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Inflammatory Profiles and Immune Responses to High-Altitude Acclimatization

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DEDICATION

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ABSTRACT OF THE DISSERTATION

Inflammatory Profiles and Immune Responses to High Altitude Acclimatization

by

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High altitude is a physiologically stressful environment due to oxygen limitation and low atmospheric pressure. Despite these conditions, over 160 million people live, work, or travel to high altitude annually. Several systemic physiological changes in response to hypoxia promote acclimatization to high altitude. However, even in acclimatized individuals, there is still a significant risk of developing high-altitude illnesses (HAIs), such as acute mountain sickness, high-altitude pulmonary edema, or high-altitude cerebral edema. While it is clear that these pathologies result from high altitude/hypoxia, gaps in our knowledge remain regarding the underlying mechanisms that drive HAI development.

The molecular pathways that control hypoxia responses are evolutionary conserved and have significant crosstalk to essential mechanisms that drive inflammation. While these responses may be key for acute adaptation to hypoxia, they may become maladaptive if not properly mediated. For example, native highlanders show evidence of natural selection for traits that promote adaptation to chronic hypoxia, but different native high-altitude populations display distinct adaptations. However, not all these adaptations are beneficial. Most notably, Andean highlanders have a high prevalence of Chronic Mountain Sickness (CMS), an incapacitating syndrome induced by lifelong exposure to hypoxia. The underlying mechanisms behind CMS are also unknown, however it has been suspected that inflammation may play a role in CMS pathogenesis.

The goal of my dissertation research was to determine how high-altitude hypoxia induces changes in inflammatory profiles and immune cell populations. Furthermore, I aimed to investigate if immune cells at high altitude are sensitized to inflammatory stimuli, and if these responses are dependent on hypoxia inducible factor (HIF) activity. To accomplish these goals, I employed several techniques such as multi-parameter flow cytometry, multiplex immunoassays, *in vitro* stimulation experiments, and an unbiased transcriptomic approach. These studies demonstrate that acute high-altitude exposure increases inflammatory expression, as well as promotes a pro-inflammatory immunophenotype. Furthermore, HIF plays a role in immune cell surface markers, most notably CD14. As sojourners acclimatize, the inflammatory profile favors an anti-inflammatory phenotype. Overall, these studies provide important insights into the role of inflammation in high altitude acclimatization and characterize a potential mechanism underlying hypoxia-induced immune sensitization.

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Chapter 1: Introduction

1.1 High-Altitude: Acclimatization and Adaptation

High altitude environments are extremely physiologically stressful due to low atmospheric pressure and oxygen limitation. To maintain oxygen homeostasis at high altitudes, humans rapidly acclimatize via several systemic physiological changes that occur in response to systemic hypoxia (Beall, 2006; Scheinfeldt *et al.*, 2012; Simonson, 2015; Moore, 2017). These physiological responses occur on a timescale of minutes to months. The most important physiological responses include increased ventilation and increased red blood cell production, both essential to promote oxygen delivery following high altitude induced hypoxemia. In short, ventilatory acclimatization to hypoxia is the increase in baseline ventilation and sensitivity of the ventilatory chemoreflexes during chronic sustained hypoxia exposure (Moore, 2001; Lenfant & Sullivan, 2010; Pamenter & Powell, 2016). This functions to promote recovery of the alveolar and arterial oxygen partial pressures, which also promotes recovery of oxygen saturation and total arterial oxygen content. Additionally, red blood cell production, as well as cardiac output, increases in order to maintain oxygen delivery to tissues (Savourey *et al.*, 2004). Together, these physiological changes compensate for the low oxygen tension at high altitude by increasing oxygen uptake and delivery (West, 2004).

While these physiological changes are crucial for acclimatization, these changes are adaptive in the context of lowlanders who travel to high altitude acutely, and some of these phenotypes are blunted over long exposure times (months to years). In comparison, native highlanders who have survived in these hypoxic environments for thousands of years exhibit distinct physiological adaptations. For example, the native Tibetan and Andean populations have both lived in severe high-altitude hypoxic environments.

However, each population has developed distinct adaptations to promote survival and fitness. Not all of these adaptations, however, are considered beneficial. When comparing these two groups, the Tibetan population are considered as one of the most well-adapted populations to high-altitude hypoxia, while the native Andean highlanders, while also well adapted to this environment in many ways, may have also developed some maladaptive physiological responses to life in hypoxia (Beall, 2006; Bigham *et al.*, 2013; Jeong *et al.*, 2014; Simonson, 2015). These two distinct populations therefore represent an incredible natural experiment in which both groups experienced similar environmental stressors but developed unique adaptations to these environments, likely based on genetic traits. One of the most significant and stark differences in the Tibetan versus Andean adaptations are the differences in ventilation. As previously mentioned, one of the first and most crucial responses in lowlanders to high altitude is the immediate increase in minute ventilation to promote increased oxygen consumption (Moore, 2001; Lenfant & Sullivan, 2010). This increased ventilation response is typically maintained for weeks but begins to decline slightly over months of exposure in a process called hypoxic ventilatory decline. While there are no long-term studies measuring hypoxic ventilatory decline over years of exposure, it is possible that the hypoxic ventilatory response may be nearly completely lost over years of exposure as a means to conserve energy by reducing the need for constant elevated ventilation. Interestingly, the elevation in resting ventilation and the hypoxic ventilatory response have been sustained in Tibetan populations, whereas the Andean populations have a low resting ventilation and nearly complete loss of the hypoxic ventilatory response (Beall, 2006, 2007). Hematocrit is also significantly different in Andeans versus Tibetans. One of the hallmarks of Andean highlander physiology is the significantly higher red blood cell concentration. While an increased red blood cell

production and hemoconcentration initially seems beneficial to promoting oxygen delivery to tissues, it can quickly become maladaptive, and lead to significant worsening problems due to the impacts of elevated blood viscosity.

While these acclimatization responses in lowlanders to high altitude and adaptive responses in native highlanders have been well documented through extensive research (Beall *et al*., 1990, 2012; Savourey *et al*., 2004; Hoit *et al*., 2005; Vij *et al*., 2005; Beall, 2006, 2007; Simonson, 2015; Pham *et al*., 2021; Yu *et al*., 2022; Mallet *et al*., 2023), the molecular mechanisms that drive these responses are poorly understood. Current research has identified several molecular mechanisms that respond to systemic hypoxia, and how the maladaptation of these mechanisms may lead to the development of high-altitude pathologies. Furthermore, little is known about how the immune system adapts to chronic hypoxemia at high altitude in sojourners or high-altitude native populations. As a result, my research investigates the mechanisms underlying immune adaptations to chronic hypoxemia at high altitude across different timescales, from acute exposure to lifelong exposure. I also explore the mechanisms underlying these immune adaptations and inflammatory responses to high altitude, and their potential links to maladaptive phenotypes and high-altitude illnesses.

1.2 High-Altitude Illness

High-altitude illnesses (HAIs) are a common risk for sojourners to high altitude (>2,500 m elevation). Within the first week of exposure, sojourners often present with Acute Mountain Sickness (AMS). However, in serious cases, sojourners may develop severe and potentially fatal illnesses such as high-altitude pulmonary edema (HAPE), high-altitude pulmonary hypertension (HAPH), or high-altitude cerebral edema (HACE) (Gallagher & Hackett, 2004; Mehta *et al.*, 2008; Luks *et al.*, 2017). The incidence and

severity of HAPH, HAPE and HACE vary depending on multiple factors, such as ascent time, altitude, time to initial recognition, and treatment (Gallagher & Hackett, 2004). Despite several decades of examining the physiology of AMS, HAPH, HAPE, and HACE, some questions remain regarding the pathophysiology of these conditions and the extent to which inflammation contributes to their onset and progression.

1.2.1 Acute Mountain Sickness

Acute Mountain Sickness (AMS) affects 25% of all travelers to high altitude above 2500m, and is characterized by headache, nausea, fatigue, and gastrointestinal issues (Roach *et al.*, 2018). AMS typically resolves within 1-3 days with acclimatization; however, severity and duration of symptoms depend on elevation and ascent time to high altitude ((Gallagher & Hackett, 2004). Acute Mountain Sickness (AMS) development results from a complex network of physiological responses to hypoxia (i.e., inflammation, vasogenic edema, acidosis) as well as anatomical factors (i.e., insufficient cerebrospinal fluid production, varied cerebral venous blood flow) (West, 2004; Luks *et al.*, 2017). While there is substantial research on potential contributors to AMS (Imray *et al.*, 2010; Luks *et al.*, 2017), the exact biological pathways and molecular mechanisms behind AMS development remain unclear.

1.2.2 High-Altitude Cerebral Edema

High-altitude cerebral edema (HACE) is a severe and potentially fatal complication that can occur in individuals who travel above 2000m. HACE is accompanied by symptoms including headache, ataxia, decline in cognitive function, and can lead to seizures (Hackett, 1999). As with AMS, HACE is most common with rapid ascent. HACE is frequently preceded by some AMS symptoms and both illnesses are influenced by cerebral hemodynamics. As a result, HACE is sometimes considered a more severe form

of AMS, however additional distinct pathophysiological mechanisms contribute to HACE development (Hackett & Roach, 2004; Brugniaux *et al.*, 2007; Li *et al.*, 2018). HACE can develop spontaneously at very high altitudes in acclimatized individuals (Clarke, 1988). HACE appears to occur as a result of hypoxia-mediated cerebral vasodilation and a subsequent impairment of the autoregulation of cerebral blood flow, loss of blood brain barrier integrity, and a rise in intracranial pressure (Hackett, 2000). Ultimately, cytotoxic and vasogenic edema leads to microvascular disruption and microbleeds (Hackett *et al.*, 2019).

1.2.3 High-Altitude Pulmonary Hypertension and High-Altitude Pulmonary Edema

High-altitude pulmonary hypertension (HAPH) occurs due to general hypoxic pulmonary vasoconstriction (HPV). Under typical conditions, local HPV aids in redistributing blood flow away from lung regions with poor ventilation and improves pulmonary gas exchange. However, at high altitude, global reductions in alveolar oxygen partial pressure can lead to HPV throughout the lung and increase mean pulmonary artery pressures (Swenson, 2013). HAPH is estimated to impact up to 10% of high-altitude residents (León-Velarde *et al.*, 2005). The clinical presentation of HAPH includes dyspnea, general fatigue, exercise intolerance, and in severe cases, chest pain and mental alterations, and ultimately cor pulmonale. While acute HAPH is primarily driven by increased vasomotor tone, chronic hypoxic stress and persistent pulmonary hypertension can produce vascular remodeling and exacerbate pulmonary artery pressures (Groves *et al.*, 1987; Wilkins *et al.*, 2015). The hallmark of vascular remodeling in chronic hypoxia is increased vessel muscularization (Arias-Stella & Saldana, 1963; Hislop & Reid, 1976).

High-altitude pulmonary edema (HAPE) presents with dyspnea, chest congestion, confusion, and drowsiness. HAPE incidence ranges from 0.5 - 15% of travelers at high

altitude but occurs most commonly during rapid ascent (Paralikar, 2012). This is due to exaggerated hypoxic pulmonary vasoconstriction which causes acute pulmonary hypertension, increased capillary permeability, and alveolar fluid buildup (Talbot *et al.*, 2005; Dunham-Snary *et al.*, 2017; Swenson, 2020; Brito *et al.*, 2020; Sydykov *et al.*, 2021).

1.2.4 Chronic Mountain Sickness

While AMS, HAPH, HAPE, and HACE can occur in all high-altitude travelers, unique pathologies can occur in long-term or lifelong high-altitude residents. Chronic Mountain Sickness (CMS) is a progressive, fatal disease that occurs in about 5-10% of all high-altitude populations (Villafuerte & Corante, 2016). CMS is characterized by excessive red blood cell (RBC) production (excessive erythrocytosis (EE); [Hb] ≥ 21 g/dL in men and ≥ 19 g/dL in women), severe hypoxemia, endothelial dysfunction, sleep disturbance, and neurological and cardiovascular impairments (Monge, 1943; Monge *et al.*, 1989; León-Velarde *et al.*, 2005). While there are multiple high-altitude populations with genetic and physiological adaptations to chronic hypoxia, CMS is prevalent in a large proportion of native Andean highlanders (30% of Andean men by age 60 and 77% of post-menopausal women), but rare in other high-altitude native populations (Villafuerte & Corante, 2016).

1.3 Possible Contributors to High-Altitude Illnesses

While it is clear that HAIs result from the high-altitude hypoxic environment, the precise underlying mechanisms of these pathologies remain unknown. Known risk factors for HAI development include the speed of ascent, the height of ascent, exertion of ascent, and if the individual had prior HAI susceptibility (Bärtsch & Swenson, 2013; Luks *et al.*, 2017). Contrary to popular belief, there is no clear evidence that pre-existing medical problems, such as asthma, diabetes, and obesity, increase susceptibility to HAIs, and general fitness is not associated with development of, or confer protection

from, these pathologies (Honigman *et al.*, 1993; Ri-Li *et al.*, 2003). Several studies have reported other risk factors that may impact HAI susceptibility, such as sex differences, history of AMS or migraines, and age, however there is as many conflicting and controversial studies that disagree (Richalet *et al*., 2012; Bärtsch & Swenson, 2013; Canouı¨- *et al*., 2014; Mcdevitt *et al*., 2014; Santantonio *et al*., 2014; Yang *et al*., 2015a; Gonggalanzi *et al*., 2016; Shen *et al*., 2020). The most prominent feature of HAIs is hypoxemia due to the low oxygen tensions. However, this alone does not account for HAI development, as some individuals are more susceptible and may have more exacerbated hypoxemia with worsening conditions. Current research has identified several possible contributors to the development of HAIs, such as lower ventilatory drive, impaired gas exchange, fluid retention, and altered metabolism (Senn *et al.*, 2006; Mehta *et al.*, 2008; Bailey *et al.*, 2009*b*; Richalet *et al.*, 2012; Bärtsch & Swenson, 2013; Luks *et al.*, 2017). These factors may impact cerebral blood flow and vascular permeability that would, in turn, lead to HAI development; however, these hypotheses remain inconclusive and controversial.

Research on AMS development has found several potential contributors from both physiological responses to hypoxia (i.e., inflammation, hypoxemia, vasogenic edema, acidosis) as well as anatomic factors (i.e., insufficient cerebrospinal fluid production, varied cerebral blood flow) (Hackett, 2000; Luks *et al.*, 2017). Previous research has found no correlation between differences in cerebral blood flow and AMS, and while increased vascular permeability may be due to the hypoxia-inducible factor (HIF) and the downstream activation of the hypoxia-response pathway, there is conflicting results regarding the correlation between evidence of cerebral edema and AMS symptoms (Reeves *et al.*, 1985; Bailey *et al.*, 2006; Kallenberg *et al.*, 2007).

Furthermore, CMS has also been extensively studied and possible contributors were identified, such as exacerbated hypoxemia due to blunted hypoxic ventilatory chemosensitivity (Severinghaus *et al.*, 1966; León-Velarde & Richalet, 2006) and sleep disordered breathing (Spicuzza *et al*., 2004; Pham *et al*., 2017; Heinrich *et al*., 2020). However, these contributors do not tell the whole story of CMS development, as chemosensitivity is blunted in a majority of Andeans and some people develop EE with no sleep disordered breathing. A rising interest has focused on the role of inflammation and pathological immune responses in HAI development (**Figure 1.1**). While the underlying mechanisms behind the inflammatory response to hypoxia as well as the potential adaptive benefit from changes in the inflammatory profile in both the acute and chronic hypoxia exposure are still under investigation, there are several studies that have reported the inflammatory profiles at high altitude.

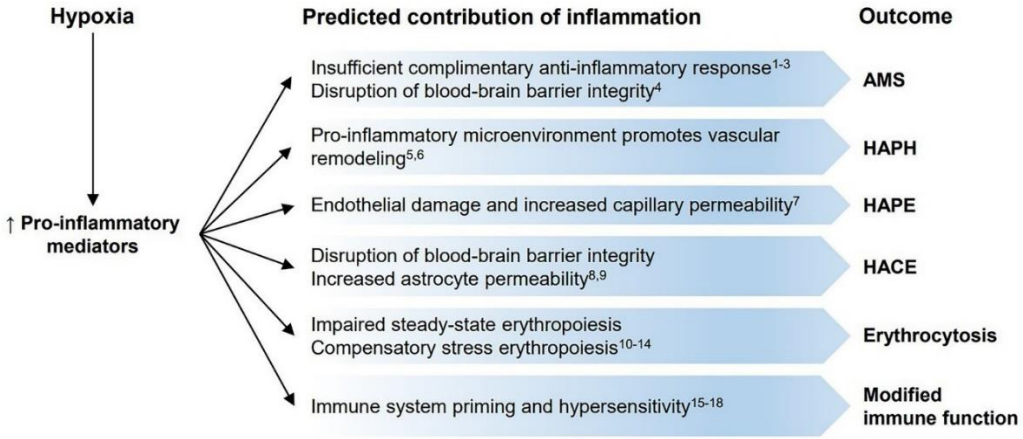

Figure 1.1. Predicted contributions of inflammation in high-altitude illnesses, erythrocytosis, and immune function. Hypoxic conditions lead to increases in pro-inflammatory mediators, which may play a role in the development of high-altitude illnesses (AMS, HAPH, HAPE, HACE) and erythrocytosis, or modify immune function. **References**: (1) Julian et al. 2011; (2) Liu et al. 2017; (3) Wang et al. 2018; (4) Vararttharaj and Galea 2017; (5) Wilkins et al. 2015; (6) Brito et al. 2020; (7) Mishra et al. 2016; (8) Song et al. 2016; (9) Zhou et al. 2017; (10) Jackson et al. 2010; (11) Liao et al. 2018; (12) Bennett et al. 2019; (13) Imagawa et al. 1990; (14) Haase et al. 2013; (15) Kvamme et al. 2013; (16) Baze et al. 2011; (17) Facco et al. 2015; (18) Feurecker et al. 2019.

