1α activity induced by its stabilization via DMOG within normoxic *in vivo* conditions. Normoxic mice lungs where HIF-1α was stabilized by intratracheal instillation of 1mg/ml DMOG, depict an increase in *CA9* transcripts within 6 hours compared to lungs of mice intratracheally instilled with vehicle controls (Figure 42, $p<0.05$).

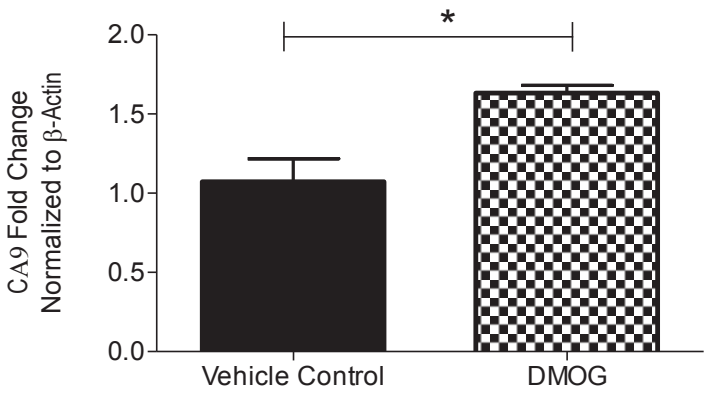

Figure 42. *In Vivo* Stabilization Of HIF-1α In Normoxic Lungs Results In Induction Of *CA9* Transcription. Transcription levels for *CA9* were up-regulated in DMOG (1mg/ml) treated SAECs compared to vehicle control cells. β-actin was used as internal loading control. Data represents mean±SEM; n=3; **p*<0.05 vs non-treated cells (0hr controls); two-tailed unpaired t-test.

Intratracheal Instillation of DMOG Results in Lung CD55 Down-regulation During Normoxia

We next investigated the role of HIF-1α in regulating CD55 expression during normoxia. Analogous to our *in vitro* DMOG studies, stabilization of HIF-1a via intratracheal instillation of 1 mg/ml DMOG in mice resulted in transcriptional down-regulation of *CD55* within 6 hours (Figure 43A, $p<0.01$) in lungs of DMOG instilled normoxic mice. Notably, densitometry analysis of CD55 protein expression also show down-regulation in lungs within 6 hours post- DMOG instillation in these normoxic mice compared to normoxic mice lungs instilled with vehicle control at the same time-point (Figures 43B, C, $p<0.01$). Airway epithelial specific CD55 suppression was also observed by IHC analysis in these DMOG treated mice compared to controls (Figure 43D). Together, these *in vivo* DMOG studies support the results obtained from DMOG in vitro studies that suggest that expression of HIF-1α, a transcription factor potently induced by hypoxia, is adequate to modulate airway epithelial CD55 expression in hypoxic, as well as, in non-hypoxic conditions. However, down-regulation of CD55 by HIF-1α stabilization in normoxic conditions did not induce local complement activation (Figure 44).

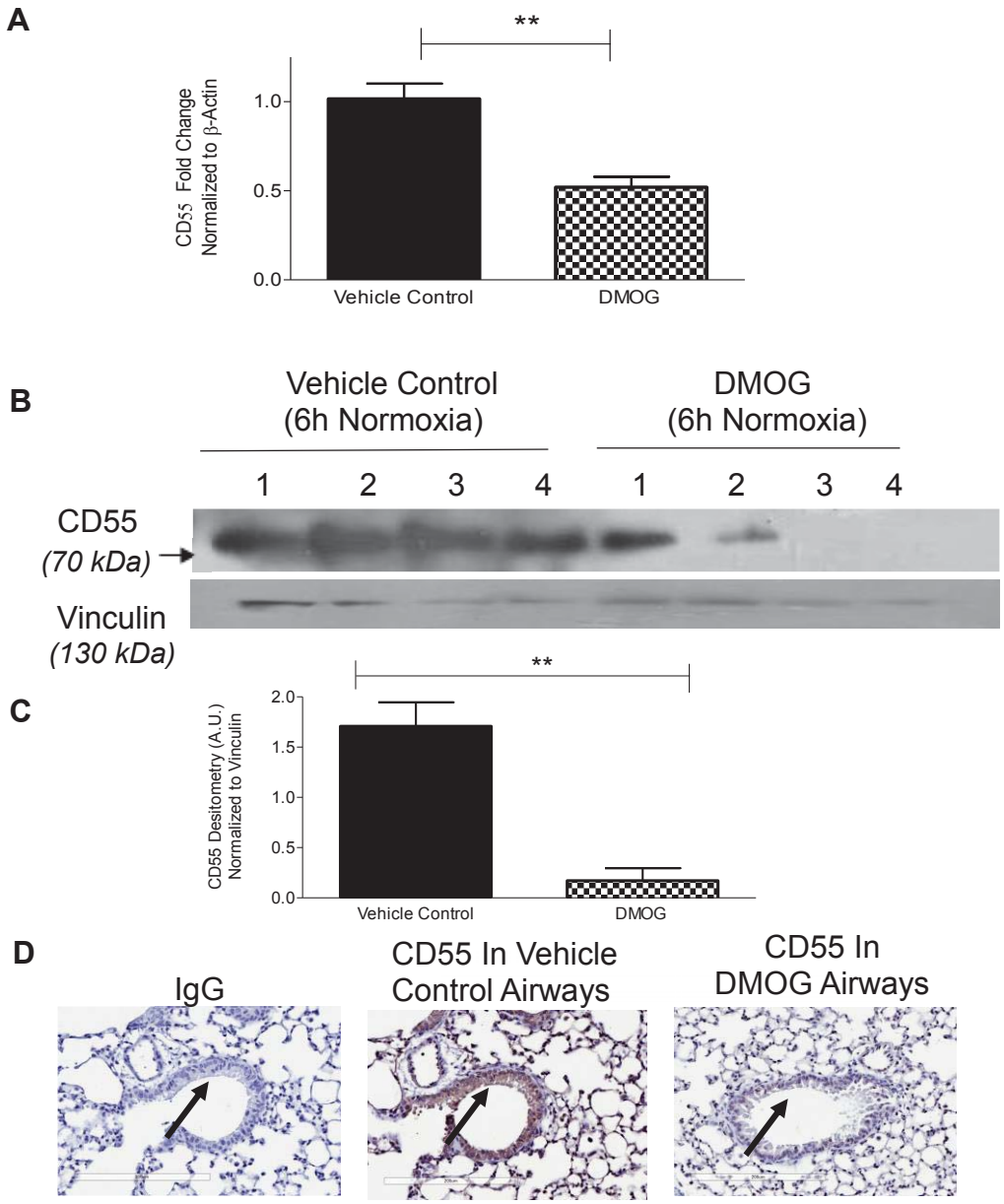

Figure 43. Intratracheal Induction Of HIF-1α In Normoxic Control Mice Down-regulates CD55 Expression In Lungs. **(A)** *CD55* transcripts were down-regulated in mice lungs intratracheally instilled with for 6h DMOG (1mg/ml) compared to vehicle controls. Data represent mean±SEM, n=3, $^{**}p<0.01$ vs vehicle controls; two-tailed unpaired t-test. **(B)** CD55 expression was probed in mice lungs 6h post intratracheal instillation with DMOG (1mg/ml) or vehicle controls. Vinculin was used as internal loading control. **(C)** Densitometry analysis indicated CD55 down-regulation in mice lungs intratracheally instilled with DMOG (1mg/ml) or vehicle controls. Values represent mean±SEM, n=4, $^{**}p<0.01$ vs vehicle controls; two-tailed unpaired t-test. **(D)** IHC shows CD55 down-regulation within 6h post-DMOG instillation vs vehicle control airways.