1.3.1 Role of Inflammation in Acute Mountain Sickness

Recent studies support a potential role of inflammation in AMS. There is a general consensus that pro-inflammatory cytokines and other inflammatory markers (most notably C-Reactive protein (CRP), IL-1β, and IL-6) are increased in individuals acutely exposed to hypoxia or high altitude (Hartmann *et al.*, 2000; Song *et al.*, 2016; Lundeberg *et al.*, 2018; Wang *et al.*, 2018; Malacrida *et al.*, 2019; Kammerer *et al.*, 2020). In some cases, inflammatory mediator expression appears to differ across individuals who develop AMS and those who do not. For example, individuals who develop AMS have been reported to show decreased plasma levels of interleukin-10 (IL-10) and increased IL-1β, IL-6, and TNF-α compared to non-AMS controls (Liu *et al.*, 2017; Wang *et al.*, 2018). Furthermore, the association between plasma IL-6 and AMS score has been identified by multiple independent groups (Boos *et al.*, 2016; Wang *et al.*, 2018). While these studies indicate that some circulating pro-inflammatory markers may be associated with AMS development, it is not yet clear what specific role they may play. It is possible that hypoxia initiates the release of inflammatory and angiogenic mediators which disrupt the blood brain barrier and promote vasogenic edema.

Alongside the changes in the inflammatory cytokine profile, hypoxia-mediated oxidative stress is also implicated in the pathophysiology of high-altitude maladaptation, as high-altitude hypoxic stress results in increased tissue oxidative stress and radical oxygen species (ROS) production (Vij *et al.*, 2005; Malacrida *et al.*, 2019). Not only is oxidative stress a well-known feature of the inflammatory response, as it promotes the generation of pro-oxidant species, but high altitudes also exacerbate these responses, since high altitude exposure significantly challenges human homeostasis (Sarada *et al.*, 2008; Bailey *et al.*, 2009*a*; Himadri *et al.*, 2010; Julian *et al.*, 2011; Yi *et al.*, 2021; Pham

et al., 2021; Mallet *et al.*, 2023). Furthermore, ROS can play a role in the progression of inflammation, particularly in a hypoxic environment (Mittal *et al.*, 2014). This mutual activation between inflammatory and oxidative stress responses has been called OxInflammation. Several studies have found different oxidative stress biomarkers were positively correlated with AMS and may be a contributing factor to the development of AMS (Araneda *et al.*, 2005; Bailey *et al.*, 2009*a*; Arulselvan *et al.*, 2016; Lüneburg *et al.*, 2016; Irarrázaval *et al.*, 2017). Furthermore, studies have also found decreases in antioxidant activity that also correlated with AMS severity (Arulselvan *et al.*, 2016; Irarrázaval *et al.*, 2017; Fei *et al.*, 2018). Since the antioxidant pathway and inflammatory pathway are interconnected in their responses to hypoxic stress, this inflammatory/oxidative environment promotes a dangerous feedback loop that could synergistically cause damage and lead to pathogenesis (Arulselvan *et al.*, 2016).

A complementary hypothesis is that protection from AMS may be driven by a robust anti-inflammatory response which protects against potential increased blood brain barrier permeability caused by acute systemic inflammation (Varatharaj & Galea, 2017). This is supported by data from Julian et al. (2011) who found higher levels of anti-inflammatory marker (IL-1RA, HSP70, adrenomedullin) expression in AMS-resistant compared to AMS-susceptible individuals (Julian *et al.*, 2011). Finally, both steroids and nonsteroidal anti-inflammatory drugs (NSAIDS) equally reduce AMS incidence despite their different modes of action (Dumont *et al.*, 2000; Gertsch *et al.*, 2012; Zheng *et al.*, 2014). This suggests that both COX-2 mediated inflammation as well as analgesic mechanisms that mediate nociception contribute to AMS symptomology (Hartmann *et al.*, 2000; Song *et al.*, 2016; Kanaan *et al.*, 2017). Indeed, inflammatory mediators released during tissue injury can activate nociceptors and can contribute to pain hypersensitivity

(Kidd & Urban, 2001). However, Lundeberg et al. (2018) notes no change in AMS symptoms in individuals receiving Ibuprofen at normal recommended doses of 400 mg three times a day (Lundeberg *et al.*, 2018), perhaps due to the lower dosage compared to other studies which did observe a reduction in AMS symptoms with a higher Ibuprofen dose (600 mg three times a day) (Gertsch *et al.*, 2010, 2012; Lipman *et al.*, 2012). The role of antioxidant activity is also supportive of a beneficial anti-inflammatory response, as studies have also shown that pretreatment with antioxidant drugs have demonstrated increased antioxidant production and activity in rats at high altitude (Lu *et al.*, 2020; Pena *et al.*, 2022). Overall, there is strong supporting evidence of an inflammatory response at high altitude that could be triggered by both the hypoxic environment as well as increase in oxidative stress. These factors can synergistically trigger and/or exacerbate AMS development.

1.3.2 Role of Inflammation in High-Altitude Cerebral Edema

While some aspects of HACE pathophysiology remain uncertain, the underlying mechanisms producing HACE are likely similar to those of AMS and inflammation may play a role in its development (Bailey *et al.*, 2009*a*; Song *et al.*, 2016; Zhou *et al.*, 2017). In both mouse and rat models, pro-inflammatory cytokines are significantly increased in the brain cortex after hypoxia exposure. Furthermore, when pre-treated with bacterial lipopolysaccharide (LPS) to produce a systemic inflammatory response, subsequent hypoxia exposure results in cerebral edema (Song *et al.*, 2016; Zhou *et al.*, 2017). It is proposed that pre-existing inflammation increases aquaporin 4 (AQP4) activity in astrocytes via toll-like receptor 4 (TLR4), mitogen-activated protein kinase (MAPK), and NF-κB signaling, thereby increasing blood-brain barrier permeability. Together, these data indicate that when challenged with a combination of hypoxia and inflammation, the

combination of increased pro-inflammatory cytokines and increased blood vessel permeability produces vasogenic edema (Song *et al.*, 2016; Zhou *et al.*, 2017). To reiterate the importance of hypoxia-induced oxidative stress and inflammation, previous studies have reported that free radicals can damage the blood-brain barrier, which can contribute to HACE development (Bakonyi & Radak, 2004; Bailey *et al.*, 2019). In synergy with inflammation, the increase in oxidative stress can also lead to increases in NF-kB, the master transcription factor of the inflammatory pathway, and trigger upregulation of pro-inflammatory cytokines (Himadri *et al.*, 2010; Sarada *et al.*, 2015). This has previously been reported in a rat model at altitude, where rats exposed to acute hypobaric hypoxia had increased NF-kB expression in the brain (Himadri *et al.*, 2010). Taking the oxidative stress and inflammatory response together, these studies indicate that hypoxia-induced inflammation and oxidative stress work in tandem to contribute to HACE pathogenesis. Although this data provides intriguing support for the role of hypoxia-induced inflammation and HIF-NF-kB crosstalk in HACE onset, it remains unclear if inflammation is a proximal cause of AMS or HACE. Nonetheless, inflammation likely plays a contributing role in susceptibility and progression of these illnesses. Further work is required to reach a unified explanation for HACE development.

1.3.3 Role of Inflammation in High-Altitude Pulmonary Hypertension and High-Altitude Pulmonary Edema

In HAPH, it is predicted that inflammation plays an important role in increased vessel muscularization remodeling process (Arias-Stella & Saldana, 1963; Hislop & Reid, 1976). Chronic hypoxia produces a proinflammatory microenvironment in pulmonary artery walls (Burke *et al.*, 2009). Resident fibroblasts, immune cells, and progenitor cells in the vascular adventitia respond to this local cellular stress by releasing additional

inflammatory mediators and growth factors which impact vascular wall cell phenotypes and contribute to increased muscularization (Stenmark *et al.*, 2013). This inflammatory microenvironment also promotes recruitment, retention, and differentiation of additional inflammatory cells (Burke *et al.*, 2009). Some studies have also suggested that inflammation contributes to HAPE susceptibility. Individuals with a history of HAPE are more susceptible to developing HAPE again upon re-entry to high altitude (Lakshminarayan & Pierson, 1975; Bärtsch, 1999; Gallagher & Hackett, 2004). This increased susceptibility is attributed to higher baseline chronic inflammation (Mishra et al., 2016; Cai et al., 2019). Furthermore, one report suggests that HAPE-susceptible individuals also demonstrate reduced lung function compared to HAPE-resistant individuals (Gupta *et al.*, 2017), which would limit the adaptive compensatory ventilatory response to hypoxia. The reduced lung function in these individuals is further correlated with increased plasma CRP (Shaaban *et al.*, 2006; Hancox *et al.*, 2007). This association between chronic inflammation and poor lung function is hypothesized to contribute to HAPE-susceptibility, although it does not explain the direct cause of onset. Additionally, pre-existing pulmonary vascular remodeling, driven partially by inflammation, may exacerbate pulmonary pressures and blood flow distribution and thereby increase HAPE susceptibility (Wilkins *et al.*, 2015).

As mentioned with the case of HACE, exposure to acute hypobaric hypoxia leads to an increase in ROS levels due to oxidative stress, as well as increase in pro-inflammatory cytokine expression, such as TNFα, IL1b, and IL6, as well as cell adhesion molecules that would promote migration into inflamed tissue, such as VCAM1, ICAM1, P-selectin, and E-selectin (Sarada *et al.*, 2008). Furthermore, a study has shown that lowlander travelers to high altitude with mutations in the mitochondrial DNA complex I

subunits had reduced oxidative phosphorylation efficiency, and when acute hypobaric hypoxia exposure induced oxidative stress, the mitochondrial dysfunction is suspected to contribute to HAPE susceptibility (Sharma *et al.*, 2021). Overall, these two components of inflammation and oxidative stress drive a positive regulatory feedback loop that can lead to HAPE development.

1.3.4 Role of Inflammation in Chronic Mountain Sickness

In most recent years, research regarding the inflammatory profile in native highlanders has been becoming a focal interest in the field. Studies on Andean highlanders have found that plasma interleukin-6 (IL6), a marker of inflammation, is significantly elevated in Andean men with EE when compared to healthy Andean men. Furthermore, IL6 plasma concentration is also positively correlated with the Qinghai CMS severity score (Heinrich *et al.*, 2018). In Han Chinese highlanders with CMS, S100A8, an important pro-inflammatory mediator of the innate immune response, is also significantly elevated in CMS Han Chinese individuals compared to healthy controls (Yi *et al.*, 2021). Gene ontology analysis of significantly differentially expressed proteins have also found that several pathways involved in the inflammatory response were enriched, such as the acute inflammatory response. To complement the inflammatory hypothesis in Chronic Mountain Sickness, native Andean highlanders with CMS were reported with elevated oxidative stress markers as well as low antioxidant markers in the blood (Bailey *et al.*, 2013, 2019). This dysregulated balance between free radical and antioxidants is indicative of systemic and chronic oxidative stress and inflammation. This suggests that while inflammation is an initial adaptive response to hypoxia, chronic inflammation in response to chronic hypoxia may be a maladaptive response.

In conclusion, there is significant evidence that indicates the role of inflammation in the development and/or exacerbation of symptoms of high-altitude illnesses.

1.4 Hypoxia-Induced Inflammation

Inflammation plays a key role in the physiological response to hypoxic stress. Tissues experience hypoxia during injury, infection, hypoperfusion, ischemia, or hypoxemia secondary to sleep apnea, pulmonary disease, anemia, high-altitude exposure, or other causes (Celli *et al*., 2004; McNicholas, 2009; Brill & Wedzicha, 2014; Hirota, 2015; Couzin-Frankel, 2020; Xie *et al*., 2020; Tobin *et al*., 2020b). Cellular hypoxia can trigger the expression of several inflammatory mediators which signal tissue damage and initiate survival responses. However, while hypoxia-induced inflammation may serve a protective role by initiating an immune response and promoting tissue healing, it can also contribute to several pathologies, particularly in the context of chronic hypoxia. At short timescales, and at the tissue level, inflammatory signaling in response to hypoxia is an adaptive mechanism which evolved to promote cell survival during infection, injury, or oxygen limitation (Walmsley *et al.*, 2014). However, chronic and/or systemic hypoxia can produce maladaptive inflammation which can contribute to disease development. For example, in a clinical context, the crosstalk between hypoxia and inflammation may contribute to several inflammation-mediated metabolic and cardiovascular comorbidities that accompany hypoxia-promoted diseases such as chronic obstructive pulmonary disease or obstructive sleep apnea (Tasali & Ip, 2008; Quercioli *et al.*, 2010; Cavaillès *et al.*, 2013). This can also be investigated in the context of high-altitude exposure, where inflammatory signaling pathways and immune function must respond and adapt to acute, chronic, or lifelong hypoxemia.

1.4.1 HIF and NF-κB Crosstalk

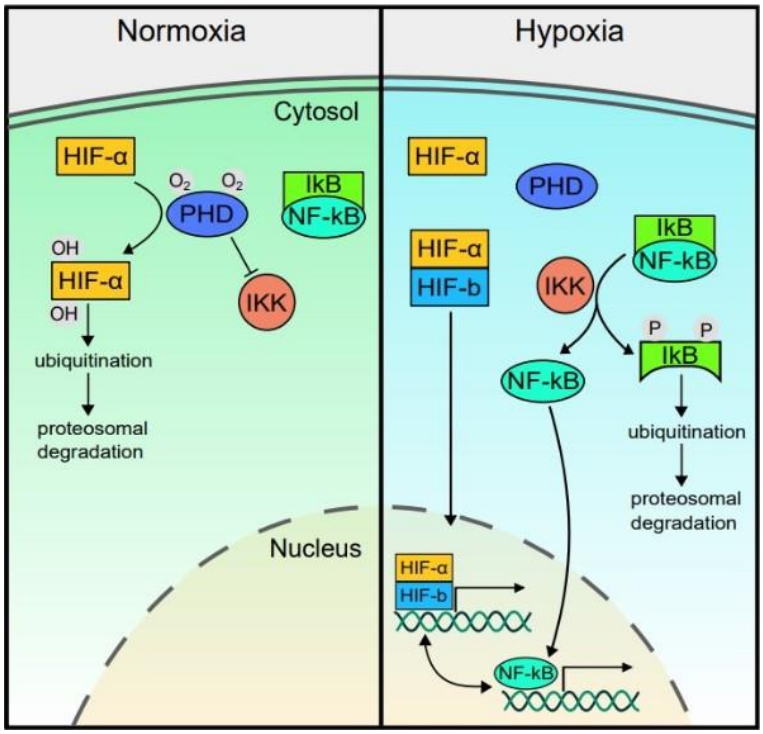

Figure 1.2. HIF-NF-kB crosstalk. In normoxic conditions, PHD hydroxylates HIF-a and the IKKβ subunit of the IKK complex, marking them for degradation and thereby reducing transcriptional activity of HIF and repressing (but not completely blocking) NF-kB activity. In hypoxia, PHD activity decreases since it utilizes O2 as a cofactor. Therefore, HIF-α is stabilized and can dimerize with the constitutively active HIF-β subunit. The complex translocates to the nucleus to upregulate expression of genes involved in the hypoxia response. In hypoxia, with reduced PHD activity, the rate of IKK degradation of IκB increases, releasing repression of NF-κB and allowing it to translocate to the nucleus at higher rates and upregulate inflammatory gene expression. PHD: prolyl hydroxylase; HIF: hypoxia-inducible factor; NF-κB: Nuclear factor kappa B; IKK: IκB kinase complex (composed of IKK-α, IKK-β, and IKK-γ subunits); IκB: nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor.

The hypoxia response and the inflammatory response pathways share significant crosstalk (**Figure 1.2**). The transcriptional response to hypoxia is controlled by the hypoxia-inducible factor (HIF) signaling cascade (Semenza, 2009). HIF is a heterodimer protein composed of an oxygen-sensitive alpha subunit and constitutively expressed beta subunit (Biddlestone *et al.*, 2015). The three HIF isoforms (HIF-1, HIF-2, and HIF-3) have some overlapping roles but also demonstrate distinct functions in different cell types (Carroll & Ashcroft, 2006; Dengler *et al.*, 2014; Watts & Walmsley, 2019). Under normoxic conditions, HIF-α is hydroxylated by oxygen-dependent prolyl hydroxylases (PHDs 1-3 in humans). Since PHD requires oxygen as a co-substrate, its activity decreases under

hypoxic conditions, allowing HIF-α to dimerize with HIF-β and translocate to the nucleus. The HIF complex can then bind to hypoxia response elements (5′-RCGTG-3′) in gene promoters to regulate expression of at least 100 genes to coordinate increased oxygen supply to hypoxic tissue (Kaelin & Ratcliffe, 2008; Semenza, 2009). HIF pathway activity is associated with activation of genes involved in metabolic adaptation, such as phosphoglycerate kinase (*PGK*) and lactate dehydrogenase A (*LDHA*), vascularization via vascular endothelial growth factor (*VEGF)*, as well as red blood cell production via erythropoietin (*EPO)* and its receptor's availability (*EPOR)*, and several other gene networks involved in improving oxygen delivery and use efficiency (Dengler *et al.*, 2014; Villafuerte *et al.*, 2014). While the HIF pathway primarily responds to hypoxia, HIF expression is also increased in response to non-hypoxic stimuli including bacterial lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), reactive oxygen species, hepatocyte growth factor, and interleukin-18 via crosstalk with the nuclear factor-κB (NF-κB) pathway (Figueroa *et al.*, 2002; Zhou *et al.*, 2003; Frede *et al.*, 2006; Taylor, 2008).

The NF-κB transcription factor is a master regulator of inflammation. NF-κB is kept localized in the cytoplasm by inhibitory IκB proteins (Lawrence, 2009; Oeckinghaus & Ghosh, 2009), which are regulatory proteins that interact with NF-κB to inhibit DNA binding of NF-κB as well as retain NF-κB in the cytoplasm (Beg & Jr, 1993; Mitchell *et al.*, 2016). In response to inflammatory stimuli and microbial products, the IκB kinase (IKK) complex phosphorylates IκB, leading to IκB ubiquitination and proteasomal degradation (Israël, 2010). With the degradation of IκB, NF-κB can translocate to the nucleus and upregulate key downstream inflammatory pathways (Hayden & Ghosh, 2004; Perkins, 2006; Mitchell *et al.*, 2016). The NF-κB pathway can upregulate HIF-1α (BelAiba *et al.*, 2007). NF-κB subunits bind to the NF-κB binding element within the HIF-1α gene promoter region and

induce HIF-1α mRNA expression (Van Uden *et al.*, 2008). Several studies support this NF-κB-dependent HIF-1α expression. For example, in cell culture models (HEK293 cells and pulmonary artery smooth muscle cells), NF-κB transfection resulted in increased HIF-1α mRNA and protein expression. Additionally, when these cells were co-transfected with a mutated dominant negative IκB (which cannot be phosphorylated by IKK) to reduce NF-κB translocation to the nucleus, HIF-1α mRNA and protein expression decreased (Bonello *et al.*, 2007; BelAiba *et al.*, 2007; Görlach & Bonello, 2008). In addition to this direct link, the HIF and NF-κB pathways also share common regulators. Like HIF-α, the IKK complex responsible for regulating NF-κB activity is also a target of PHD and therefore its NF-κB regulatory activity is oxygen dependent. In normoxic conditions, PHD hydroxylates IKKβ, therefore repressing NF-κB nuclear translocation and transcriptional activity. When PHD is rendered inactive in hypoxic conditions, the IKK complex can proceed to remove IκB from NF-κB, allowing it to translocate to the nucleus and upregulate inflammatory gene expression (Cummins *et al.*, 2006; Taylor, 2008).

1.5 Impacts of Hypoxia on Immune Function and Inflammatory Signaling

Immune cells are exposed to hypoxia when they are recruited to sites of inflammation. In physiological immunological niches (bone marrow, placenta, gastrointestinal tract mucosal surfaces, and lymph nodes), the maintenance of sustained and moderate physiological hypoxia is an adaptive mechanism to regulate metabolic pathways and immune homeostasis. However, in pathological immunological niches (tumors and chronically inflamed and ischemic tissue), severe and unregulated hypoxia can lead to maladaptive inflammation and disease development (Taylor & Colgan, 2017). In cases of systemic hypoxia seen during high-altitude exposure, the inflammatory

response is potentially a crucial aspect to the acclimatization process, and in case of maladaptation, the development of HAIs.

1.5.1 Immune Cell Mobilization and Activation

Inflammation is a natural and protective response to tissue damage and infection which is often associated with directing immune responses. In response to injury or infection, surrounding cells in the inflamed tissue release chemotactic factors, such as IL-8, that mobilize and promote migration of immune cells to the site of inflammation. IL-8 is a chemotactic pro-inflammatory cytokine involved in immune cell mobilization and infiltration into inflamed sites (Harada *et al.*, 1994). In the presence of pro-inflammatory stimuli, such as lipopolysaccharide (LPS), TNF, and IL1b, IL8 can be released by a wide variety of cells, such as monocytes, granulocytes, lymphocytes, endothelial, and epithelial cells (Baggiolini & Clark-Lewis, 1992; Harada *et al.*, 1994; Matsushima *et al.*, 2022). Because IL-8 can be produced in a large variety of cells, ranging from immune cells to epithelial cells to dermal fibroblasts, IL-8 plays a pivotal role in host defense and inflammation. In addition to the chemotactic ability, IL-8 also triggers neutrophilic activity and promotes the release of granule enzymes (Baggiolini *et al.*, 1989). In hypoxic conditions, IL-8 has been found to be significantly upregulated as well as positively correlated with HIF-1α expression (Walmsley *et al.*, 2005; Fei *et al.*, 2018).

HIF is a transcription factor that acts as the master regulator of the hypoxia response, and because physiological or pathological hypoxic conditions influence the immune response, HIF also plays a critical role in regulating the immune response (Krzywinska & Stockmann, 2018; Chen & Gaber, 2021). Several studies demonstrate the significance of HIF signaling in immune function. HIF-1α deletion in myeloid cells (granulocytes and monocytes/macrophages) impairs their mobility, aggregation,

antibacterial activity, and survival (Cramer *et al.*, 2003; Walmsley *et al.*, 2005). On an organismal level, HIF-1α deletion in macrophages can reduced mortality in LPS-induced sepsis in mice (Peyssonnaux *et al.*, 2007). HIF-2α can also directly regulate pro-inflammatory cytokine expression in myeloid cells (Imtiyaz *et al.*, 2010). In addition to HIF's essential role in immune cell function, NF-kB is also critical to cell survival in hypoxia. Walmsley et al. (2005) demonstrated that the transcription of the NF-κB p65 subunit is regulated by hypoxia. Neutrophils cultured in hypoxia and treated with NF-κB inhibitors had reduced survival rates, suggesting that activation of the NF-κB pathway promotes neutrophil survival in hypoxia (Walmsley *et al.*, 2005).

1.5.2 Toll-Like Receptor 4 Signaling Pathway

The innate immune response is the first line of defense against exogenous and/or endogenous insults. Within the innate immune response, the family of Toll-like receptors (TLRs) are a critical component in detecting evolutionary conserved patterns that are shared by large groups of microorganisms and initiating an immune response once triggered (Akira *et al.*, 2001, 2006; Kawai & Akira, 2007). The TLR family consists of 13 receptors that work individually or in conjunction with each other to respond to a variety of stimuli, such as lipopolysaccharide (LPS), an integral component found on Gram-negative bacteria, or HMGB1, a protein normally sequestered away from the extracellular environment and is released following cell stress, damage, or death. Upon activation, the TLR signaling pathway induces subsequent activation of the NF-kB pathway, the master regulator of pro-inflammatory cytokine expression.

The Toll-like receptor 4 pathway is essential for the response against LPS. However, TLR4 alone is not enough to confer activation of the signaling pathway. Additional molecules, such as CD14 and MD-2, are required in conjunction with TLR4 to

recognize LPS and initiate downstream activation of NF-kB signaling pathway. CD14 is a glycoprotein pattern-recognition receptor (PRR) essential for LPS-mediated signaling through TLR4. CD14 is expressed on a subset of myeloid cells, such as leukocytes, monocytes, and macrophages, and plays a crucial role in immune recognition against LPS (Landmann & Zimmerli, 2000). Previous studies have reported that LPS induces physical proximity between CD14 and TLR4 before initiating NF-kB translocation to the nucleus (Jiang *et al.*, 2000; Da Silva Correia *et al.*, 2001). Furthermore, CD14 controls the trafficking and signaling cascade of TLR4, and is essential for LPS-induced endocytosis of TLR4 (Zanoni *et al.*, 2011). Alongside CD14, MD-2 is another essential co-receptor of TLR4 that plays a critical role in LPS recognition (Kobayashi *et al.*, 2006). MD-2 is physically associated with TLR4 and confers LPS responsiveness (Shimazu *et al.*, 1999). In summary, LPS binds to CD14, which then binds to a MD-2/TLR4 complex. This promotes the final activated heterodimer complex (LPS/MD-2/TLR4) that is endocytosed into the cell to initiate downstream activation of the NF-kB pathway (Shimazu *et al.*, 1999; Da Silva Correia *et al.*, 2001; Kobayashi *et al.*, 2006; Akashi-Takamura & Miyake, 2008; Park *et al.*, 2009; Kuzmich *et al.*, 2017) (**Figure 1.3**).

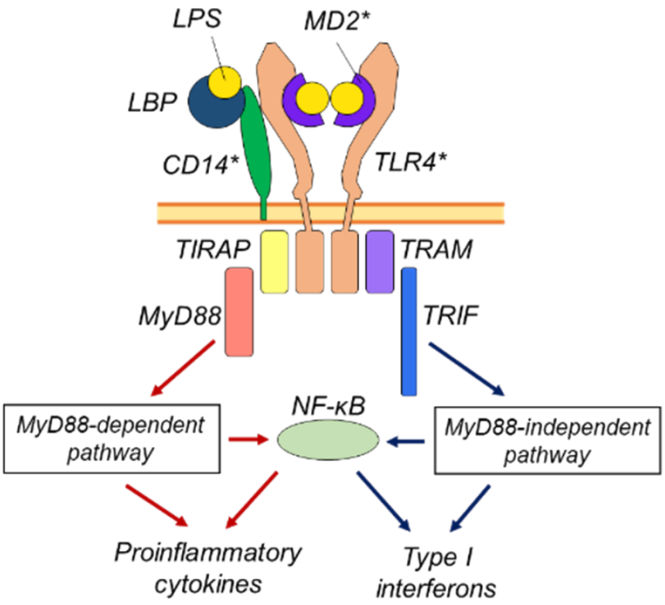

Figure 1.3: Toll-Like Receptor 4 (TLR4) Signaling Pathway.
An important component of the innate immune response to lipopolysaccharide (LPS). Important co-receptors include CD14 and MD2. TLR4 activation triggers downstream inflammatory responses via NF-kB activation.

1.5.3 Alarmins

Evolutionary conserved surveillance and defense mechanisms, such as the TLR signaling pathways, that recognize and respond to damage associated molecular patterns (DAMPs) are crucial components of the immune response (Akira *et al.*, 2006; Li & Wu, 2021). These “danger signals” can arise from exogenous pathogens that share recognizable pathogen-associated molecular patterns (PAMPs), as well as from endogenous sources that are released in the event of cellular stress or tissue damage (alarmins) (Bianchi, 2007). In both cases, these PAMPs and alarmins have highly conserved motifs that trigger activation of the immune response when recognized. While PAMPs are indicative of a pathogenic invasion, alarmins arise from within host cells that are experiencing stress or death, which may be a result of a “sterile insult”, such as exposure to a high-altitude hypoxic environment.

Alarmins have dual roles that are dependent on their location (Bianchi, 2007; Vénéreau *et al.*, 2015). Because their original function is within the cell, they are normally sequestered away from pattern recognition receptors (PRRs) (Akira *et al.*, 2006; Li & Wu, 2021) When exposed to the extracellular environment, alarmins are recognized by PRRs, most notably toll-like receptors, and mount an immune response (Takeda & Akira, 2015). While this response is crucial to the physiological response and resolution to injury, immune activation via DAMPs may initiate a positive feedback loop that exacerbates the inflammatory response. Indeed, sustained upregulation of alarmins and excessive inflammation have been found to play a role in the pathogenesis of pathologies, such as cancer and COVID (Cen *et al.*, 2018; Li & Wu, 2021; Santos-Sierra, 2021). These include HMGB1 and S100 proteins, which operate as critical alarmins and mediators in the immune response. Interestingly, DAMPs have also been found to be significantly

upregulated under hypoxemic conditions, such as in cases of acute respiratory disease syndrome, or exposure to a systemic hypoxic environment (Pham *et al.*, 2022; Mirchandani *et al.*, 2022).

1.6 Conclusion

This collective study identifies high-altitude induced changes in inflammatory profiles and immunological cell balance in humans and how immune cells at high altitude may have altered responses to inflammatory stimuli. To accomplish these goals, several techniques were used, such as multi-parameter flow cytometry, multiplex immunoassays, *in vitro* stimulation experiments, and an unbiased transcriptomic approach. These studies demonstrate that acute high-altitude exposure increases inflammatory gene and protein expression, as well as promotes a pro-inflammatory immunologic balance. Transcriptomic analyses have also identified novel inflammation-related genes that may be involved in immune system sensitization. As sojourners acclimatize, the inflammatory profile favors an anti-inflammatory phenotype. Additionally, *in* vitro work using PBMCs treated with HIF activators or inhibitors demonstrates that HIF plays a role in altering immune cell surface markers, most notably CD14. Overall, these studies provide important insights into the role of inflammation in high altitude acclimatization and characterize a potential mechanism underlying hypoxia-induced immune sensitization.

Chapter Two

Inflammatory gene expression during acute high-altitude exposure

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Key point summaries:

- Inflammation plays a critical role in the physiological response to hypoxia.
- High-altitude hypoxia exposure causes alterations in the inflammatory profile that may play an adaptive or maladaptive role in acclimatization.
- In this study we characterized changes in the inflammatory profile following acute high-altitude exposure.
- We report upregulation of novel inflammation-related genes in the first three days of high-altitude exposure which may play a role in immune system sensitization.
- These results provide insight into how hypoxia-induced inflammation may contribute to high-altitude pathologies and exacerbate inflammatory responses in critical illnesses associated with hypoxemia.

2.1 ABSTRACT

The molecular signaling pathways that regulate inflammation and the response to hypoxia share significant crosstalk and appear to play major roles in high-altitude acclimatization and adaptation. Several studies demonstrate increases in circulating candidate inflammatory markers during acute high-altitude exposure, however significant gaps remain in our understanding of how inflammation and immune function change at high altitude and if these responses contribute to high-altitude pathologies, such as Acute Mountain Sickness. To address this, we used an unbiased transcriptomic approach, including RNA sequencing and direct digital mRNA detection with NanoString, to identify changes in the inflammatory profile of peripheral blood throughout three days of high-altitude acclimatization in healthy sea-level residents (N=15; 5 women). Several inflammation-related genes were upregulated on the first day of high-altitude exposure, including a large increase in *HMGB1* (High Mobility Group Box 1), a danger associated molecular pattern (DAMP) molecule which amplifies immune responses during tissue injury. Differentially expressed genes on the first and third days of acclimatization was enriched for several inflammatory pathways including NF-kB and toll-like receptor (TLR) signaling. Indeed, both *TLR4* and *LY96*, which encodes the lipopolysaccharide binding protein (MD-2), were upregulated at high altitude. Finally, *FASLG* and *SMAD7* were associated with AMS scores and pulse oxygen saturation levels on the first day at high altitude, suggesting a potential role of immune regulation in response to high-altitude hypoxia. These results indicate that acute high-altitude exposure upregulates inflammatory signaling pathways and may sensitize the TLR4 signaling pathway to subsequent inflammatory stimuli.

2.2 INTRODUCTION

High altitude is a physiologically stressful environment due to low oxygen availability, low temperatures, and low humidity. Since maintenance of oxygen homeostasis is essential for survival, rapid physiological adaptations occur upon high-altitude exposure to increase tissue oxygen delivery, including increased ventilation and red blood cell production (Beall, 2006; Scheinfeldt *et al.*, 2012; Simonson, 2015; Moore, 2017). While many of the mechanisms that modulate high-altitude acclimatization are well described, it remains unclear how acute high-altitude exposure influences inflammatory signaling and immune function.

Under typical conditions in a healthy individual, cellular hypoxia is experienced primarily during infection or tissue injury. Local hypoxia triggers an inflammatory response that initiates tissue protection and repair mechanisms (Walmsley *et al.*, 2014). Due to this vital link between inflammation and hypoxia, the signaling pathways that control these responses have evolved to share significant crosstalk (Görlach & Bonello, 2008; Bandarra & Rocha, 2013; D'Ignazio *et al*., 2016; Corcoran & O'Neill, 2016; Pham *et al*., 2021). The Hypoxia-Inducible Factor (HIF) is a transcription factor that regulates gene expression in response to hypoxic stress (Semenza, 2009). HIF activity is regulated by oxygen-sensitive prolyl hydroxylase domain proteins (PHDs) and factor inhibiting HIF (FIH). As a result, under normal oxygen tensions, HIF-α is hydroxylated by PHD, leading to its degradation. However, decreased PHD activity in hypoxia allows HIF-α to accumulate, bind to HIF-β subunits, and translocate to the nucleus to initiate expression of hypoxia-response genes. One downstream target of HIF is Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-kB), a master regulator of inflammation. Conversely, NF-kB expression also upregulates *HIF1A* mRNA expression

(BelAiba *et al.*, 2007; Van Uden *et al.*, 2008). As a result, several studies have demonstrated that the HIF and NF-kB pathways share an interdependent relationship (Zhou *et al.*, 2003; Frede *et al.*, 2006; Bonello *et al.*, 2007; BelAiba *et al.*, 2007; Taylor, 2008). Furthermore, PHD is also involved in regulating NF-kB nuclear translocation through its action on IKK activity, providing an additional direct mechanism by which hypoxia regulates inflammatory signaling (Cummins *et al.*, 2006).