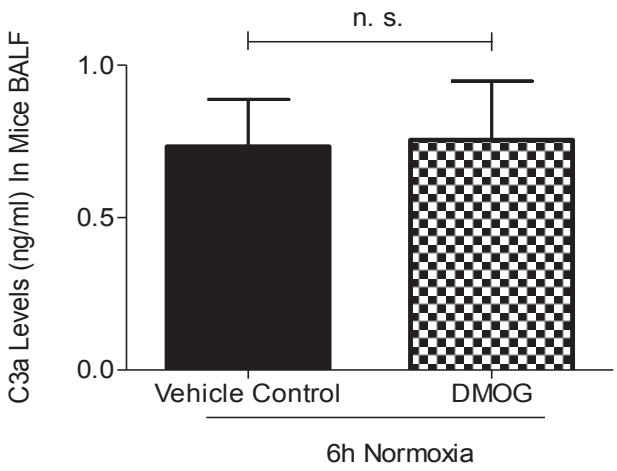

Figure 44. Down-regulation Of Airway Epithelial CD55 By DMOG, Does Not Result In Local Complement Activation. C3a levels are not statistically significant (n. s.) in BALF of vehicle control or DMOG 1 mg/ml intratracheally instilled mice within 6h in normoxia. Values represent mean$\pm$SEM; n$\geq$3; n. s. vs vehicle control; two-tailed unpaired t-test.

Gene Therapy-induced Expression of HIF-1α in SAECs by HIF-1α Adenoviral Vector, AdCA5

Pharmacological stabilization of HIF-1α in normoxic *in vitro* and *in vivo* conditions, down-regulate CD55. In a complementary approach, we utilized adenoviral vectors to induce constitutive HIF-1α expression in SAECs during normoxia. SAECs were transduced with an adenoviral vector, AdCA5, containing an insert to express constitutively activated HIF-1α (155). AdLACZ was used as the control vector (155). To confirm that HIF-1α expression was stabilized in normoxic SAECs following 50 PFUs/cell transduction of AdCA5, western blot analysis was performed. Compared to the control AdLACZ transduced normoxic SAECs, protein quantification of HIF-1α expression through densitometric analysis show induction of HIF-1α in AdCA5 transduced normoxic SAECs 24 hours post- transduction (Figures 45A, B, $p<0.05$).

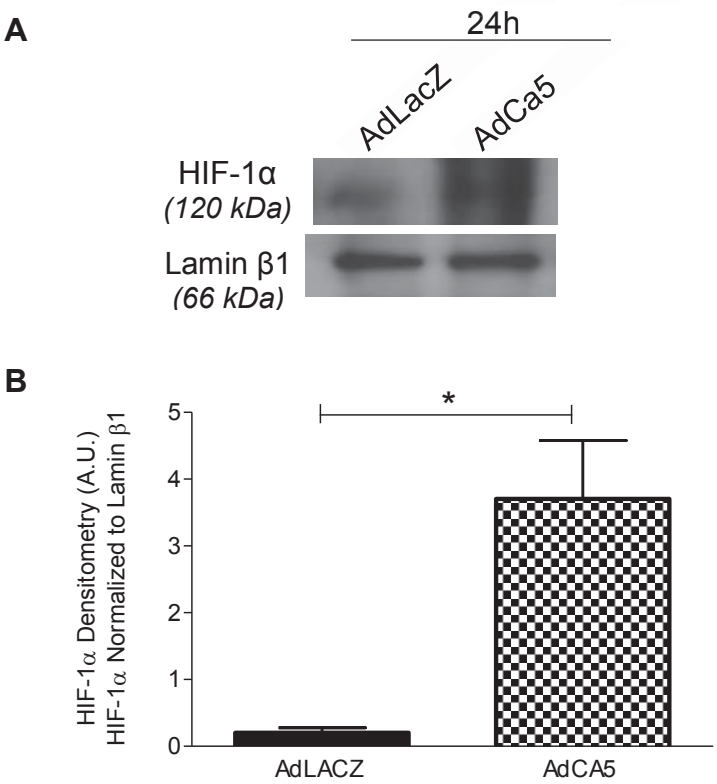

Figure 45. AdCA5 Mediated HIF-1α Induction In Normoxic SAECs Results In CD55 Down-regulation. **(A)** AdCA5 (50 PFUs/cell) transduced SAECs for 24h were probed for HIF-1α protein expression. Lamin β1 was used as internal loading control. **(B)** Densitometry analysis indicated significant induction of HIF-1α within 24h of AdCA5 transduced SAECs compared to cells with control AdLACZ (50 PFUs/cell) transduction. Values represent mean±SEM, n=3, **p*<0.05 vs AdLACZ; two-tailed paired t-test.

CA9 Expression was Induced in AdCA5 Transduced Normoxic Human SAECs

CA9 transcripts were assessed to confirm that AdCA5 mediated induction of HIF-1α in normoxic SAECs also resulted in HIF-1α transcriptional activity. Compared to *CA9* transcripts observed in SAECs transduced with control adenoviral vector, AdLACZ, AdCA5 transduced normoxic SAECs show increase expression of *CA9* transcripts. Thus, confirming that even in normoxic conditions, AdCA5 can induce HIF-1α expression which leads to activation of its transcriptional activity (Figure 46, $p<0.001$).

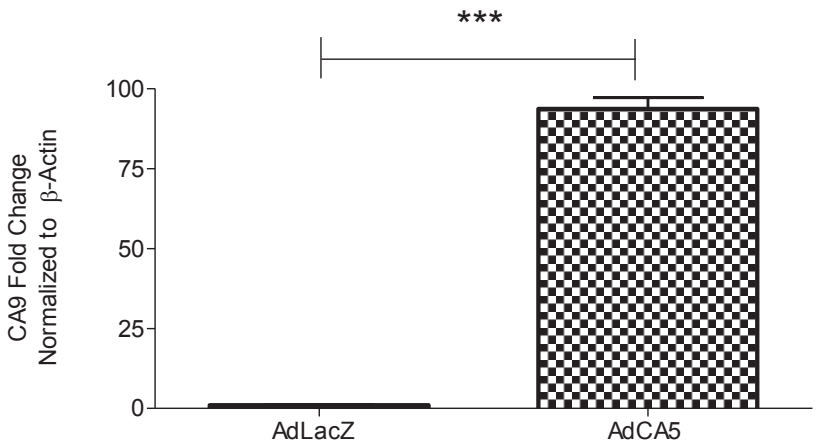

Figure 46. HIF-1α Stabilization By AdCA5 In Normoxic SAECs Induces *CA9* Transcription. *CA9* transcript levels were significantly elevated in normoxic SAECs transduced with AdCA5 (50 PFUs/cell) for 24h vs SAECs transduced with AdLACZ (50 PFUs/cell). β-actin was used as internal loading control. Values represent mean±SEM; n=3; ****p*<0.001 vs AdLACZ; two-tailed paired t-test.

CD55 Expression was Down-regulated in AdCA5 Transduced Normoxic Human SAECs

DMOG provided indirect evidence of HIF-1α regulating CD55 expression on airway epithelium, therefore, we also investigated CD55 expression in AdCA5 transduced normoxic SAECs. Western blot and densitometric analysis confirmed that transduction of normoxic SAECs with 50 PFUs/cell of AdCA5 for 24 hours, resulted in CD55 down-regulation compared to its expression in observed in the control AdLACZ transduced normoxic SAECs (Figures 47A, B, $p<0.05$). Collectively, these studies demonstrate that induction of HIF-1α, in the presence or absence of hypoxia, negatively regulates CD55 expression in airway epithelial cells.

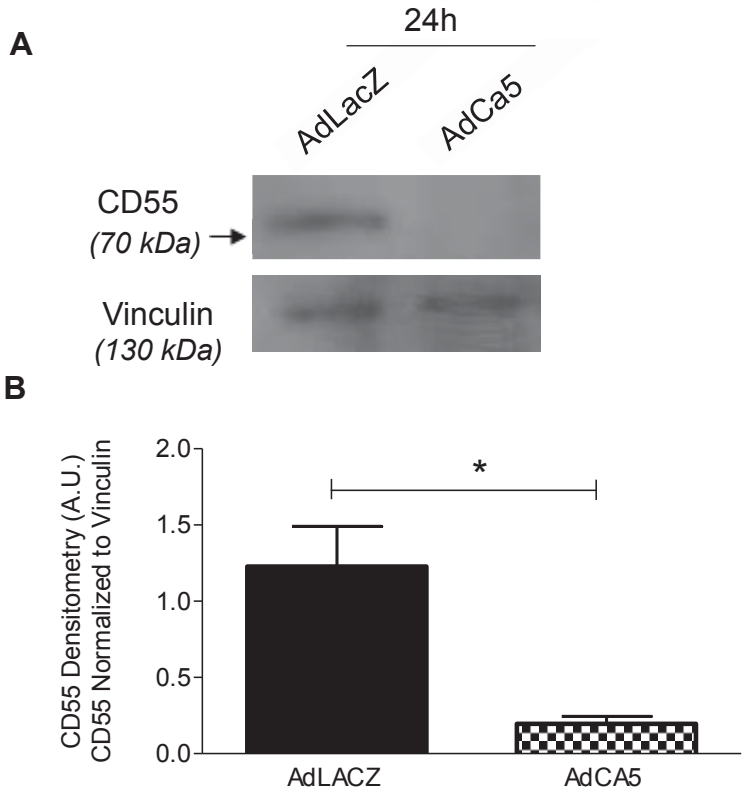

Figure 47. AdCA5 Mediated HIF-1α Stabilization Down-regulates CD55 In Normoxic SAECs. **(A)** CD55 expression was probed for in SAECs transduced with AdCA5 (50 PFUs/cell) for 24h. Vinculin was used as internal loading control. **(B)** Densitometry analysis show significant down-regulation of CD55 in 24h SAECs transduced with AdCA5 (50 PFUs/cell) compared to expression in AdLACZ (50 PFUs/cell) transduction. Values represent mean±SEM; n=4; *p<0.05 vs AdLACZ; two-tailed paired t-test.

IV. DISCUSSION

A. Summary

The key findings in these *in vitro* and *in vivo* studies, highlight the importance of HIF-1α in regulating airway epithelial CD55 expression. As indicated in part I and II, induction of HIF-1α coincided with CD55 down-regulation in hypoxic SAECs, as well as, in hypoxic mice lungs. Thus, to determine if the presence of HIF-1α correlated with suppression of CD55 on airway epithelium, HIF-1α expression was manipulated in airway epithelium, and CD55 expression was analyzed *in vitro* and *in vivo*. Notably, to confirm that HIF-1α induced during hypoxia mediates down-regulation of CD55, but not other membrane-bound CRPs, we assessed expression of other membrane-bound CRPs such as CD46 and CRRY in our hypoxic *in vitro* and *in vivo* studies, respectively. While *CD46* and *CRRY* transcript levels were down-regulated *in vitro* and *in vivo* respectively, neither of them were down-regulated at the protein level. These results suggest that CD46 and CRRY may be differentially regulated at the mRNA and protein level in hypoxic conditions. Another level of complexity for CD46 and CRRY regulation may involve the fact that although they are functionally and structurally similar, they are not identical which may also contribute to their differential regulation along with their location being species restricted. Therefore, our studies primarily focus on CD55 because it is affected by hypoxia, and its expression is not species restricted. Interestingly, we also observed difference in CD55 and CRRY expression between two different strains of mice. C57BL/10 mice when exposed to hypoxia for 24 hours

had down-regulation of CD55 at the transcript and protein level, but *CRRY* was only transcriptionally down-regulated. However, when C57BL/6 mice were exposed to hypoxia for 24 hours, no change in CD55 or CRRY expression was observed on the lungs.

Experimental findings in part III indicate that inhibiting HIF-1α in hypoxic SAECs by chetomin treatment or by transfection of HIF-1α siRNA, resulted in recovery of CD55. Notably, HIF-1α inhibition, *in vivo*, by intratracheal instillation of chetomin or HIF-1α siRNA also restored CD55 expression in hypoxic mice lungs. However, as suggested by part IV, induction of HIF-1α in normoxic human SAECs through AdCA5 transduction or treatment with DMOG, resulted in CD55 down-regulation. Similarly, *in vivo* HIF-1α stabilization mediated by intratracheal instillation of DMOG, caused CD55 down-regulation in normoxic mice lungs. To confirm HIF-1α specificity of chetomin and DMOG, transcript levels for *CA9* was assessed. Due to the short half-life of HIF-1α, *CA9* expression was also analyzed in the *in vivo* model to confirm HIF-1α activity.

Correlation between local complement activation and down-regulation of the CRP, CD55, on airway epithelium has been observed in these hypoxic *in vivo* conditions. Following the exposure of mice to 24 hours hypoxia, airway epithelial down-regulation of CD55 and local complement activation was observed in our *in vivo* hypoxic model. These experimental findings provide insight into HIF-1α mediated regulation of CD55. Although, the precise molecular mechanism by which HIF-1α regulates CD55 expression still remains undefined.

B. Cell-specific Effects of HIF-1α Mediated CD55 Regulation

While numerous studies have tried to elucidate mechanisms and signaling pathways that regulate CD55 expression (80), (190), only limited studies have focused their investigation on the role of hypoxia, and in particular, the role of HIF-1α, in regulating cell surface expression of CD55. Hypoxia-mediated down-regulation of CD55 has been identified in other studies with the human neuronal cell line, NT2N (191). However, to the best of our knowledge, this is the first study to show HIF-1α mediated negative regulation of CD55 on airway epithelial cells. On the contrary, prior studies by Colgan and Stahls' group have reported HIF-1α or hypoxia-mediated induction of CD55 expression on either human colorectal adenocarcinoma cells (80) or human umbilical vein endothelial cells (190), respectively. These contrasting reports on CD55 regulation by HIF-1α or hypoxia, may be attributed to the variability associated with the utilization of different cell types. In the current study we have assessed effects of HIF-1α on CD55 expression in the context of primary human airway epithelial cells. Whereas, prior reports exploited immortalized cell lines or utilized endothelial cells for analysis of hypoxia or HIF-1α dependent CD55 expression. In addition, the use of different cell types may add another factor of variability for hypoxia or HIF-1α mediated CD55 regulation, because it has been reported that different cell types have distinct time-frames during which hypoxia mediated- HIF-1α expression is induced (80), (190). Therefore, it is possible that the differences temporal regulation of HIF-1α expression among various cell types can differentially regulate CD55. Other factors that may contribute to the differential