Due to the demonstrated links between HIF and NF-kB signaling, it is reasonable to suspect that hypoxemia induced by high-altitude exposure may result in a systemic inflammatory response. Indeed, hypoxemia and systemic inflammation commonly occur simultaneously in critical illnesses such as sepsis and acute respiratory distress syndrome (ARDS) and, while *in vivo* data is limited, pre-clinical data suggests a key role of oxygen status in modulating inflammatory and immunological outcomes in these cases (Kiers *et al.*, 2016). Furthermore, acute increases in inflammatory cytokine expression may contribute to, or be a downstream consequence of, high-altitude illnesses such as Acute Mountain Sickness (AMS). Several previous studies provide evidence that candidate inflammatory mediators are upregulated in peripheral blood during the first few days of acclimatization (Faquin *et al.*, 1992; Hartmann *et al.*, 2000; Eltzschig, Holger K, 2011; Scholz & Taylor, 2013; Lundeberg *et al.*, 2018; Heinrich *et al.*, 2018). However, whether these changes are associated with phenotypes including AMS severity remains inconclusive.

In the current study, we expand on this work with a broad, unbiased transcriptomic approach to improve our understanding of how inflammatory signaling is altered in peripheral blood mononuclear cells (PBMCs) during acute exposure to high altitude. We utilize RNA sequencing and NanoString direct digital detection of mRNA to identify broad

patterns in inflammatory gene expression. We hypothesized that pro-inflammatory gene expression would increase upon acute high-altitude hypoxia exposure and that larger inflammatory cytokine expression levels would be associated with more severe hypoxemia and AMS severity.

2.3 METHODS

Ethical approval

This study was approved by the University of California, Riverside Clinical Institutional Review Board (HS 19-076). All participants were informed of the study's purpose and risks. Participants provided written informed consent in their native language (English). The work was conducted in accordance with the *Declaration of Helsinki*, except for registration in a database.

Participants

The study included 15 healthy participants (N=5 women, 10 men) between 19 and 32 years of age. Participants were recruited by word of mouth and flyers on the UC Riverside campus. All participants reported no known history of cardiopulmonary disease or sleep disturbances, including obstructive sleep apnea, and displayed no abnormal findings on electrocardiogram (ECG) or pulmonary function testing. Mean age was 25 ± 4 years for men and 26 ± 5 years for women and BMI was 26.7 ± 5.4 kg/m^2 for men and 28.4 ± 6.9 kg/m^2 for women. Exclusion criteria included travel above 8,000 feet within one month of the first measurements, a previous history of high-altitude pulmonary or cerebral edema, smoking, and pregnancy.

Experimental design and physiological measures

In the two weeks prior to ascent to high altitude, participants completed initial screening for eligibility at UC Riverside, at approximately 400 m elevation (Riverside, CA, USA). Demographic information including age, height, weight, and blood pressure were collected. Participants also answered questions about their ancestral background (to determine presence of high-altitude ancestry) and medical history including current medications. Participants then completed pulmonary function testing and ECG to verify absence of lung or heart disease.

Participants returned to UC Riverside in the early morning on the day of ascent. Baseline (sea-level, SL) physiological measures were collected at this time, including blood pressure, pulse oximetry (SpO_2), heart rate, end-tidal carbon monoxide (CO), and AMS scores via the 2018 Lake Louise scoring criteria with an experimenter asking participants each question (Roach *et al.*, 2018). Fasting blood samples were then collected via standard venipuncture procedures. Breakfast was provided to participants following blood sampling, prior to travel.

The group then traveled by car to Barcroft Station (3800 m elevation) in the White Mountain Research Center (Bishop, CA, USA) over a period of approximately 6.5 hours. At the field station, fasting blood samples and morning measurements were collected each day within 1 hour of waking and before 9 am to keep timing consistent with sea level measures. Physiological measures and fasting blood samples were collected every morning for 3 consecutive days (HA1, HA2, HA3), while end-tidal CO was measured at night. End-tidal CO concentration was measured with a Micro$^+$ Basic Smokerlyzer (CoVita, Santa Barbara, California, USA). Participants held their breath for 15 seconds then exhaled through the device to residual volume. Measurements were taken in

triplicate and averaged. The analyzer was calibrated with 50 ppm CO prior to each day's measurements. Pulse oximetry and heart rate values were collected using a Nellcor N-600 pulse oximeter (Medtronic, Minneapolis, MN, USA). Participants sat upright in a chair without their legs crossed and rested, breathing normally, for 3 minutes until values stabilized. Blood pressure measurements were collected in duplicate while participants rested in an upright seated position using a manual sphygmomanometer.

Participants abstained from taking anti-inflammatory medications or other agents that may interfere with acclimatization, such as acetazolamide (Basaran *et al*., 2016). Participants were permitted to consume caffeine in moderation after completing morning measurements but were asked to abstain from caffeine after noon. Three meals per day were provided and participants did not complete any strenuous physical activity. Participants did not consume alcohol, and fluid intake was supervised to ensure participants remained hydrated.

Gene expression

RNA isolation

Peripheral blood was collected in PaxGene Blood RNA tubes (QIAGEN, Germantown, MD, USA) using standard venipuncture procedures. Samples collected at sea level were incubated at room temperature for 30 minutes then frozen at -20°C and stored at -80°C until further processing. Samples collected at high altitude were stored at room temperature for 30 minutes, frozen at -20°C at the field station, then transported to UC Riverside in liquid nitrogen and frozen at -80°C until further processing. Prior to RNA isolation, PaxGene Blood RNA tubes were allowed to thaw and incubate for 4 hours at room temperature as per the manufacturer's instructions. RNA was isolated using a PaxGene Blood RNA Kit (QIAGEN, Germantown, MD, USA) following manufacturer

protocols. RNA quantity and quality were verified via Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA USA). RNA samples with RIN values greater than 8 were utilized for downstream sequencing.

RNA sequencing

RNA samples were prepared using the NEBNext Ultra ll Directional RNA Library Prep Kit (New England BioLabs, Ipswich, Massachusetts, USA) following manufacturer protocols with the following adjustments: 0.8x beads were used during the first purification step after second strand synthesis, the adaptor was diluted 1:15, 0.7x beads were used for purification after adaptor ligand, 13 cycles of enrichment were conducted, and a dual bead size selection (0.5x and 0.7x) was used for size selection of adaptor ligated RNA. Samples were then pooled and checked for quality via qPCR and Agilent 2100 Bioanalyzer. Samples were stored at -80°C and transported on dry ice to the University of California San Diego Institute for Genomic Medicine for sequencing via Illumina NovaSeq 6000 (Illumina Inc., San Diego, USA), which generates 50 bp paired-end reads.

Raw sequence data was aligned using *Rsubread* in RStudio (RStudio, Boston, Massachusetts, USA) (R version 4.02) using the reference genome GRCh38/hg38 (Liao *et al.*, 2019) and soft-clipping of unmapped read bases and adaptors with the *align* function. *featureCounts* from the *Rsubread* package was used to assign and count mapped fragments (Liao *et al.*, 2014). Normalization and differential gene expression analysis was conducted with *DESeq2* following the workflow described by Love et al. (2014) (Love *et al.*, 2014). Paired contrasts were made for the first (HA1) and third (HA3) mornings at high altitude versus sea level (SL) separately. P-values were adjusted with

the Benjamini-Hochberg adjustment method for a false discovery rate of 5% and genes with adjusted p-values less than 0.01 were considered significantly differentially expressed. Differentially expressed genes were examined for gene set enrichment in Enrichr using GO Biological processes (2021) for gene ontology and Panther 2016 for pathway enrichment (Chen *et al.*, 2013; Kuleshov *et al.*, 2016; Xie *et al.*, 2021). Significantly enriched GO terms were clustered and visualized with REVIGO (Supek *et al.*, 2011).

NanoString

To obtain a more precise measure of inflammatory gene expression, we also measured mRNA levels of 250 key inflammation-related genes with the NanoString SPRINT Profiler (NanoString Technologies, Seattle, WA, USA). Since we aimed to determine if these expression levels were associated with phenotypes, this analysis was conducted on samples collected at sea level and after one night at high altitude (HA1) since AMS scores were highest, SpO_2 was lowest, and both were more variable across subjects on HA1. RNA profiling was conducted with 50 ng of total RNA, quantified using Nanodrop 2000. Samples were prepared for codeset hybridization with NanoString-prepared reporter and capture probes specific for the Inflammatory Panel CodeSet Human V2 following manufacturer protocols. Paired participant samples were placed on the same cartridge to eliminate replicate bias.

Normalization and differential expression analysis were performed with the Advanced Analysis add-on to nSolver software (version 4.0). Counts were normalized to the following normalization probes: *PGK1, CLTC, GADPH, GUSB, TUBB,* and *HPRT1.* These normalization probes are automatically selected using *geNorm* algorithm, which selects probes that minimize pairwise variation statistic during housekeeping gene

selection. Genes within a sample with mRNA counts lower than or equal to 2x the maximum background (the average of the negative controls' housekeeping gene count) in more than 20% of all samples were excluded from analysis. Differential expression analysis included location (SL v. HA1) as a predictor and subject as a confounder. P-values were adjusted with the Benjamini–Yekutieli method. Adjusted p-values less than 0.05 were considered significant. Since the NanoString gene expression panel included a pre-selected group of inflammation-related genes, we conducted enrichment analysis with GOrilla, which allowed a custom background gene set including only genes on the nCounter Human Inflammation panel (Eden *et al.*, 2009). The nSolver Advanced Analysis software's Probe Descriptive module was used to examine gene-by-gene correlations with location set as a covariate (SL or HA1) and interval ID, and participant as a series ID. Expression correlation for each pair of genes is expressed as the overall Pearson correlation coefficient and p-value.

Statistical analysis

Statistical analyses were conducted in R (version 4.1.0) (R Foundation). To identify changes in physiological variables at high altitude compared to baseline sea-level measures, we used repeated measures ANOVA and post-hoc pairwise t-tests with Bonferroni corrections. To determine if fold changes in gene expression were associated with physiological measures at high altitude (SpO_2, AMS Score), Pearson correlation coefficients and p-values were obtained with the *rcorr* function from the *Hmisc* package in R. Data is presented throughout the paper as mean ± standard deviation.

2.4 RESULTS

Physiological measures

Table 2.1 provides an overview of physiological measures at sea level and over three days of acclimatization to high altitude. On the first morning at high altitude, 8 out of 15 subjects indicated mild AMS (AMS score 3-5 with headache) and one participant indicated severe AMS (AMS score 6-9 with headache). By the third day, only 1 out of 15 subjects scored positive for AMS. SpO_2 decreased by about 10 percentage points on the first day at high altitude and remained lower than sea-level values throughout day 3. This was coupled with an increase in heart rate at high altitude. There was no significant increase in diastolic or systolic blood pressure at high altitude. Exhaled end-tidal carbon monoxide levels were elevated on average at high altitude, although not significant by repeated measures ANOVA.

Gene expression at high altitude

RNA sequencing

3958 genes were differentially expressed (adj $p<0.01$) on HA1 (2177 upregulated and 1781 down regulated) and 4219 genes were differentially expressed on HA3 (2190 upregulated and 2029 downregulated) compared to sea level. When applying a fold change threshold of 1, 88 genes were upregulated and 75 downregulated on HA1 and 234 were upregulated and 51 downregulated on HA3. The top 20 differentially expressed genes on each day are provided in **Table 2.2**. **Figure 2.1** demonstrates that genes most differentially expressed on HA1 remain differentially expressed throughout HA3. However, genes most differentially expressed on HA3 are slower responding and are typically not differentially expressed on HA1. Several genes associated with inflammation and the immune response are in the top 20 upregulated genes on HA1

including *BCL2A1*, *S100A8*, *HMGB1*, and *B2M*. Additional genes likely involved in the acclimatization process, including *PDCD10* which is involved in vascular development, were also in the top 20 differentially expressed genes. On HA3, several genes associated with acclimatization were upregulated including *BPGM* (2,3-DPG), *CA1* (carbonic anhydrase 1), *FECH* (ferrochelatase), *HEMGN* (hemogen), and *PDCD10*, as well as several other genes associated with inflammation and immune function including *GYPA*, *IFIT1B*, and *RIOK3*.

A gene ontology analysis revealed that on the HA1, differentially expressed genes were enriched for biological processes including regulation of autophagy, proteasome-mediated ubiquitin-dependent protein catabolic processes, endomembrane system organization, and I-kappaB kinase/NF-kappaB signaling (**Figure 2.2A**). There were also 8 significantly enriched pathways including apoptosis signaling, CCKR signaling, ubiquitin proteasome pathway, PDGF signaling, T cell activation, toll-like receptor signaling, Ras pathway, and FAS signaling (**Table 2.3**). On HA3, the I-kappaB kinase/NF-kappaB signaling GO biological process remained enriched in addition to other inflammation and immune function processes including neutrophil activation involved in immune response (**Figure 2.2B**). 19 pathways were significantly enriched on day 3 including several involved in inflammation and immune function and angiogenesis (VEGF signaling pathway) (**Table 2.3**).

Following the gene ontology analysis, we examined key genes of interest which play significant roles in the top enriched GO pathways. In particular, we were interested in genes involved in immune pathway activation, such as *TLR4, HMGB1, Ly96,* and *IL8* (**Figure 2.3**). We found that there was a significant upregulation in *TLR4* and *HMGB1* mRNA counts across all participants. However, *Ly96* was not found to be significantly

differentially expressed and had low counts. Additionally, we were interested in *IL8* gene expression since neutrophil activation was highly enriched. We found there was significant *IL8* upregulation upon high-altitude exposure in all but one participant.

Nanostring analysis of inflammatory pathway genes

To further examine specific changes in inflammation-related gene expression, we conducted a NanoString analysis on samples collected on HA1. Of the 250 genes on the human inflammation panel, 18 genes showed significant upregulation (**Figure 2.4; Figure 2.5**). Of the 18 genes identified as differentially expressed by NanoString, 13 were also detected with RNA sequencing. However, 5 differentially expressed genes were identified by NanoString alone, including *LY96*. This may be because the NanoString nCounter targets all isoforms of each gene and does not need to convert RNA to cDNA for amplification. 7 of the 18 significantly differentially expressed genes are involved in the significantly enriched GO pathway "Regulation of binding (GO: 0051098)" (adj $p < 0.0001$; *TGFBR1*, *IFIT2*, *IFIT1*, *MAPK8*, *DDIT3*, *HMGB2*, *HMGB1*). Other processes that significantly upregulated genes are involved in include positive regulation of apoptotic processes (*MAPK8*, *DDIT3*, *HMGB1*, *TGFBR1*, *IFIT2*), regulation of DNA binding (*MAPK8*, *HMGB2*, *HMGB1*) and DNA conformational change (*HMGB2*, *HMGB1*), and positive regulation of endothelial cell proliferation (*HMGB2*, *HMGB1*, *TGFBR1*).

Since our inflammation gene panel included the key hypoxia-response gene, *HIF1A*, we looked for significant correlations between *HIF1A* expression and expression of our top 18 differentially expressed genes to identify possible relationships between HIF signaling and inflammatory gene expression *in vivo*. *HIF1A* expression was significantly associated with expression of *PTK2*, *MAPK1*, *HMGB1*, *TLR8*, and *NFE2L2* (**Figure 2.6**).

Phenotype associations

Since the highest AMS scores and lowest SpO_2 levels were observed on the first day at high altitude, we looked for significant correlations between phenotypes on the first day at high altitude and inflammatory genes measured by NanoString. Resting oxygen saturation (SpO_2 %) was significantly associated with *FASLG, SMAD7, PTGER4, and TRAF2,* with a trend toward a correlation with *IL8* (p=0.05) (**Table 2.4**). AMS Score was significantly associated with *TNFSF14, FASLG, IL18, CD40LG,* and *PTGER4, MAPKAPK2, HLADRB1, SMAD7, AGER, MAFK, and IRF5* (**Table 2.4**).

2.5 DISCUSSION

This study examined how high-altitude acclimatization influences inflammatory signaling using RNA sequencing and NanoString transcriptome analyses in whole blood of healthy participants. Our results demonstrate that upon acute high-altitude exposure, many inflammation-related genes are significantly upregulated. These upregulated genes are enriched in pathways involved in stress responses and regulation of inflammatory signaling (**Figure 2.2**, **Table 2.3**), and the expression of several inflammation-related genes including *FASLG* and *SMAD7* were associated with AMS scores and SpO_2 levels (**Table 2.4**). The release of cellular stress markers during tissue hypoxia are known to trigger downstream mechanisms that promote host defense, such as the toll-like receptor (TLR) signaling pathway (Schaefer, 2014). Therefore, it is not surprising that we found TLR signaling pathways to be impacted by acute high-altitude exposure. Many of the differentially expressed genes we identified also included damage associated molecular patterns (DAMPs) (*HMGB1*, *HMGB2*, *S100A8*), interferon-stimulated genes (*IFIT1*, *IFIT1B*, *IFIT2*, *IFI44*), and markers of DNA damage (*DDIT3*) (**Table 2.2, Figure 2.5**) (Schaefer, 2014, Diamond, 2014, Yang et al., 2017). Here we

will discuss the impact of DAMPs, particularly *HMGB1* and its synergistic role with *LY96* in TLR4 activation.