CD55 expression observed among our studies and studies reported by Colgan (80) or Stahl's group (190), may involve the amount of oxygen concentration used to mimic hypoxia, or the time-frame of hypoxia implemented on these various cell types. Notably, studies reported by Stahl's group involve the assessment of CD55 under hypoxia and reoxygenated conditions. The involvement of reoxygenation initiates several other signaling pathways and mechanisms that may be distinct from those only seen in hypoxic conditions. This may result in variability of CD55 expression. Furthermore, while HIF-1α is potently induced by hypoxia, other down-stream signaling cascades such as activation of NFkB or MAPK are also initiated and play a pivotal factor in regulating responses associated with hypoxia (192), (193). Collectively, this suggests that while studies by Stahl's group show hypoxia-driven CD55 induction on human umbilical vein endothelial cells, they do not specifically illustrate the role of HIF-1α in regulating its expression (190). Additionally, unlike prior studies performed by Colgan (80) or Stahl's group (190), we confirmed the observed results from our *in vitro* data by translating them into *in vivo* conditions. Thus, demonstrating HIF-1α dependent down-regulation of CD55 in mouse airway epithelium.

C. Potential Role of Other HIF Isoforms in Regulating CD55

While the focal point of our studies was to understand and elucidate the role of HIF-1α in regulating CD55 expression in airway epithelium, other hypoxia inducible factor isoforms, such as hypoxia- inducible factors 2 alpha or 3 alpha

(HIF-2α and HIF-3α), might also have a role in CD55 regulation. HIF-1α was the primary hypoxia-inducible factor selected to assess CD55 in hypoxic *in vitro* and *in vivo* conditions, due to its ubiquitous and uniformly inducible nature, as well as, the wide array of studies available on its molecular mechanism and regulation (134), however, recent advances have highlighted the importance of HIF-2α and HIF-3α in hypoxic conditions (194), (175). Notably, while chetomin and DMOG are widely known to target HIF-1α they also have an effect on other HIF isoforms. Therefore, the indirect evidence of airway epithelial CD55 expression being regulated by chemical inhibitor or stabilizer of HIF-1α such as chetomin or DMOG, respectively, may also suggest that other HIF isoforms could also possibly play a role in airway epithelial CD55 expression. To date, no direct evidence is available for HIF-2α or HIF-3α mediated regulation of CD55. However, due to the ability of HIF-2α and HIF-3α to bind to HREs present on promoter regions of HIF-1α dependent genes, and the fact that these HREs comprise part of the promoter region for CD55, it is possible that the negative regulation of CD55 observed by HIF-1α may also incorporate regulation mediated by HIF-2α or HIF-3α. As previously mentioned, a splice-variant of HIF-3α is a dominant negative regulator of HIF-1α (175), (195). Therefore, it is also possible that the HIF-1α mediated inverse regulation of CD55 may, in part, be driven by presence of splice variant for HIF-3α (195). Notably, while the isoforms of these hypoxia-inducible factors are structurally similar, they are not functionally identical (135), (140). Therefore, these HIF isoforms possess the ability to cooperatively work together. Interestingly, these HIF isoforms still have

distinct mechanisms to regulate hypoxic responses in specific cell types (194). Expression of all three HIF isoforms may be temporally and differentially regulated depending on the specific cell type (135), (140). Along with expression of HIF-1α, HIF-2α (174) and HIF-3α (175) have also been identified to be induced in lung epithelium under hypoxic stimuli. Thus, while the possibility of other HIF-1α isoforms has not been directly assessed in our experiments, their role in regulating CD55 on airway epithelium is certainly plausible.

D. Positive and Negative Transcriptional Regulation by HIF-1α

Conventionally, the HIF-1α transcription factor is more commonly well-known for its role as a transcriptional activator of genes. However, in certain conditions, as indicated by our experimental studies, HIF-1α can also mediate transcriptional repression of genes. As previously mentioned, regulation of HIF-1α occurs at the post-translational level, whereby, the protein is stabilized and protected against proteosomal-degradation (134). Notably, expression of HIF-1α may be observed in our normoxic conditions as seen in our normoxic controls due to its inducible nature. Following its stabilization, HIF-1α translocates to the nucleus and binds to the constitutively expressed co-subunit, HIF-1β. This heterodimeric complex can co-assemble either with co-activators or co-repressors, and bind to HREs located within the promoter regions of the target gene to mediate its transcriptional activation or repression, respectively (134). While the specific mechanisms by which HIF-1α represses *CD55* transcription still remains to be elucidated, HIF-1α mediated epigenetic modifications on DNA

sequence of CD55 may contribute to its transcriptional down-regulation. Co-activators and co-repressors possess functional characteristics for modulating epigenetic changes on the target genes. While co-activators can initiate transcriptional activation of target genes via mechanisms such as histone acetylation (196), co-repressors can repress transcriptional activation by mechanisms such as deacetylation of histones and making them less accessible for transcription (197). This histone deacetylase (HDAC) mediated co-repressor activity, may be one potential mechanism that is responsible for HIF-1α mediated transcriptional down-regulation of *CD55*. Studies have indicated a role for several HDACs binding to HIF-1α including histone deacetylases 1, 2, 3, 4, 5, 7 (HDAC1, HDAC2, HDAC3, HDAC4, HDAC5, HDAC7), and Sirtuin-3 (SIRT3) (198), (199), (200), (201). However, the type of HDAC-HIF-1α complex formed varies depending on the cell type, the gene targeted for repression, and the extracellular or intracellular conditions in the context where repression of the target gene is necessary (202), (203). On the contrary, certain HIF-1α and HDAC interactions can also result in transactivation of some genes (201), (204). Thus, deacetylation of histones may not be the only mechanism that potentially represses *CD55* transcription. Other functional characteristics of the co-repressors, such as methylase or demethylase activity, may also be playing a role (205).

Inverse regulation of CD55 by HIF-1α may also regulated by its competition with other transcription factors to bind to co-activators for transcriptional activation of genes such as CD55. Due to the limited availability

of co-activators present in the nucleus, binding of these coactivators to HIF-1α may, in fact, result in less available coactivators to bind to transcription factors that could possibly induce CD55 transcription in hypoxic airway epithelium. Notably, it is also known that other transcription factors compete with HIF-1α to bind to co-activators for transcriptional activation (206), which may result in less binding of co-activators to HIF-1α. Thus, providing a possible explanation for the observed induction of CD55 transcripts that surpass the normoxic basal transcripts seen in chetomin and siRNA *in vitro* studies. Notably, the 3' untranslated region of CD55 mRNA is comprised of AU-rich elements that can destabilize CD55 transcripts, unless the stabilizing protein HuR is bound to it (207). Thus, it is possible that HIF-1α could prevent HuR binding to the AU-rich elements in CD55 mRNA which may result in decrease CD55 transcript levels (207). Recent advances have also suggested that the orientation of the HREs present on the antisense strand of the target gene may also contribute to repression of genes mediated by HIF-1α (208), (209). However, further studies will need to be conducted to determine the precise mechanism by which the HRE orientation may regulate HIF-1α dependent CD55 transcriptional down-regulation in airway epithelial cells.

E. Putative Mechanisms for HIF-1α Mediated Suppression of CD55

Our studies have reported transcriptional and post-translational down-regulation of CD55 on airway epithelial cells, where HIF-1α expression is induced in the presence or absence of hypoxia. However, a temporal lag is observed

between transcriptional and post-translational CD55 down-regulation. While this interval between HIF-1α dependent suppression of CD55 mRNA and protein expression on airway epithelium, may result from amount of time it takes to go through the eukaryotic transcription-translation process (210), (211), which are temporally and spatially distinct, the differential regulation of *CD55* transcripts or protein expression can also contribute to the observed lag period. Transcriptional repression of *CD55* in airway epithelial cells can inhibit the translation of CD55 protein. However, other HIF-1α dependent mechanisms must be present in order to down-regulate the existing membrane-bound CD55 already present on the surface of these airway epithelial cells.

Due to the presence of GPI-anchor on CD55, their down-regulation may be mediated by enzymatic cleavage through proteolytic proteins that are up-regulated in hypoxic conditions such as PIPLC (212), or MMPs (77). Internalization of CD55 by oxidative stress can also result in its down-regulation. Notably, hypoxic conditions within the lung can initiate oxidative stress by the production of reactive oxygen species (ROS) (213) or reactive nitrogen species (RNS) such as nitric oxide (NO) (214). Thus, internalization of CD55 via oxidative stress may serve as another potential mechanism by which the CD55 protein is down-regulated (215). S-nitrosylation by NO can result in CD55 mRNA degradation by inhibiting HuR, a stabilizing protein, from binding AU-rich elements on the 3' untranslated region of CD55 mRNA (207). Other proteins reported to mediate down-regulation of membrane-bound CRPs, such as CD55, include IL-17A and activated complement proteins (76), (96). In the context of

IPF and OB, airway epithelial expression of CD55, along with other membrane-bound CRPs, were down-regulated by IL-17A or activated complement proteins (76), (96). However up-regulation of IL-17A and complement activation has also been reported in lung IRI. Particularly, since hypoxia is a component of ischemia (108), it is plausible that the cell surface down-regulation of CD55 seen in our experimental studies may be regulated by hypoxia induced IL-17A or complement activation (76).

F. Regulation of Complement Activity by Other CRPs in Airway Hypoxia

Functionally characterized as a CRP, one major role for CD55 is to regulate complement activation. Interestingly, local complement activation was observed in our *in vivo* hypoxic model at the same time-point as the down-regulation of airway epithelial CD55 down-regulation was observed. However, complement activation was not inhibited in hypoxic mice BALF where CD55 was restored due to HIF-1α silencing. Thus, it is also possible that presence of serine proteases from other mechanisms initiated in the hypoxic environment, such as activation of the coagulation system, could maintain complement activation (216). It is also possible that complement activation can also be initiated by serine proteases present in leukocytes, such as neutrophils, that may be activated in hypoxia- induced inflammation.

Stabilization of HIF-1α in normoxic mice by DMOG, does not result in complement activation despite the down-regulation of CD55. Therefore, it is also

possible that presence of other CRPs; such as the mouse-specific CRRY; as well as, other membrane-bound or soluble CRPs may also play a role in modulating the activity of complement. Thus, while hypoxia induced HIF-1α-dependent mechanisms down-regulate CD55, other CRPs may provide protection against complement-mediated *in vivo* injury in airway hypoxia by compensating for the loss of CD55. Activation of complement within 24 hours post-hypoxia in our *in vivo* model, suggests that other hypoxia-driven mechanisms may attenuate the role for these other CRPs to control complement activity. However, we have not directly investigated the role of other CRPs in our studies. Contrary to our *in vivo* functional data, we did not assess complement activation in our *in vitro* studies, because although airway epithelial cells can synthesize complement proteins, *in vitro* hypoxic conditions, alone, will not induce complement activation since the presence of serine proteases are critical.

G. Therapeutic Relevance for Understanding HIF-1α Dependent CD55 Regulation on Airway Epithelium

To date, only limited studies have reported or identified mechanisms that regulate CD55. However, to the best of our understanding, the current data is the first to identify the role of HIF-1α negatively regulating CD55 expression on airway epithelium. Elucidating mechanisms that regulate expression of CD55 in the context of airway hypoxia, are of importance due to its role in several pulmonary pathologies such as OB, IPF, allergic asthma, and lung IRI (36) where hypoxia is a factor (117), (118), (121), (125). In these lung diseases where

hypoxia is known to occur, complement activation is also observed (76), (96). Notably, in OB and IPF down-regulation of CD55 and other membrane-bound CRPs correlate with the local complement activation which may contribute to the pathogenesis of these diseases (76), (96). Therefore, understanding how to regulate the expression of CRPs, such as CD55, may help attenuate some of the complement-mediated lung injuries associated with some of the hypoxia and complement mediated pulmonary diseases like OB or IPF.

Our studies provide insight into potential therapeutic interventions for preserving CD55 expression in pulmonary pathologies where down-regulation of CD55 and local complement activation are observed. Through our experimental studies, we have shown that airway hypoxia induced HIF-1α mediates CD55 down-regulation on airway epithelium. Thus, one possible mechanism to preserve CD55 expression in pulmonary diseases; such as asthma, OB, IPF, or lung IRI; where hypoxia occurs, is to inhibit the expression of HIF-1α. However, the caveat to this is that HIF-1α plays a major role in other biological processes, such as angiogenesis and metabolism, that help adapt cells and tissues to these hypoxic conditions at the cellular and physiological level (135). Therefore, while inhibiting HIF-1α expression may preserve CD55 expression and protect against complement activation, it may also ultimately result in cell death due to the lack of the cells' ability to adapt to hypoxia by mediating physiological responses that are critical for survival. On the contrary, HIF-1α does not always promote survival in hypoxic conditions. Depending on the severity of hypoxia, HIF-1α can also induce cell apoptosis. Therefore, due to the various cellular effects that HIF-

1α has in hypoxia, other options may be more suitable for potential therapeutic interventions that could regulate complement activity in hypoxic pulmonary pathologies.

Over-expression of CD55 may be the more appropriate therapeutic option in these pulmonary diseases where local CD55 down-regulation and complement activation are observed. To protect and attenuate some of the complement-mediated inflammation observed in lungs of asthmatic individuals, CD55 protein containing SCRs could be directly delivered into the lungs in the form of aerosolized proteins via inhalers or nebulizers as seen in other treatments used for asthma (217), (218). In terms of IPF, several pharmacological treatments are utilized to prevent or manage IPF. However, the efficacy of the drugs were variable, and none of them specifically targeted complement activation. Therefore, the only successful and promising treatment known to prolong survival in individuals with IPF is lung transplantation (219). Interestingly, however, even during or after lung transplantation, injury of the lungs are observed in the form of lung IRI or OB, respectively. Notably, it is possible that complement-mediated injury seen in lung IRI may predispose the lung into developing OB. Therefore it is important to target complement activation during the transplant procedure to mitigate complement-mediated lung damage, and improve the outcome of lung transplant survival.

As previously mentioned, while several inflammatory mechanisms play a role in the onset of OB and lung IRI, complement activation is a common factor in both. More so, down-regulation of CRPs, such as CD55, is also observed in OB.