TLR4 pathway upregulation at high altitude

LY96 is a potential indicator of immune system priming (Kim *et al.*, 2010; Park & Lee, 2013). The *LY96* gene encodes for MD-2, a coreceptor with toll-like receptor 4 (TLR4), a key player in innate immunity defense. *LY96* plays an essential role in the TLR4-mediated inflammatory response to lipopolysaccharide (LPS) (Da Silva Correia *et al.*, 2001; Park *et al.*, 2009). TLR4 activation by LPS, a pathogen associated molecular pattern (PAMP), triggers the expression of inflammatory cytokines and chemokines (Park & Lee, 2013). Since hypoxia has been shown to increase *TLR4* expression, this may lead to exacerbated inflammation in response to subsequent inflammatory stimuli (Kim *et al.*, 2010). In this *in vivo* study, both *TLR4* and *LY96* gene expression were significantly upregulated after 1 day of high-altitude exposure (**Figure 2.5**). While *TLR4* upregulation is only found significant in the RNA-seq data, *TLR4* expression approaches significance in the NanoString data (adj.p = 0.057). This data suggests that over 1-3 days of hypoxia exposure, hypoxic stress primes the innate immune response and may increase the inflammatory response to infection. This is supported with *in vitro* work by Kim et al., (2010) in which hypoxia exacerbated TLR4-mediated inflammation in response to LPS in murine RAW 264.7 macrophages. However, the duration of hypoxia exposure, as well as the model used, have varying effects on *TLR4* expression. In short term exposure (2-4 hours) in macrophages, *TLR4* is noted to have increased expression (Kim *et al.*, 2010). In longer hypoxic exposure (> 24 hours) in murine dendritic cells, there is no significant change in *TLR4*, however there was a significant increase in *TLR2* and *TLR6* expression (Kuhlicke *et al.*, 2007). Therefore, while it is clear that hypoxic

stress likely impacts TLR4 signaling in peripheral blood cells, the details and time domains of this effect requires further study.

To complement *LY96* and *TLR4* upregulation, *HMGB1* also showed one of the strongest signals of increased expression at high altitude (**Table 2.2**). *HMGB1* encodes the High Mobility Group Box 1 protein which directly interacts with TLR4 and functions as a damage associated molecular pattern (DAMP) mediator of inflammation. HMGB1 activity is dependent on its location and cell type. Intracellular cytosolic HMGB1 has been shown to inhibit apoptosis as well as activate the autophagic response, particularly in response to oxidative stress (Tang *et al.*, 2011; Zhu *et al.*, 2015). Upon release after cell death or active secretion, extracellular HMGB1 can act as a pro-inflammatory mediator by binding to other proinflammatory molecules, such as LPS or IL-1β, to activate TLR4 receptors and initiate downstream inflammatory signaling (Yang *et al.*, 2020). This mode of TLR4 activation is particularly important in immune cell types, such as monocytes, macrophages, and neutrophils. In monocytes and macrophages, extracellular HMGB1 can bind to MD-2, which forces two TLR4 chains to form a complex and initiate downstream signaling and induces cytokine and chemokine production, such as TNF (Yang *et al.*, 2015*b*, 2020). In neutrophils, HMGB1-TLR4 signaling increases reactive oxygen species production through activated neutrophil NADPH oxidase activity (Fan *et al.*, 2010; Yang *et al.*, 2020; Billiar *et al.*, 2021).

In addition to HMGB1, *S100A8* was also significantly upregulated following one day of acute high-altitude exposure (**Figure 2.1**). Like HMGB1, S100A8 is an endogenous DAMP that is actively secreted from phagocytes in response to stress (Ehrchen *et al.*, 2009). S100A8, along with S100A9, are highly expressed in neutrophils as well as in phagocytes in inflammatory conditions, such as inflammatory bowel and lung diseases

(Zwadlo *et al.*, 1988; Rugtveit *et al.*, 1994). The S100A8/S100A9 heterodimer have previously been found to be endogenous activators of TLR4 (Foell *et al.*, 2007; Vogl *et al.*, 2007; Ehrchen *et al.*, 2009; Ma *et al.*, 2017). Furthermore, S100A8 has been found to interact directly with the TLR4-MD2 complex to initiate downstream signaling (Vogl *et al.*, 2007). Previous research has also found that S100A8/S100A9 significantly increases proinflammatory cytokine secretion, such as TNF-α and IL-6, in cultured BV-2 microglial cells. When TLR4 was inhibited, the proinflammatory cytokine secretion was blunted following S100A8/A9 stimulation (Ma *et al.*, 2017). Additionally, in a mice model that lacked the S100A8/A9 complex, these mice were protected from endotoxin-induced lethal sepsis (Vogl *et al.*, 2007). Together, this data further supports the hypothesis that the TLR4 signaling pathway is sensitized to subsequent inflammatory stimuli following systemic hypoxic stress, at least following 1-3 days of high-altitude exposure.

TLR4 activation may also contribute to the release of IL8, a chemotactic cytokine that recruits and mobilizes neutrophils to sites of infection. Previous studies show that damage to the extracellular matrix following cellular stress contributes to a positive feedback loop that drives a TLR-dependent chronic inflammation, including chronic IL8 expression (Valenty *et al.*, 2017). Our data reveals that *IL8* mRNA expression is significantly upregulated at both the first and third day at high altitude (**Figure 2.3**), and approaches significance for a negative association with SpO_2 (**Table 2.4**). This falls in line with previous research in acute respiratory distress syndrome (ARDS), where Hirani et al. (2001) found IL8 levels negatively correlated with arterial oxygen saturation in patients with severe ARDS and that *in vitro* hypoxic stimuli significantly upregulate IL8 production in human monocyte-derived macrophages.

Overall, the upregulation of several DAMPs and chemokines essential for immune cell mobilization demonstrate that acute high-altitude exposure may prime the immune system to subsequent inflammatory stimuli. This hypoxia-induced immune sensitization supports “the danger model” theory (Gallucci & Matzinger, 2001; Matzinger, 2003; Pugin, 2012), where these DAMPs serve as an endogenous danger signal to activate the innate immune system. “The danger model” theory proposes that the immune system requires two signals to activate: one from the foreign antigen itself, and one arising from tissue injury (Gallucci & Matzinger, 2001; Pugin, 2012). In response to hypoxemia and/or tissue hypoxia, cellular stress triggers the upregulation and release of DAMPs. This massive release of danger signals could have several negative implications, such as the development of systemic inflammatory response syndrome (SIRS) (Bone, 1992; Pugin, 2007; Chakraborty & Burns, 2022) SIRS is an exaggerated inflammatory response to a stressor, such as trauma or acute inflammation, in an attempt to resolve the endogenous or exogenous source of the insult, where patients have an increase in both pro- and anti-inflammatory mediators in circulation (Bone, 1992; Dinarello *et al.*, 1993; Chakraborty & Burns, 2022). More importantly, if followed by an infection of foreign bacterial antigens, this could trigger a synergistic activation of the immune system, leading to a devastating result of septic shock and multi-organ failure (Pugin, 2007). Furthermore, DAMPs, particularly HMGB1 and S100A8/S100A9, have been previously used as biomarkers for risk of death in septic shock patients (Karakike *et al.*, 2019; Dubois *et al.*, 2019). This elevated level of HMGB1 and S100A8 expression found in sojourners to high altitude potentially indicates that systemic hypoxia causes immune system sensitization and a potential exaggerated response to subsequent stimuli (**Figure 2.1**), although this requires further study. Interestingly, one previous

report finds that pancreatitis patients who also had high-altitude polycythemia (and associated baseline hypoxemia) developed more severe case of SIRS compared to patients who only had acute pancreatitis (Zhu *et al.*, 2020). This indicates that patients with hypoxemia may be at higher risk for an exaggerated systemic inflammatory response.

AMS and inflammatory marker expression

AMS commonly manifests in sojourners to high altitude (>2500m) (Hackett, 2000; Gallagher & Hackett, 2004; Julian *et al.*, 2011; Luks *et al.*, 2017). A majority of AMS affected individuals develop headaches, nausea, insomnia, and shortness of breath that resolve on their own within a few days. Current research has noted that AMS develops as a result of a complex network of physiological responses to hypoxia (i.e., inflammation, hypoxemia, vasogenic edema, acidosis) as well as anatomical factors (i.e., insufficient cerebrospinal fluid production, varied cerebral venous blood flow) (Hackett, 2000; Luks *et al.*, 2017). Several studies support the role of inflammation in AMS development. For example, multiple groups report that select pro-inflammatory cytokines and inflammatory marker (most notably CRP, IL-1β, and IL-6) concentrations are increased in individuals acutely exposed to high-altitude compared to their sea-level concentrations (Hartmann *et al.*, 2000; Song *et al.*, 2016). Some groups also find associations between AMS incidence and expression of these candidate inflammatory markers (Klausen *et al.*, 1997; Boos *et al.*, 2016; Song *et al.*, 2016; Liu *et al.*, 2017; Malacrida *et al.*, 2019; Kammerer *et al.*, 2020). Accordingly, Dumont et al., (2000) show a reduction in AMS incidence and severity with anti-inflammatory drug treatment. Both steroids and nonsteroidal anti-inflammatory drugs (NSAIDS) reduce AMS incidence despite their different mechanisms of action (Rock *et al*., 1989; Zheng *et al*., 2014; Tang

et al., 2014; Nepal *et al*., 2020; Gertsch *et al*., 2010, 2012; Lipman *et al*., 2012; Kanaan *et al*., 2017). However, other studies demonstrate no significant association in pro-inflammatory cytokine concentration and AMS incidence (Swenson *et al.*, 1997; Lundeberg *et al.*, 2018). As a result, questions remain about how high-altitude induced inflammation may contribute to AMS.

In our study, many typical inflammatory markers were not included in the top differentially expressed genes via RNA-seq or NanoString after 1 or 3 days at high altitude and were not associated with AMS severity. This is not unexpected since many studies show that inflammatory markers such as IL6 and TNF-α resolve rapidly after expression. Previous studies have also reported that while IL-6 and TNF-α plasma protein expression significantly increased following acute vigorous exercise, there was no change in mRNA expression in PBMCs (Ostrowski *et al.*, 1998, 1999; Bernecker *et al.*, 2013). Thus, while protein levels may remain elevated following 24 hours of exposure, mRNA expression levels may have resolved by this time. Nonetheless, we identified other significant components of inflammatory signaling and immune system regulation that were activated at high altitude and significantly associated with AMS severity at these time points.

Of the genes most significantly associated with AMS scores, we found that *FASLG* is not only negatively associated with AMS severity ($R= -0.071$; $p<0.01$), but also positively associated with SpO_2 ($R=0.68$; $p<0.01$) (**Table 2.4**). While most of the participants in our study had mild AMS symptoms, this may indicate that *FASLG* and the FAS/FASLG pathway may play a role in modulating the physiological response to acute high-altitude hypoxia exposure. *FASLG* encodes the Fas ligand and the FAS/FASLG pathway plays a critical role in protection against autoimmunity as well as tightly regulating immune

system activation by activation-induced cell death (AICD) (Griffith *et al.*, 1995; Nagata & Golstein, 1995; Brunner *et al.*, 2003; Strasser *et al.*, 2009). This process is crucial to dampen the immune response. Our findings show that participants with lower AMS scores had increased *FASLG* mRNA expression compared to their baseline sea-level expression. Furthermore, we also found the FAS signaling pathway was enriched with significantly differentially expressed genes following the first day of high-altitude exposure (**Table 2.3**). This may have multiple implications. First, evidence suggest that endothelial cells produce soluble Fas ligand in hypoxia, which protects them from AICD (Mogi *et al.*, 2001). Additionally, pro-inflammatory cytokines (such as TNF-α) can induce Fas ligand expression on tissue cells, and in turn trigger apoptosis in activated T cells by binding to the FAS receptor on the T cell surface (Brunner *et al.*, 2003). Indeed, pro-inflammatory cytokine protein levels have been found to be elevated in plasma upon hypoxic exposure (Julian *et al.*, 2011; Boos *et al.*, 2016; Song *et al.*, 2016). Thus, this initial elevation in pro-inflammatory cytokine concentration upon high altitude exposure may induce Fas ligand expression on tissue cells and cause apoptosis in activated T cells to dampen the immune response. However, since the majority of our RNA sample should be derived from PBMCs, this may not explain the increased *FASLG* expression we observed. A final interpretation is that elevated *FASLG* expression may be reflective of T cells inducing mutual AICD amongst the T cell population (Brunner *et al.*, 2003).

Our data also shows that *FASLG* mRNA expression is also positively correlated with SpO_2. Mogi et al. (2001) show that hypoxia stimulates the release of the soluble Fas ligand. In contrast to the membrane bound Fas ligand, soluble Fas ligand would inhibit the apoptotic signal in FAS^+ cells. Upon high altitude exposure, Higher SpO_2 and elevated *FASLG* expression could indicate that their physiological response to hypoxia

quickly adapts and appropriately blunts the immune response. This would also protect tissue cells from apoptosis. However, there is conflicting research regarding the role of Fas ligand in hypoxia. Kosanovic *et al*. (2019) show that there is a significant reduction of circulating Fas ligand in sojourners to high altitude as well as in native highlanders. It is important to note that circulating plasma protein may come from multiple sources, such as endothelial cells, and we are measuring gene expression in immune cells. Additionally, they also show that highlanders with pulmonary hypertension had a significantly lower circulating Fas ligand in plasma compared to lowlander controls (Kosanovic *et al.*, 2019; Sydykov *et al.*, 2021). Overall, these results indicate a potential role of FAS signaling in high-altitude acclimatization and adaptation and highlight the importance of immune system regulation in hypoxia. These promising new findings warrant future study as they suggest an important role of immune regulation in high-altitude acclimatization and adaptation.

Concurrent anti-inflammatory profile

Our data also demonstrate a concurrent upregulation of anti-inflammatory elements. One such element is *NFE2L2,* which encodes for Nuclear factor erythroid 2-related factor 2 (NRF2), an important transcription factor involved in regulating and attenuating oxidative damage and toxic insults by regulating the expression of cytoprotective genes (Kobayashi *et al.*, 2004) (**Figure 2.5**). Previous studies clearly illustrate effects of hypobaric hypoxia exposure on radical oxygen species (ROS) production and biomarkers of oxidative damage (Chandel *et al.*, 1998; Waypa & Schumacker, 2002; Malacrida *et al.*, 2019). One downstream gene target of the NRF2 pathway is heme oxygenase-1 *(HO-1),* a cytoprotective rate-limiting enzyme that is crucial for heme degradation into equimolar amounts of Fe^{2+}, biliverdin, and carbon monoxide (CO), all of

which play roles as antioxidants and regulators of inflammation, apoptosis, and angiogenesis (Tift *et al.*, 2020). Our study shows a non-significant trend for increased end-tidal CO after one day at high altitude (**Table 2.1**). Since the only known source of endogenous CO is the heme-oxygenase pathway, increased CO upon acute high-altitude exposure may indicate increased heme degradation. Furthermore, elevated CO levels may provide tissue protective effects such as those described in models of acute inflammation in ischemia-reperfusion injury, vascular injury or disease, and sepsis (Minamino *et al.*, 2001; Knauert *et al.*, 2013; Ryter & Choi, 2016; Tift *et al.*, 2020).

LIMITATIONS

One limitation of our study is our moderate sample size (n=15), although the paired design allowed us to identify changes in numerous inflammatory pathway markers. Also, while our study group included both men and women, we did not examine sex-specific changes in inflammatory gene expression patterns due to the low number of women in our sample (N = 5 women). Future work exploring potential differences in the impact of high-altitude on immune function in women and men will be essential. Furthermore, only one participant developed severe AMS following acute high-altitude exposure. Therefore, while we were still able to identify significant associations between expression of select inflammation-related genes and AMS scores, a larger sample size with a wider range of AMS severity will provide stronger power to identify and validate these potential associations. Finally, the RNA samples utilized in our study were collected from peripheral whole blood, and therefore our data is representative only of changes occurring in peripheral blood cells. However, these findings are significant because they shed light on the impact of high-altitude on immune cell function and inflammatory status.

2.6 CONCLUSION

In conclusion, we demonstrate that acute high-altitude hypoxia exposure triggers significant changes in inflammation-related gene expression. Specifically, our analysis has led to the identification of several inflammatory-related genes that may be involved in immune system sensitization, such as components of the TLR4 signaling pathway. Clearly, hypoxemia and high-altitude exposure have significant impacts on inflammatory signaling, but further studies are essential to elucidate the mechanism behind hypoxia-induced inflammation *in vivo* and how high-altitude exposure impacts immune cell function. Future research studies should investigate how concurrent hypoxic and inflammatory stimuli may exacerbate pro-inflammatory cytokine production in PBMCs *in vitro*. Additionally, investigating the potential role of *Ly96* and *HMGB1* in immune system sensitization may expand our understanding of how hypoxia and inflammatory response pathways lead to an exacerbated response to subsequent inflammatory stimuli. This work will provide valuable insights into how hypoxemia modulates inflammatory responses in critical and chronic illnesses such as ARDS and COVID-19.