Thus, over-expressing CD55 during the transplant procedure via ex vivo lung perfusion (EVLP), may attenuate some of the complement-mediated injury seen in lung IRI or OB. EVLP is a technique that involves improving the state and condition of the donor lungs prior to transplant by flushing them with acellular normo-thermic perfusate (220), thereby, controlling complement-dependent injuries associated with lung IRI or OB by over-expressing CD55 during the EVLP procedure. This can be achieved by flushing the lungs during EVLP with soluble CD55. This enables the soluble CD55 to inhibit local complement activation by preventing or dissociating C3 and C5 convertases in the lung. Following lung transplant, alloimmune-mediated mechanisms may also initiate local and systemic complement activation in OB. Over-expression of CD55 as a therapeutic intervention in these conditions may involve using aerosolized CD55 delivery as indicated for asthmatic patients for local complement activation, or the possibility of injecting soluble CD55 intravenously in transplant recipients to regulate systemic complement levels.

Despite the innovative concept of CD55 over-expression as a therapeutic treatment for several hypoxia-mediated pulmonary diseases where activation of complement and CD55 down-regulation are observed, the exogenous over-expression of CD55 may not result in complete abrogation of the lung injury. This is due to the fact that many other inflammatory mechanisms, besides complement activation, also regulate the pathogenesis of OB, IPF, asthma, and lung IRI. While down-regulation of the CRP, CD55, can correlate with the increased complement activation observed in the lung diseases, presence of

other membrane-bound or soluble CRPs also regulate complement activity. Therefore, the most promising therapeutic intervention would be one that targets not only the complement activation via over-expression of CRPs, such as CD55, but one that also focuses on targeting other inflammatory mechanisms like blocking of adhesion molecules. Interfering with the adhesion molecules on the endothelium can disrupt the recruitment of inflammatory leukocytes like neutrophils to the site of injury that contribute to exacerbated inflammation. Thus, targeting multiple factors that cause lung injury may serve to attenuate or lessen the injury/inflammation. Although, our studies of HIF-1α mediated CD55 down-regulation in hypoxic airways do not provide direct therapeutic relevance in the clinical setting, we did highlight a new mechanism by which airway epithelial down-regulation of CD55 occurs. Consequently, the therapeutic relevance of our experimental studies lies in understanding the mechanisms to preserve CD55 expression or over-express them in the lungs to attenuate complement-mediated pulmonary injuries.

V. CONCLUSIONS

CD55, a membrane-bound CRP, is constitutively expressed on the surface of many cell types including airway epithelium (69), (76). However, airway epithelial down-regulation of CD55 observed in several pulmonary diseases associated with hypoxia, led us to hypothesize that hypoxic conditions may be responsible for suppression of CD55. In particular, hypoxia-induced transcription factor, HIF-1α, may be regulating the expression of CD55. Notably, by assessing expression of other membrane-bound CRPs such as CD46 or CRRY in our *in vitro* and *in vivo* studies, we recognized the hypoxia mediated protein down-regulation of membrane- bound CRP is specific to CD55 not CD46 or CRRY (Figures 23, 24, 25). In our studies we further demonstrated the role for HIF-1α in mediating CD55 down-regulation in airway epithelial cells. Exposing SAECs or our mouse model to hypoxic conditions, resulted in down-regulation of CD55 in airway epithelium (Figures 22, 24). Interestingly this effect was only seen in C57BL/10 mice and not C57BL/6 mice, which may give rise to the possibility that various strains of mice may possess differential expression of CRPs like CD55 (Figure 26). Thus, to verify the presence of hypoxia, we reported the expression of a potent hypoxia-induced protein, HIF-1α *in vitro* (Figure 20). However, assessing HIF-1α expression in our hypoxic mice model was difficult due to its short half-life. Therefore, we further confirmed the presence of hypoxia *in vitro* and *in vivo*, by assessing transcriptional expression of *CA9*, an indicator of HIF-1α transcriptional activity (Figures 20C, 21). To verify that the hypoxia-induced HIF-1α, was responsible for suppression of *in vitro* and

in vivo airway epithelial CD55, we inhibited expression of HIF-1α using chetomin (Figures 28, 30) or HIF-1α siRNA (Figures 31, 34). Notably, HIF-1α inhibition via chetomin (Figures 28, 30) or HIF-1α siRNA (Figure 33, 35), resulted in recovery of CD55 expression on airway epithelial cells in our *in vitro* and *in vivo* studies. Inhibition of HIF-1α was further confirmed by data depicting that *CA9* transcripts were down-regulated in hypoxic SAECs or hypoxic mice lungs where HIF-1α was inhibited by chetomin (Figures 29, 30D) or silenced by siRNA (Figures 32, 35). Furthermore, we verified that hypoxia-mediated CD55 down-regulation that was occurring, was dependent on HIF-1α rather than other hypoxia-driven mechanisms. This was achieved by stabilizing HIF-1α expression in normoxic *in vitro* and *in vivo* conditions via DMOG treatment (Figures 38, 41) or by transducing normoxic SAECs with adenoviral vector that expresses constitutively activated HIF-1α (Figure 45). Even in the absence of hypoxia, *in vitro* or *in vivo* stabilization of HIF-1α resulted in CD55 down-regulation (Figures 40, 43, 47). Increased transcript levels of *CA9* was observed in normoxic SAECs (Figures 39, 46) or mice lungs (Figure 42) where HIF-1α was stabilized. Thus, confirming the induction of HIF-1α in these normoxic conditions. Interestingly, down-regulation of CD55 expression in our hypoxic *in vivo* models within 24 hours, correlated with complement activation observed in BALF of these 24 hour hypoxic mice.

These results support our hypothesis that HIF-1α regulates expression of CD55 on airway epithelial cells. From these data, we can hypothesize potential mechanisms by which HIF-1α represses CD55 mRNA. One possible mechanism is that HDACs enable HIF-1α-coactivator complex to bind to rHRE regions on

genes like CD55 where HIF-1α acts to repress their mRNA (Figure 48). Therefore, in hypoxia where HDAC, HIF-1α, or HIF-1α- coactivators are inhibited via trichostatin A, chetomin, or HIF-1α siRNA respectively, then we see that CD55 mRNA is restored to basal levels (Figure 49). HIF-1α is also known to induce expression of proteolytic enzymes like MMPs (221). Thus, while it is possible that CD55 mRNA repression which occurs by HIF-1α transcription as suggested by our data, prevents translation of further CD55 protein, the down-regulation of the already present GPI-anchored CD55 protein on airway epithelial cell surface in hypoxia may occur by other HIF-1α dependent mechanisms. One such mechanism may involve MMPs that could cleave CD55 protein from cell surface, hence, why we see CD55 down-regulation. It is possible that HIF-1α induces transcription of MMPs which then go on to be translated and mediate proteolytic cleavage of CD55 from cell surface. This could provide the possible explanation as to why there is a temporal difference between down-regulation of CD55 mRNA and CD55 protein in our *in vitro* studies.