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Disclosures

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

Author Contributions

K. Pham and ECH conceived and designed the research. K.Pham, SF, NP, BO, and ECH assisted in sample collection. K.Pham, K.Parikh, and ECH analyzed data, interpreted the results of experiments, and prepared figures. K.Pham drafted the manuscript. K.Pham, K.Parikh, and ECH edited and revised the manuscript. K.Pham, K.Parikh, SF, NP, BO, and ECH approved the final version of the manuscript.

Data Availability Statement

The data supporting these findings will be made publicly available in the Dryad data repository, and a link provided, immediately following publication.

FIGURES

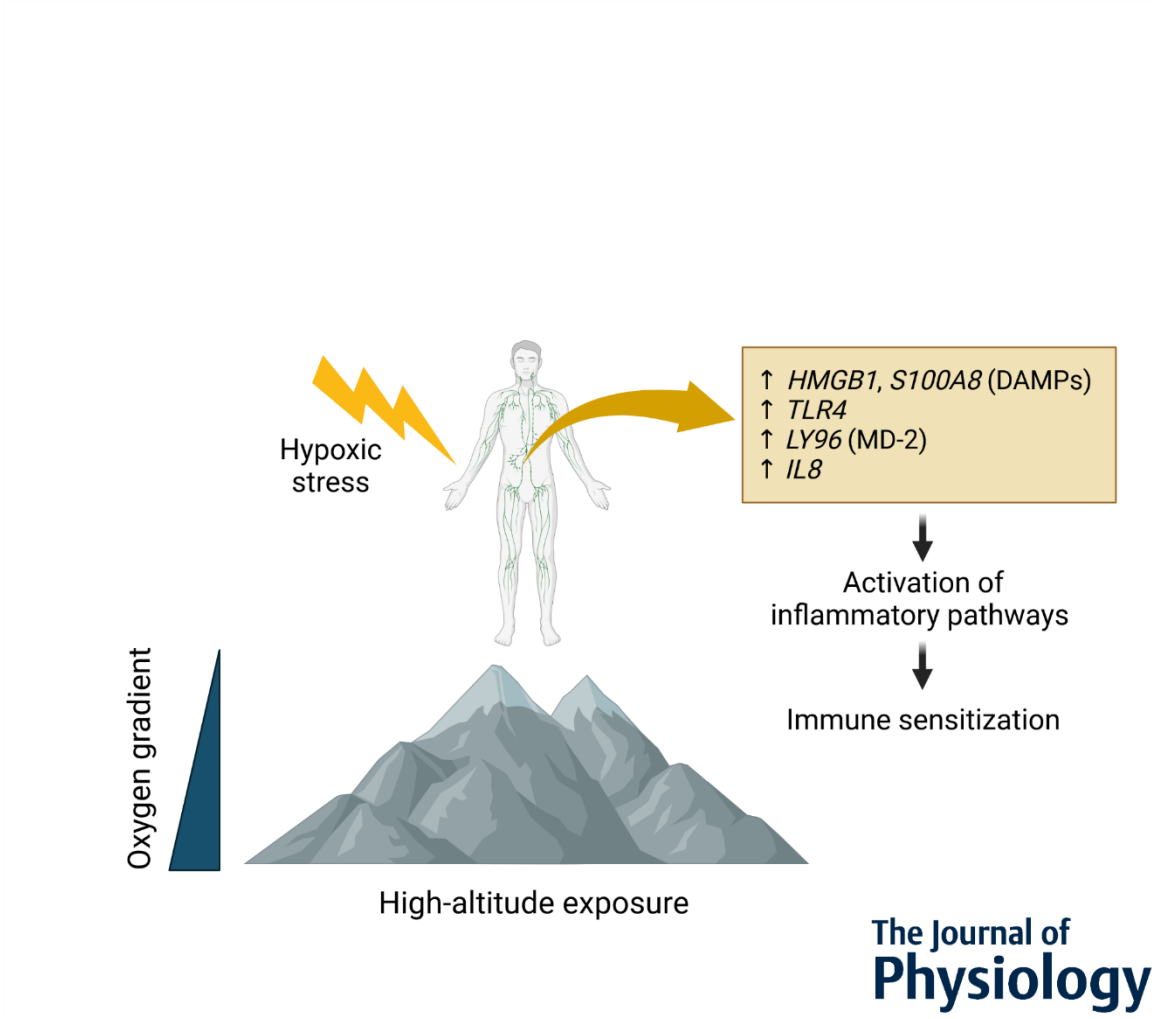

Figure 2.1. Schematic representation of the proposed impact of high-altitude exposure on inflammatory signaling. Acute high-altitude exposure (1-3 days) causes systemic hypoxic stress. In this study, we found that this is associated with upregulation of genes encoding DAMPs, TLRs, and chemotactic factors in peripheral blood. These changes are suspected to result in enhanced immune responses to subsequent inflammatory stimuli over this timeframe.

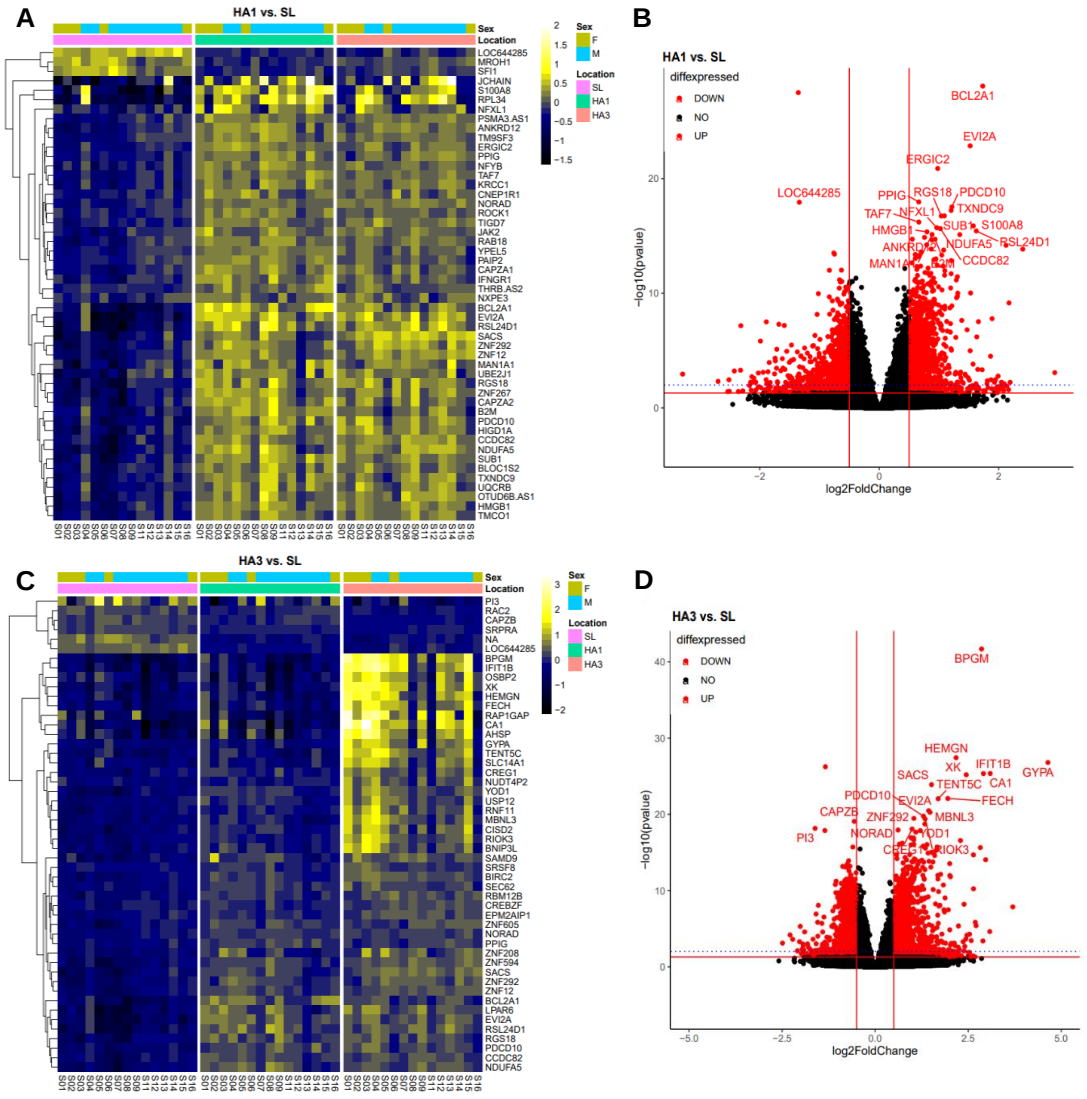

Figure 2.2. Top differentially expressed genes at high altitude. Heat maps with hierarchical clustering of top 50 differentially expressed genes and volcano plots from HA1 **(A-B)** and HA3 **(C-D)** versus sea-level baseline. **(A, C)** Columns represent data for individuals. Sample locations are identified by pink (sea level; SL), green (high altitude day 1; HA1), and red (high altitude day 3; HA3) markers at the top of each row. Sex differences are identified by green (female) or blue (male). Colors represent relative log2 fold changes from sea level. **(B, D)** Red points represent genes with adjusted p-values less than 0.01 and absolute log2 fold change greater than 0.5.

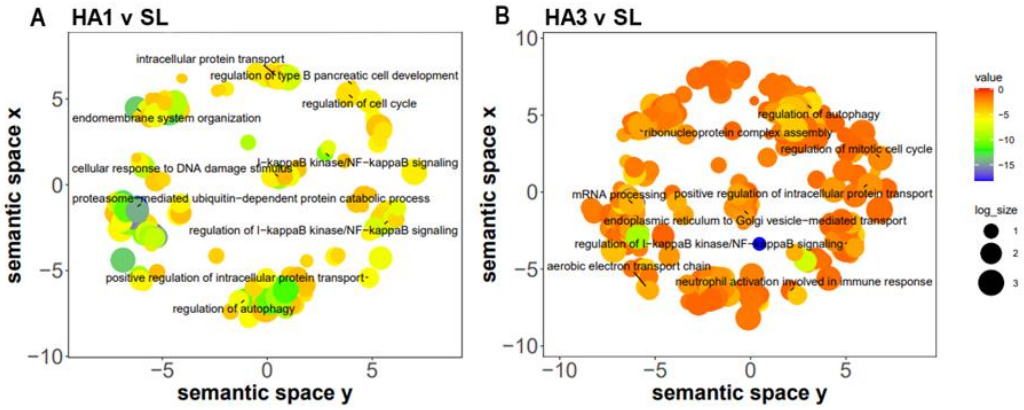

Figure 2.3. Enriched GO terms with highly variable genes. Clustering of significantly enriched GO terms into representative subsets using semantic similarities. Significantly enriched GO terms are provided for genes differentially expressed on day 1 **(A)** and day 2 **(B)** at high altitude compared to a sea-level baseline. Bubble color indicates the log10 p-value for each term and bubble size indicates the frequency of the GO term in the underlying GOA database (more general terms are larger).

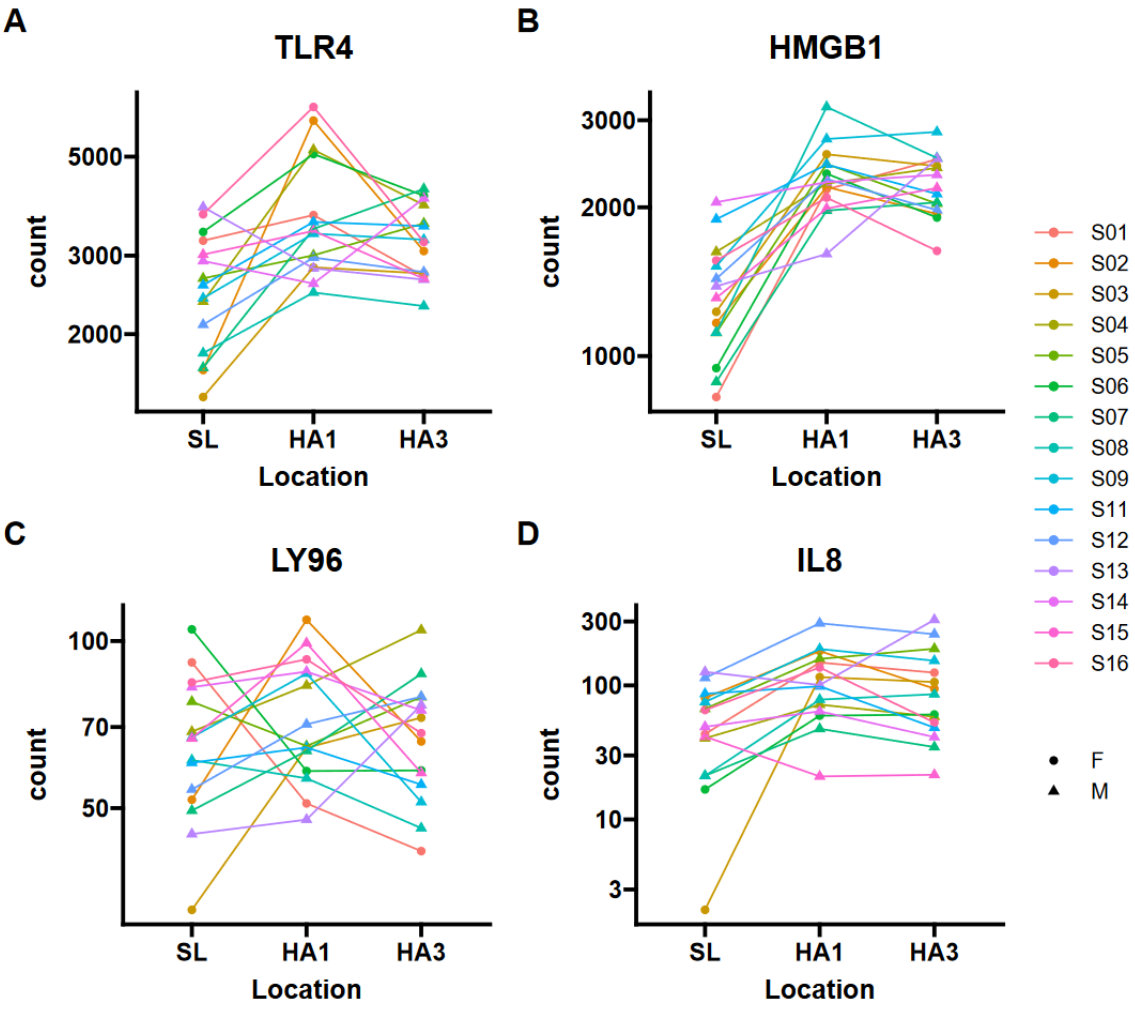

Figure 2.4. Normalized individual gene counts of RNA-seq data. Normalized individual RNA counts at sea level (SL), first day at high altitude (HA1) and third day at high altitude (HA3) for **(A)** *TLR4*, **(B)** *HMGB1*, **(C)** *LY96*, and **(D)** *IL8*. Y-axis is a log10 scale. Subject ID is coded by color and sex by shape.

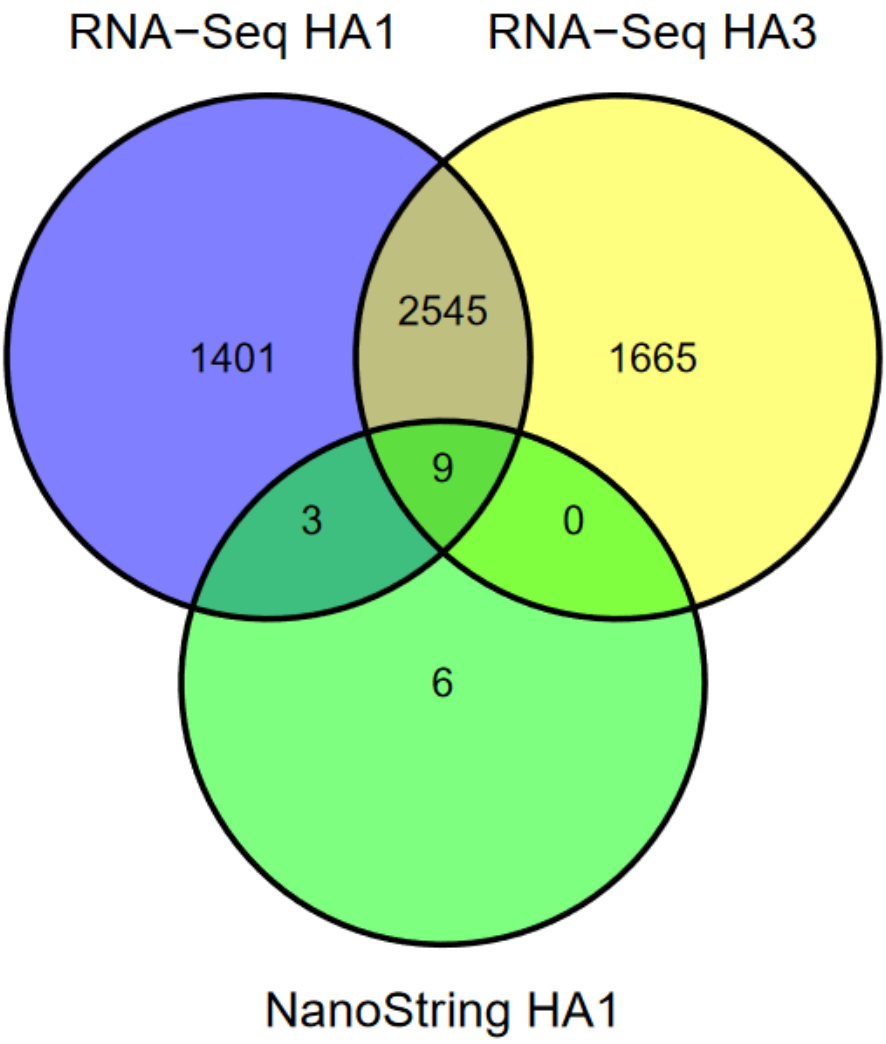

Figure 2.5. Venn diagram of differentially expressed genes identified by RNA-seq and NanoString. Differentially expressed genes identified via RNA-seq on HA1 (purple) and HA3 (yellow) are compared to differentially expressed genes identified by NanoString on HA1 (green). NanoString identified 6 additional differentially expressed genes not identified by RNA-seq.