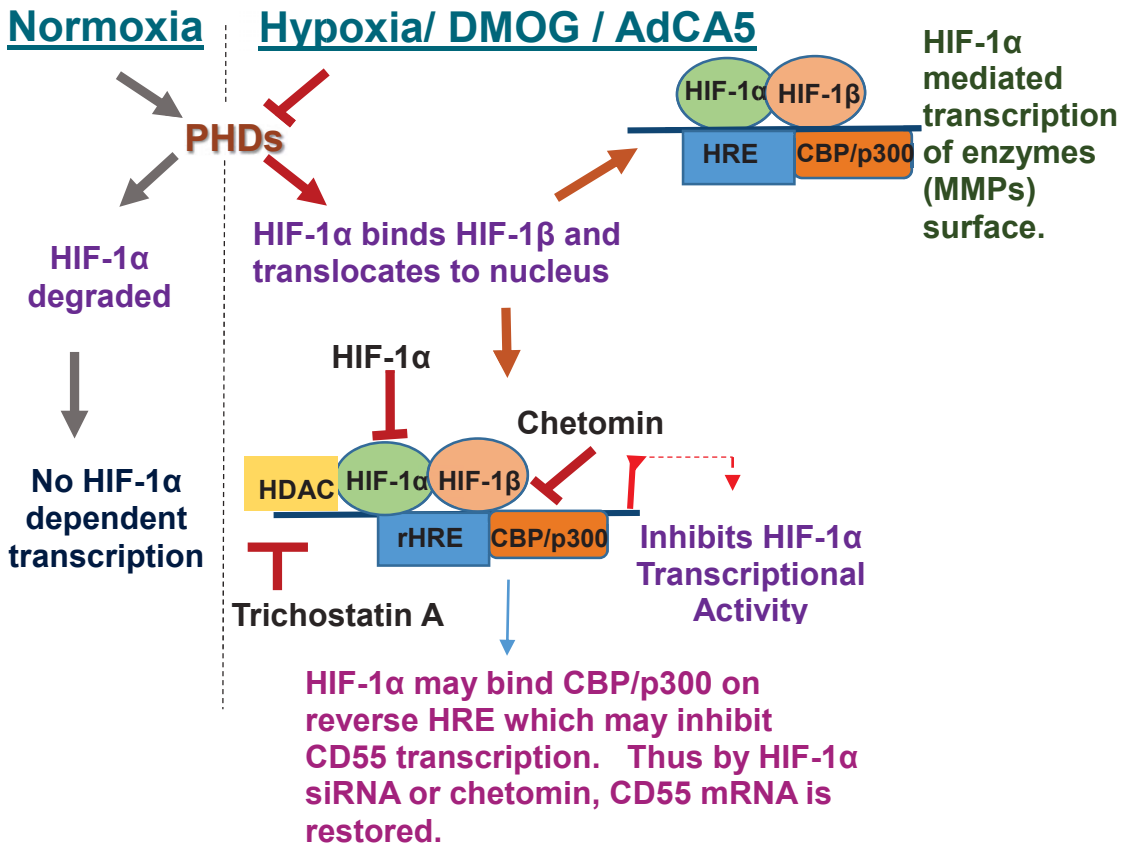

Figure 48. Proposed Mechanistic Model. In normoxic conditions, PHDs are activated and hydroxylate HIF-1α which targets it for proteosomal degradation. However, in hypoxic conditions, PHDs are not activated and HIF-1α protein is stabilized. In normoxic conditions, HIF-1α protein can also be stabilized by DMOG or AdCA5. Stabilization of HIF-1α enables it to bind to its constitutively expressed partner, HIF-1β, and the complex is translocated to the nucleus. Once in the nucleus, HIF-1α can complex with coactivators like CBP/p300, and bind to HRE regions on promoter region of genes. One proposed hypothesis is that down-regulation of CD55 mRNA may occur when HDACs bind this HIF-1α-coactivator complex which may enable it to bind to rHRE on gene of interest. However, inhibiting HIF-1α by siRNA or chetomin or inhibiting HDACs can reverse this phenomenon. HIF-1α can also induce expression of proteins like MMPs which may contribute to protein down-regulation of CD55.

VI. FUTURE STUDIES

While our findings are novel in that we have identified HIF-1α mediated negative regulation of CD55 on airway epithelial cells, some limitations that still exist within our studies result in unresolved questions that may be elucidated by future studies. Previous studies have reported a correlation between the presence of HIF-1α and induction of CD55. This was confirmed by studies demonstrating the direct binding of HIF-1α to HREs present on CD55 (80). Due to the negative regulation of CD55 by HIF-1α in our studies, we are limited in that we have not assessed direct binding of HIF-1α to the CD55 promoter region by a chromatin immunoprecipitation (CHIP) assay. However, by performing a CHIP assay using anti-HIF-1α and an antibody against a specific HDAC, we may be able to detect what co-activators or co-repressors complexes are present during the HIF-1α and CD55 interaction. This may gave insight into how HIF-1α negatively regulates CD55 (80).

Future studies will also need to be conducted to elucidate the precise mechanism by which HIF-1α negatively regulates CD55 expression in our model. It has been widely reported that HIF-1α can bind to co-repressors such as HDACs to inhibit gene expression (222). Preliminary studies suggest a role for HDACs in regulating airway epithelial CD55 expression under hypoxic conditions (Figure 49). SAECs exposed to 6 hours of hypoxia restored CD55 mRNA expression when treated with optimal dose of HDAC inhibitor trichostatin A (Figure 49, $p<0.05$, $p<0.001$).

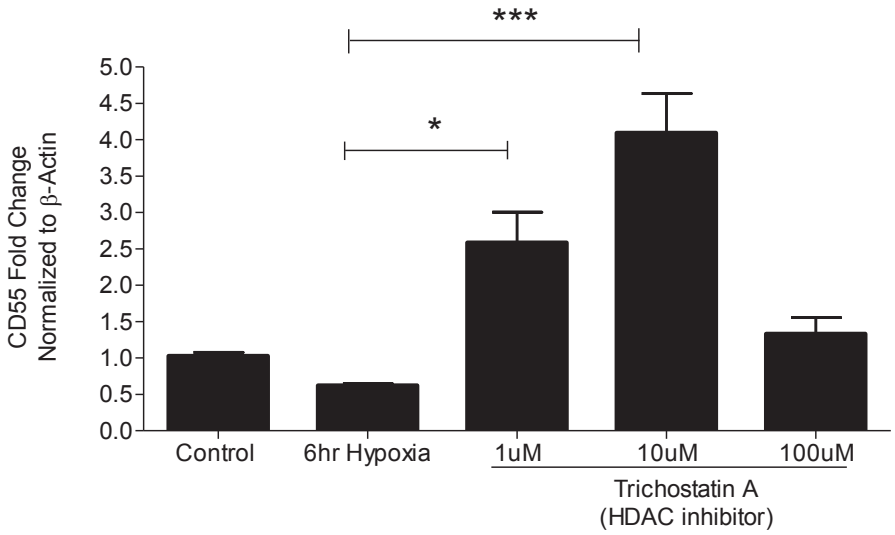

Figure 49. Inhibiting HDACs By Trichostatin A Restores CD55 Expression In Hypoxic SAECs. Hypoxic SAECs treated with trichostatin A results in recovery of CD55 mRNA. Data represents mean±SEM, n=3; ***p<0.001 vs 6h hypoxia, *p<0.05 vs 6h hypoxia; One-Way ANOVA with Bonferroni post-test.

One future study that could provide insight into how the HIF-1α mediated negative regulation of CD55 occurs on airway epithelial cells, is to screen for the presence of various HDACs in our *in vivo* and *in vitro* hypoxic versus normoxic studies. Based on these results, a CHIP assay could be utilized to confirm the recruitment of HIF-1α and the specific HDAC to the HRE in CD55 promoter region (199). From these studies, the role of the particular HDAC identified in HIF-1α mediated negative CD55 regulation, can be further assessed by examining CD55 expression when this HDAC is silenced. It has also been reported that HIF-1α mediated repression of certain genes may be regulated by orientation of HREs in the anti-sense strand (208), (209), (223). This could be assessed by performing a promoter analysis using a database search for CD55 (199), which may show if one if the sites has an inverted or reverse sequence for HRE. Furthermore, since *CD55* has at least three HRE sites (80), a series of deletion mutants can be constructed on *CD55* promoter to assess which HRE is the site responsible for the transcriptional role of HIF-1α on HREs present within *CD55* promoter (199).

Another area of this study that requires further investigation is identifying the mechanisms that lead to CD55 protein down-regulation in these hypoxic airway epithelial cells. While HIF-1α mediated *CD55* transcript down-regulation may prevent translation of more CD55 protein expression, the down-regulation of the pre-existing CD55 expression present on the cell surface may result from cleavage of the GPI anchor by proteolytic enzymes. There are several proteolytic enzymes that may contribute to CD55 down-regulation. However, it

has been reported that hypoxia, in particular, HIF-1α can induce expression of proteolytic enzymes known as matrix metalloproteinases (MMPs) (134). The role of such enzymes in regulating CD55 protein expression in hypoxic conditions, could be determined by inhibiting MMP activity and assessing if CD55 expression is preserved. To assess which particular MMP may be responsible for CD55 down-regulation, a multiplex MMP ELISA array may be used (224).

Although our studies showed a promising role for HIF-1α for airway epithelial CD55 regulation, further studies need to be conducted to elucidate how intervention in this mechanism could attenuate complement-mediated inflammation observed in hypoxia-associated pulmonary diseases. While complement activation has been observed in asthma and lung IRI, another potential study would be to assess CD55 expression on airway epithelium of these particular hypoxic pulmonary diseases, because that has not been directly assessed. A caveat to these and other hypoxic pulmonary diseases like OB and IPF, is that other mechanisms besides hypoxia could be suppressing CD55. Regardless of the mechanisms involved in airway epithelial CD55 down-regulation in these lung conditions, it would still be interesting to analyze if down-regulation of CD55 contributes to local complement activation. If the latter scenario is true, then the over-expression or preservation of CD55 could be utilized in such pulmonary diseases to control or inhibit complement activation. It has been reported that local complement activation, and CD55 down-regulation occurs in the transplanted lung with OB (76). Whereas, blocking complement activation attenuates incidence of OB (76). Therefore, it would be beneficial to

intervene in the mechanism that mediates HIF-1α-dependent CD55 down-regulation to protect the lung from complement mediated injury. This could be assessed by using the mouse OB model. Prior to the transplant, HIF-1α expression could be locally silenced in the mouse lung via intratracheal instillation of HIF-1α siRNA or chetomin. In theory, HIF-1α silencing would mitigate its induction in hypoxic conditions such as IRI that occur during lung transplant. Thus, expression of CD55 would be preserved on the airway epithelium of the transplanted lung. However, as previously mentioned, one stipulation with this study is that silencing of HIF-1α would also inhibit its expression for other processes such as metabolism or pH regulation which aid in adapting the cells or the tissues to hypoxic conditions (225). Therefore, the more appropriate method may be to locally over-express soluble CD55 containing regions critical for complement regulation in the donor mouse prior to lung transplant. In this manner, even if induction of HIF-1α occurs, and CD55 is down-regulated on airway epithelium, the exogenous presence of soluble CD55 can help protect the transplanted lung from complement injury. Additionally, we also retain the HIF-1α expression for processes critical for hypoxic adaptions. Over-expression of CD55 in the donor lung during the transplant procedure can be of clinical relevance as well. As previously mentioned, the therapeutic relevance of overexpression of CD55 during the transplant procedure can be achieved with the goal of improving the quality of the donor lungs making them more suitable for transplant. This can be mediated by the use of soluble CD55 in

perfusing the lungs during EVLP to attenuate complement activation that may occur during the hypoxic phase in lung IRI.

Detrimental effects of hypoxia are not limited to airway epithelium. Due to the ubiquitous nature of CD55 expression, further studies can also be conducted to assess how hypoxia-induced HIF-1α affects CD55 expression on other cell types present within the lungs such as lymphocytes and other leukocytes. This may shed light on other pathways that can activate complement in hypoxic lungs. While CD55 is known to regulate complement activation, recent findings have suggested its role in binding to CD97 (226). This may be beneficial in the sense that CD55 can also initiate other aspects of the immune system even if it keeps complement activation in check. It has also been reported that expression of CD55 on mucosal epithelial, can bind to CD97 on cells such as neutrophils and mediate its epithelial transmigration resulting in further inflammation (80), (227). Notably, CD55 also plays a role in adaptive immunity by being involved in $CD4^+$ T cell co-stimulation and activation (228). Therefore, it would be interesting to investigate the effects of HIF-1α mediated CD55 down-regulation on other innate or adaptive cell types present within the lung.

Since the primary focus of our studies involved HIF-1α mediated effects on CD55 expression within airway epithelium, it would also be useful to assess the effects of HIF-1α of other membrane-bound CRPs such as CRRY and CD59 on airway epithelium or on local soluble CRPs present in the interstitium of the lung. Notably, understanding how HIF-1α impacts airway epithelium or

leukocytes expressing the complement receptors, C3aR and C5aR, can also be further investigated.

Another limitation present within our studies was the amount of oxygen concentration we utilized to mimic hypoxia. While most of our *in vitro* and *in vivo* hypoxic studies were performed in 1% O_2 or 10% O_2, respectively, it would be interesting to assess what oxygen concentration induces HIF-1α, and how this affects CD55 expression under varying oxygen levels. This can be pursued by repeating all of the indicated hypoxic SAEC and animal experiments under varying oxygen concentrations. However, one caveat of assessing varying oxygen levels *in vivo* is that exposing mice to severe hypoxia (less than 10% O_2) for long time points may be prove to be lethal (184), (185). However, short-term exposure of mice to less than 10% O_2 may not be harmful. Similarly, future studies could also assess *in vitro* and *in vivo* HIF-1α and CD55 expression in hypoxic conditions over varying time-points, other than the 6 and 24 hours that we utilized in our studies.

Another aspect of this study that entails further investigation is the analysis of other HIF isoforms in regulating CD55 expression on airway epithelium. As previously mentioned, HIF-2α and HIF-3α can also be induced in hypoxic airway epithelial cells (174), (175). Notably, while we have utilized specific mechanisms to specifically intervene with HIF-1α such as HIF-1α siRNA or AdCA5, treatments with DMOG and chetomin can also target other HIF-isoforms (151), (186). Thus, it is possible that HIF-2α or HIF-3α can also play a role in regulating CD55 expression on airway epithelial cells. To specifically

investigate the role of these HIF isoforms in CD55 regulation, HIF-2α or HIF-3α siRNA can be transfected in hypoxic SAECs or be intratracheally instilled in mice prior to their hypoxic exposure and CD55 expression can be assessed.

Completion of these future studies will highlight the importance of the amount of oxygen concentration, as well as, the time-frame required for regulation of CD55 in hypoxic airway epithelium. Similarly, these suggested experiments may also indicate a role for other HIF isoforms or CRPs in regulating complement activation in hypoxia- associated pulmonary diseases. Collectively, these potential studies will provide new insight on our current research on how CD55 can be regulated in hypoxic airway epithelium.

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Paper Publications

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Abstracts

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