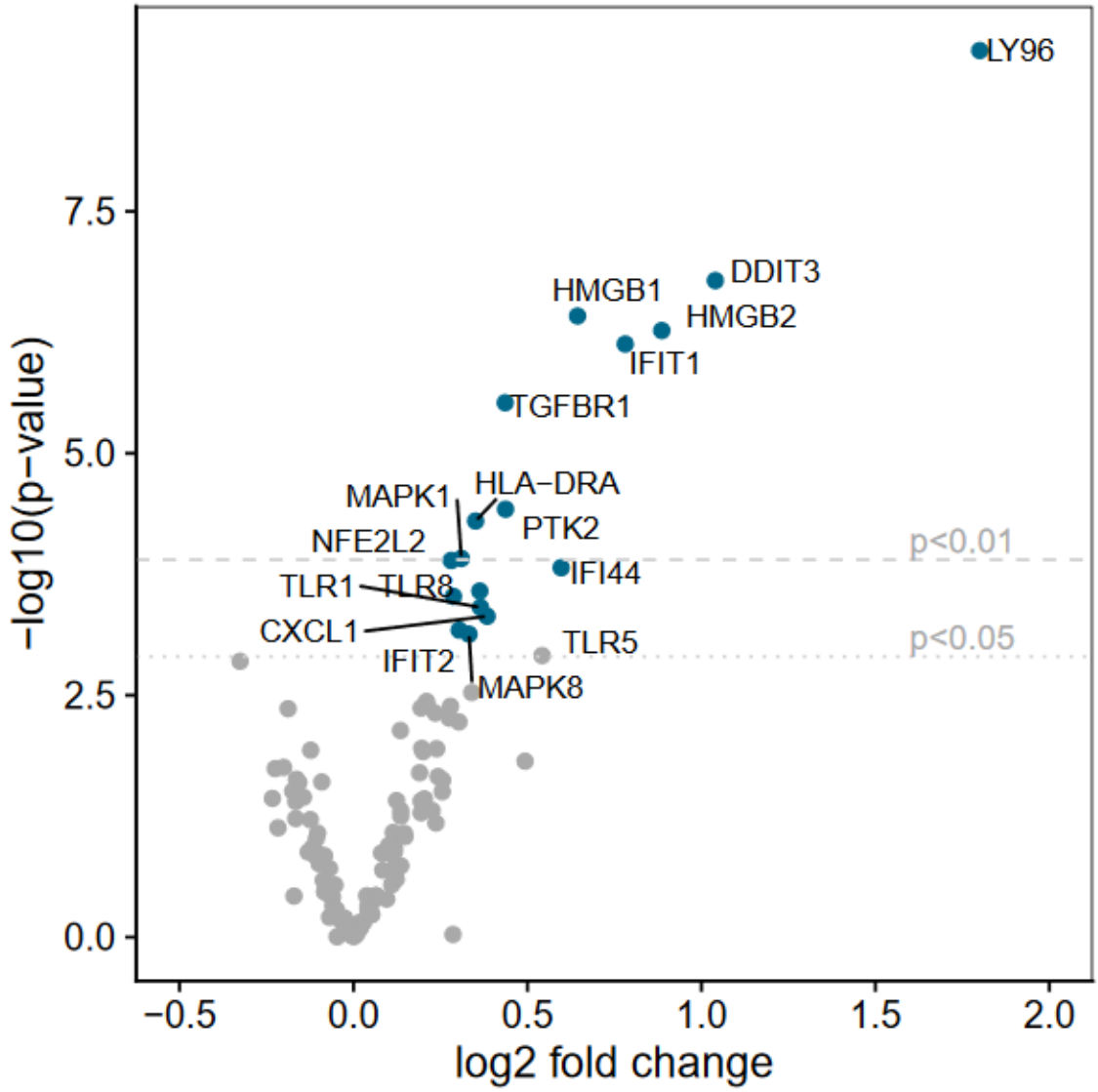

Figure 2.6. Volcano plot from NanoString data. Blue points represent significantly differentially expressed genes. Values are plotted with raw -log10 p values on the y axis with dotted grey lines indicating adjusted p-value thresholds.

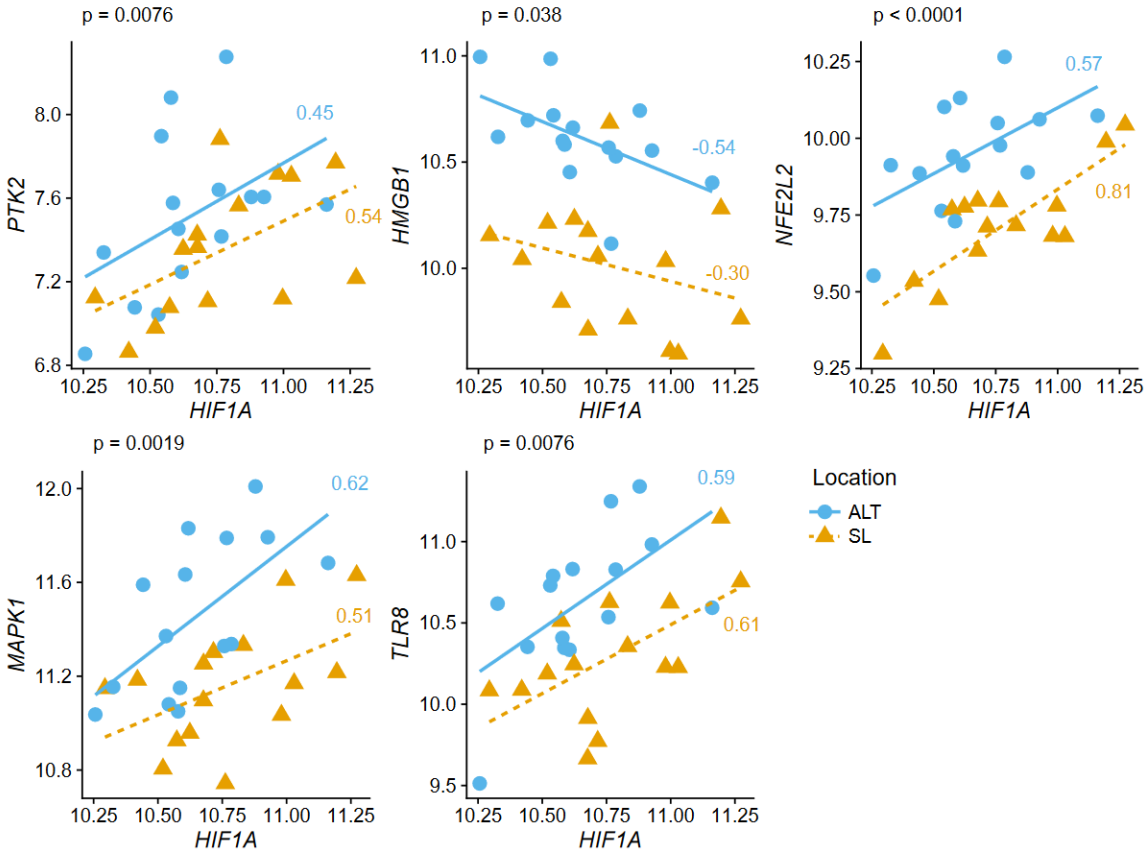

Figure 2.7. Relationships between *HIF1A* expression and upregulated inflammatory response genes at high altitude. Expression levels are reported as the normalized log2 counts. Pearson correlation coefficients for sea level and high altitude are provided independently. Orange items (triangles and dotted lines) represent sea-level expression levels and blue items (dots and solid lines) represent expression levels on the first day at high altitude.

Table 2.1 Physiological measures at baseline and over three days at high altitude.

Variable	SL	HA 1	HA 2	HA 3	ANOVA
P_{sys}	128 ± 7	125 ± 12	126 ± 45	126 ± 13	0.537
P_{dia}	79 ± 10	83 ± 9	83 ± 7	85 ± 7	0.054
HR	78.0 ± 8.1	88.3 ± 13.2	89.7 ± 12.1*	95.6 ± 12.8***	<0.001
SpO_2	94.8 ± 1.6	85.0 ± 4.4***	83.7 ± 2.5***	86.1 ± 2.5***	<0.001
AMS	0.2 ± 0.4	3.1 ± 1.8***	2.3 ± 2.0**	0.7 ± 1.2	<0.001
CO	3.9 ± 1.4	5.2 ± 1.5	5.0 ± 1.9		0.080

Variable units: P_{sys} and P_{dia} (mmHg); HR (bpm); SpO2 (%); CO (ppm). Overall p-values for repeated measures ANOVA are provided. Asterisks indicate significant differences from SL at p<0.05 (*), p<0.01 (**), and p<0.001 (***) levels via post-hoc pairwise comparisons with Bonferroni adjusted p-values.

Table 2.2. Top 20 differentially expressed genes on days 1 and 3 at high altitude.

SL vs. HA 1			SL vs. HA 3		
Gene	**FC**	**Adj. *P***	**Gene**	**FC**	**Adj. *P***
BCL2A1	1.73	1.46E-24	*BPGM*	2.86	3.65E-38
EVI2A	1.52	7.87E-20	*HEMGN*	2.18	3.33E-24
ERGIC2	0.979	5.60E-18	*GYPA*	4.64	9.63E-24
PPIG	0.663	3.25E-15	*IFIT1B*	2.91	1.35E-22
PDCD10	1.22	7.17E-15	*CA1*	3.09	1.35E-22
TXNDC9	1.20	1.28E-14	*XK*	2.45	1.65E-22
RGS18	1.04	3.09E-14	*SACS*	1.51	2.85E-21
SUB1	1.09	3.09E-14	*TENT5C*	1.69	1.61E-19
TAF7	0.66	9.58E-14	*FECH*	1.95	1.61E-19
S100A8	1.58	1.88E-13	*EVI2A*	1.44	5.45E-18
NFXL1	0.97	2.53E-13	*MBNL3*	1.47	8.16E-18
CCDC82	1.02	2.79E-13	*PDCD10*	1.30	2.45E-17
RSL24D1	1.63	4.17E-13	*ZNF292*	1.04	4.31E-17
HMGB1	0.80	4.71E-13	*RIOK3*	1.34	4.92E-17
MAN1A1	0.89	7.26E-13	*CAPZB*	-0.562	9.42E-17
NDUFA5	1.35	7.26E-13	*YOD1*	1.34	1.97E-16
ANKRD12	0.76	1.20E-12	*PI3*	-1.61	6.91E-19
B2M	0.94	1.59E-12	*CREG1*	0.993	8.01E-16
NORAD	0.55	1.59E-12	*NORAD*	0.616	1.02E-15
BLOC1S2	0.88	1.70E-12	*LOC644285*	-1.35	1.12E-15

Table 2.3. Significantly enriched pathways on day 1 and 3 at high altitude.

HA1 v SL

Term	Adj. P	Overlap
Apoptosis signaling pathway Homo sapiens P00006	0.0003	40/102
CCKR signaling map ST Homo sapiens P06959	0.0003	57/165
Ubiquitin proteasome pathway Homo sapiens P00060	0.0024	20/43
PDGF signaling pathway Homo sapiens P00047	0.0033	39/112
T cell activation Homo sapiens P00053	0.0033	28/73
Toll receptor signaling pathway Homo sapiens P00054	0.0033	21/49
Ras Pathway Homo sapiens P04393	0.0064	26/69
FAS signaling pathway Homo sapiens P00020	0.0156	14/31

HA3 v SL

Term	Adj. P	Overlap
Apoptosis signaling pathway Homo sapiens P00006	0.0000	49/102
CCKR signaling map ST Homo sapiens P06959	0.0000	62/165
T cell activation Homo sapiens P00053	0.0011	31/73
Toll receptor signaling pathway Homo sapiens P00054	0.0011	23/49
PDGF signaling pathway Homo sapiens P00047	0.0011	42/112
VEGF signaling pathway Homo sapiens P00056	0.0053	23/54
Interleukin signaling pathway Homo sapiens P00036	0.0068	32/86
Glycolysis Homo sapiens P00024	0.0103	10/17
Ras Pathway Homo sapiens P04393	0.0141	26/69
Integrin signaling pathway Homo sapiens P00034	0.0162	49/156
Ubiquitin proteasome pathway Homo sapiens P00060	0.0162	18/43
B cell activation Homo sapiens P00010	0.0171	22/57
Angiotensin II-stimulated signaling through G proteins and beta-arrestin Homo sapiens P05911	0.0176	15/34
Inflammation mediated by chemokine and cytokine signaling pathway Homo sapiens P00031	0.0232	56/188
p53 pathway Homo sapiens P00059	0.0302	25/71
Parkinson disease Homo sapiens P00049	0.0464	27/81
General transcription regulation Homo sapiens P00023	0.0464	12/18
mRNA splicing Homo sapiens P00058	0.0464	4/5
Alzheimer disease-amyloid secretase pathway Homo sapiens P00003	0.0464	20/56
EGF receptor signaling pathway Homo sapiens P00018	0.0464	34/109

Table 2.4. Relationships between phenotypes and log2 fold changes in gene expression.

SpO_2

Gene	R	p
FASLG	0.68	0.005
SMAD7	0.63	0.011
PTGER4	0.56	0.028
TRAF2	0.55	0.035
IL8	-0.51	0.051

AMS Score

Gene	R	p
TNFSF14	-0.74	0.002
FASLG	-0.71	0.003
IL18	-0.62	0.013
CD40LG	0.61	0.015
PTGER4	-0.60	0.019
MAPKAPK2	-0.58	0.024
HLADRB1	0.57	0.027
SMAD7	-0.57	0.027
AGER	-0.55	0.035
MAFK	-0.54	0.037
IRF5	-0.54	0.037

P values for Pearson correlations are provided. Negative R values indicate that increased expression levels at high altitude were associated with lower AMS scores or SpO_2 levels.

Chapter Three

Immune adaptation during acute high-altitude exposure

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A version of this chapter will be submitted for publication

3.1 ABSTRACT

The immune response to acute hypoxemia has been considered to play a critical role in high altitude acclimatization and adaptation. However, if not properly controlled, hypoxemia induced by high-altitude exposure is suspected to potentially exacerbate subsequent high-altitude pathologies, such as Acute Mountain Sickness (AMS). Furthermore, investigating the impact of high-altitude exposure on the immunological cell balance may provide insight into high-altitude illness development, or the pathology of hypoxia-related clinical conditions. Several studies report changes in immune cell subsets at high altitude, however there are still gaps in our knowledge regarding the underlying mechanisms that alter immune cell balances at high altitude, and if these alterations are beneficial or maladaptive. To address this, we performed multiparameter flow cytometry on peripheral blood mononuclear cells (PBMCs) collected throughout 3 days of high-altitude acclimatization in healthy sea-level residents (n=20). Additionally, we conducted *in vitro* stimulation assays to test if there is a synergistic effect of inflammatory stimuli and hypoxia on pro-inflammatory cytokine production, as well as analyzed how hypoxia-inducible factor (HIF) pathway activity affects components of the toll-like receptor 4 (TLR4) signaling pathway, which has previously been found upregulated in response to acute high-altitude exposure. We found several immune populations to be significantly altered in response to high-altitude exposure, including monocytes, T cells, and B cells. These changes in immune cell populations are potentially correlated with acute mountain sickness (AMS) incidence and severity. In particular, the distribution of monocyte subsets was found to be correlated with AMS severity. *In vitro* PBMC cultures stimulated with hypoxia and lipopolysaccharide (LPS), which is recognized by TLR4, showed no changes in pro-inflammatory cytokine

production. However, *in vitro* whole blood cultures showed significant increases in TNFα when stimulated with hypoxia and LPS. Lastly, we identified a potential role of hypoxia inducible factor (HIF) in CD14 expression, an essential co-receptor to TLR4, that potentially drives inflammatory responses. These results indicate that high-altitude exposure may initiate an inflammatory response that encompasses innate immune sensitization, but adaptive immune suppression.

3.2 INTRODUCTION

High-altitude hypoxia is an extremely stressful environment that triggers physiological responses to maintain oxygen homeostasis. While many physiological mechanisms that modulate high-altitude adaptation are well described, such as the impact on respiratory, cardiovascular, and metabolic mechanisms, (Bartsch *et al.*, 2002; Beall, 2006; Scheinfeldt *et al.*, 2012; Simonson, 2015; Moore, 2017), little is understood regarding immunological adaptation to high altitude.

Under typical conditions, immune cells are exposed to a wide variety of oxygen tensions as they migrate from bone marrow to blood, and throughout the arterio-venous circuit (Tsai *et al.*, 2010). However, immune cells are also mobilized to sites of inflammation or tissue injury, where hypoxia is an important feature that immune cells must accommodate. As such, the molecular hypoxia- and inflammatory-response pathways work in tandem and share significant crosstalk to mediate the response and resolution mechanisms for tissue injury or insult (Görlach & Bonello, 2008; Bandarra & Rocha, 2013; D'Ignazio *et al*., 2016; Corcoran & O'Neill, 2016; Pham *et al*., 2021). Immune cell adaptation to hypoxia is largely mediated by the hypoxia-response pathway, which is primarily controlled by transcriptional activity of the hypoxia inducible factor (HIF) (Semenza, 1998, 2009). In addition to HIF being the master regulator of the

cellular hypoxia response, HIF also plays a crucial role in immune cell metabolic function (Tao *et al.*, 2015).

The crucial crosstalk between hypoxia and inflammation is a significant feature in critical illnesses, such as sepsis and acute respiratory distress syndrome (ARDS). Indeed, hypoxemia may exacerbate inflammatory responses and subsequently worsen outcomes in these cases. Previous research has investigated how acute high-altitude hypoxia exposure upregulates inflammatory mediators in peripheral blood (Faquin *et al.*, 1992; Hartmann *et al.*, 2000; Eltzschig, Holger K, 2011; Scholz *et al.*, 2013; Kiers *et al.*, 2016; Lundeberg *et al.*, 2018; Heinrich *et al.*, 2018; Pham *et al.*, 2022), and may also impact immune cell adaptation and differentiation (Caldwell *et al.*, 2001; Tao *et al.*, 2015; Kiers *et al.*, 2016; Zhu *et al.*, 2022). While there is evidence that hypoxemia induced by high-altitude exposure may contribute to acute increases in pro-inflammatory mediators, it remains unknown if this systemic inflammatory response is a consequence of or a contributing factor to the development of high-altitude illnesses, such as Acute Mountain Sickness (AMS), high-altitude pulmonary edema (HAPE), high-altitude pulmonary hypertension (HAPH), and high-altitude cerebral edema (HACE) (Bärtsch & Swenson, 2013; Luks *et al*., 2017). Furthermore, little is known about what drives these changes in the inflammatory profile at high altitude, as well as the changes in the immunological balance, and it is unclear if these changes are beneficial or maladaptive.

In the current study, we investigate the changes in the immune cell balance by characterizing peripheral blood mononuclear cells (PBMCs) during three days of acute high-altitude hypoxia exposure in healthy sea-level residents. Furthermore, since previous research has shown that acute high-altitude exposure may sensitize the TLR4 signaling pathway to subsequent inflammatory stimuli (Pham *et al.*, 2022), we aim to

determine the role of HIF in innate immunity sensitization, particularly in the context of CD14 and TLR4 expression. We utilized flow cytometry and enzyme-linked immunosorbent assay (ELISA) to characterize PBMCs at high altitude, and if HIF affected inflammatory surface markers or inflammatory cytokine production. We hypothesized that the immune phenotype will be pro-inflammatory upon initial acute high-altitude hypoxia exposure but will shift to an anti-inflammatory profile upon acclimatization as complimentary physiological changes occur to improve oxygen delivery to tissue.

3.3 METHODS

Ethical approval

This study was approved by the University of California, Riverside Clinical Institutional Review Board (HS 22-088). All participants were informed of the study's purpose and risks. Participants provided written informed consent in their native language (English). The work was conducted in accordance with the *Declaration of Helsinki*, except for registration in a database.

Participants

The study included 20 healthy participants (N=7 women, 13 men) between 19 and 35 years of age. Participants were recruited by word of mouth and flyers on the UC Riverside campus. All participants reported no known history of cardiopulmonary disease or sleep disturbances, including obstructive sleep apnea, and displayed no abnormal findings on electrocardiogram (ECG) or pulmonary function testing. Mean age was 25 ± 7 years for women and 26 ± 6 years for men and mean BMI was 30 ± 5.4 kg/m^2 for women and 31 ± 5.3 kg/m^2 for men. Exclusion criteria included travel above

8,000 feet within one month of the first measurements, a previous history of high-altitude pulmonary or cerebral edema, current smoking, and pregnancy.

Experimental design and physiological measures

In the two weeks prior to ascent to high altitude, participants completed initial screening for eligibility at UC Riverside, at approximately 400 m elevation (Riverside, CA, USA). Demographic information including age, height, weight, and blood pressure were collected. Participants also answered questions about their ancestral background (to determine presence of high-altitude ancestry) and medical history including current medications. Participants then completed pulmonary function testing and ECG to verify absence of lung or heart disease.

Participants returned to UC Riverside in the early morning on the day of ascent. Baseline (sea-level, SL) physiological measures were collected at this time, including blood pressure, pulse oximetry (SpO_2), heart rate, and AMS scores via the 2018 Lake Louise scoring criteria with an experimenter asking participants each question (Roach *et al.*, 2018). Fasting blood samples were then collected via standard venipuncture procedures. Breakfast was provided to participants following blood sampling, prior to travel.

The group then traveled by car to Barcroft Station (3,800 m elevation) in the White Mountain Research Center (Bishop, CA, USA) over a period of approximately 6.5 hours. At the field station, fasting blood samples and morning measurements were collected each day within 1 hour of waking and before 9 am to keep timing consistent with sea level measures. Physiological measures and fasting blood samples were collected every morning for 3 consecutive days (HA1, HA2, HA3). Pulse oximetry and heart rate values were collected using a Nellcor N-600 pulse oximeter (Medtronic,

Minneapolis, MN, USA). Participants sat upright in a chair without their legs crossed and rested, breathing normally, for 3 minutes until values stabilized. Blood pressure measurements were collected in duplicate while participants rested in an upright seated position using a manual sphygmomanometer.

Participants abstained from taking anti-inflammatory medications or other agents that may influence acclimatization, such as acetazolamide (Basaran *et al.,* 2016). Participants were permitted to consume caffeine in moderation (1 cup of coffee or tea) after completing morning measurements but were asked to abstain from caffeine after noon. Three meals per day were provided and participants did not complete any strenuous physical activity. Participants did not consume alcohol, and fluid intake was supervised to ensure participants remained hydrated.

Immune cell characterization

Isolation of PBMCs and freezing

Peripheral venous blood was collected in a 10mL vacutainer tube containing EDTA (BD, Franklin Lakes, NJ, USA) and processed within 4 hours of collection. Blood was diluted 1:1 with PBS. In a separate tube, equal volume (compared to blood volume) of Lymphoprep Density Gradient Medium (1.077 g/mL density) (StemCell, Seattle, WA, USA) was added. Blood was then slowly added to be layered on top of the Lymphoprep. Tubes were centrifuged at 400xg for 30 minutes at room temperature, with slow acceleration and no brakes. PBMCs were carefully collected into a separate 15 mL tube with 8 mL of EasySep media (StemCell, Seattle, WA, USA). PBMCs were centrifuge at 400xg for 5 minutes and resuspended in 5 mL of EasySep media. The wash step was repeated twice. PBMCs were resuspended in 1.5 mL of freezing media (90% FBS, 10% DMSO), and aliquoted in 500 uL volumes. Aliquots were placed in a Mr. Frosty freezing

container (ThermoFisher, Carlsbad, USA) and into a -80 °C freezer overnight, then transferred to liquid nitrogen the following morning.

At high altitude, the same procedure was followed, with the following exceptions. Due to a lack of a -80 °C freezer at high altitude, the Mr. Frosty containers were placed in a large Styrofoam box filled with dry ice overnight. Aliquots were transferred to a transportable liquid nitrogen dewar for transport to sea level. Due to logistical constraints and timing, PBMCs collected on day 3 at altitude were first collected as buffy coat while at Barcroft Station, and then processed for PBMCs following the same protocol the following day at sea level.

PBMC thawing

PBMCs were first thawed before use. PBMCs were removed from liquid nitrogen and placed in a 37 °C water bath for 30-45 seconds, or until a small ice crystal was left. 1 mL of warmed media (RPMI 1640, 10% FBS, 100 U/mL Strep/Penicillin) was added in a dropwise manner to the tube, which was then transferred to a 15 mL falcon tube containing 5 mL of warmed media. An additional 1 mL of warm media was used to rinse PBMC cryotube and added to the 15mL falcon tube. The tubes were gently mixed by inverting, and then centrifuged at 330xg for 10 minutes at room temperature. PBMCs were then resuspended in 1 mL of warmed media and counted via hemocytometer for experiments.

Flow cytometry for immune cell characterization

PBMCs from sea level, day 1 at high altitude, and day 3 at high altitude were stained and analyzed. 3.0×10^5 PBMCs from each sample were aliquoted for the experiment. PBMCs were then centrifuged at 330xg for 10 minutes and resuspended in

100 uL of a 1:20 dilution of Human TruStain FcX (Fc Receptor Blocking Solution) (BioLegend, San Diego, USA) in FACS buffer. Cells were stained using fluorescent antibodies: anti-CD3 PerCP/Cyanine 5.5 (BioLegend, San Diego, USA; Clone OKT3), anti-CD11b APC/Cyanine 7 (BioLegend, San Diego, USA; Clone M1/70), anti-CD25 BV 420 (BioLegend, San Diego, USA; Clone BC96), anti-CD45 BV 711 (BioLegend, San Diego, USA; Clone HI30), anti-CD66b APC (BioLegend, San Diego, USA; Clone G10F5), anti-CD56 (BioLegend, San Diego, USA; Clone HCD56), anti-CD14 AlexaFluor 488 (BioLegend, San Diego, USA; Clone HCD14), anti-CD163 BV 510 (BioLegend, San Diego, USA; Clone GHI/61), anti-CD8 BV 785 (BioLegend, San Diego, USA; Clone SK1), anti-CD16 PE (BioLegend, San Diego, USA; Clone 3G8), anti-CD19 PerCP-eFluor 710 (eBioscience, San Diego, USA; Clone HIB19), anti-CD4 PE/Cyanine 5 (BioLegend, San Diego, USA; Clone A16A1), and anti-HLA-DR, DP, DQ (BD Biosciences, San Diego, USA; Clone Tu39). After staining, PBMCs were fixed with 4% PFA for 10 minutes, washed and resuspended in 2 mL FACS buffer. Samples were analyzed using the NovoCyte Quanteon flow cytometer, NovoSampler Q, and NovoExpress Software. An average of $1.0x10^5$ events were collected in total for analysis. Analysis was conducted using FlowJo software version 10.0.

Flow cytometry for TLR4 surface expression analysis

PBMCs from sea level (SL), first day (HA1) and third day (HA3) at altitude were stained with LIVE/DEAD Fixable Far Red Dead Cell Stain (Thermofisher, Carlsbad, USA) for 30 minutes at 4°C. After staining, cells were washed with FACS buffer, centrifuged, and resuspended in 100 uL of FACS buffer. Cells were stained using fluorescent antibodies: anti-CD14 AlexaFluor488 (BioLegend, San Diego, USA; Clone HCD14) and anti-TLR4 PE (BioLegend, San Diego, USA; Clone HTA125). Samples

were analyzed using the NovoCyte Quanteon flow cytometer, NovoSampler Q, and NovoExpress Software. An average of $0.7x10^5$ events were collected in total for analysis. Analysis was conducted using FlowJo software version 10.0.

HIF-dependent CD14 and TLR4 analysis

PBMC experiments

1.0×10^5 PBMCs from SL samples were used per condition and treatment and resuspended in 200 uL of media (RPMI 1640, 10% Fetal Bovine Serum (FBS), 10,000U/mL penicillin/streptomycin). PBMCs were treated with 0.5 mM DMOG (Millipore Sigma, Darmstadt, Germany), an antagonist of α-ketoglutarate cofactor and inhibitor for HIF prolylhydroxylase, or 25 uM PX478 (Cayman Chemicals, Michigan, USA), a HIF-1α inhibitor, and co-treated with 100 ng/mL lipopolysaccharide (LPS) (E. coli Serotype (026: B6) (Sigma-Aldrich, Burlington, Massachusetts, USA)). PBMCs were then cultured in normoxic conditions (21% O_2; 5% CO_2) or hypoxic conditions (1% O_2; 5% CO_2) for 6 hours in a Hypoxia Incubator Chamber (StemCell, Seattle, WA, USA). The chamber was placed inside a 37 °C incubator for the duration of the culture. Following culture, the plate was centrifuged at 330xg for 10 minutes. Supernatant was collected and stored at -80 °C for ELISA analysis. PBMCs were analyzed via flow cytometry.

Whole blood experiments

Experimental design was adapted from another study (Feuerecker *et al*., 2019). Briefly, 150 uL of whole blood was combined with 850 uL of media. Whole blood cultures were treated with 0.5 mM DMOG, 25 uM PX478, or control (PBS), and co-treated with 10 ug/mL LPS. Whole blood cultures were then cultured in normoxic conditions (21% O_2; 5% CO_2) or hypoxic conditions (1% O_2; 5% CO_2) for 24 hours in a Hypoxia Incubator

Chamber. The chamber was placed inside a 37°C incubator for the duration of the culture. Following culture, the culture was collected into microfuge tubes and was centrifuged at 330xg for 10 minutes. Supernatant was collected and stored at -80 °C for ELISA analysis.

Flow cytometry for HIF-dependent analysis

SL PBMCs were resuspended in 100 uL of a 1:20 dilution of Human TruStain FcX (Fc Receptor Blocking Solution) (BioLegend, San Diego, USA) in FACS buffer. PBMCs were then stained with LIVE/DEAD Fixable Far Red Dead Cell Stain (Thermofisher, Carlsbad, USA) for 30 minutes at 4 °C. After staining, cells were washed with FACS buffer, centrifuged, and resuspended in 100 uL of FACS buffer. Cells were stained using fluorescent antibodies: anti-CD14 AlexaFluor488 (BioLegend, San Diego, USA; Clone HCD14) and anti-TLR4 PE (BioLegend, San Diego, USA; Clone HTA125). An average of $0.7x10^5$ events were collected in total for analysis. Analysis was conducted using FlowJo software version 10.0.

ELISA analysis

According to manufacturer's instructions, the concentration of TNFα in cell culture supernatants were analyzed using a TNFα Human ELISA kit (Invitrogen, Carlsbad, USA). An automated microplate reader (Synergy Lx Multimode Reader) (Biotek, Seattle, Washington, USA) was used for the measurement of the optical density at 450 nm. The concentrations of each sample were detected based on optical density (OD) and the concentration of the standard.

Statistical analysis

Statistical analyses were conducted in R (version 4.1.0) (R Foundation) and GraphPad Prism v10.0. To identify changes in physiological variables and immune cell phenotypes at high altitude compared to baseline sea-level measures, we used repeated measures ANOVA and post-hoc pairwise t-tests with Tukey corrections. To analyze the concentration of TNFα in cell culture supernatants, we performed a paired t-test analysis. To determine if changes in immune cell phenotype were associated with physiological measures at high altitude (SpO_2, AMS Score), Pearson correlation coefficients and p-values were obtained with the *rcorr* function from the *Hmisc* package in R. Data is presented throughout the paper as mean ± standard deviation. Three participants did not have PBMCs collected for one or more days at high altitude due to blood collection complications and were excluded from immune cell characterization analysis.

3.4 RESULTS

Physiological measures

Table 3.1 provides an overview of physiological measures at sea level and over three days of acclimatization to high altitude. On the first morning at high altitude (HA1), 8 of 20 subjects indicated mild AMS (AMS score 3-5 with headache), and 6 of 20 subjects indicated moderate – severe AMS (AMS score 6+ score with headache). By the third day at altitude, 5 of 20 subjects indicated mild AMS, and 3 of 20 subjects indicated moderate – severe AMS. SpO_2 dropped by about 11 percent on the first day at high altitude and remained lower than sea-level values throughout all 3 days at altitude. This was coupled with a 17-point increase in heart rate on the first day at high altitude, which

increased a further 9 points by day 3. There was no significant increase in systolic or diastolic blood pressure at high altitude.

Acute high-altitude exposure promotes a shift in immune cell populations throughout acclimatization

Immune cell subsets were quantified to identify high altitude-induced changes over the course of three days at altitude compared to sea level values (n=17). Flow cytometry gating of peripheral blood mononuclear cells (PBMCs) was performed to quantify innate and adaptive immune cells (**Figure 3.S1**). Total monocyte frequency as a percentage of total white blood cells (% WBCs) were significantly elevated on the first day of acute high-altitude exposure (following the night of arrival) compared to sea-level values (**Figure 3.1A**, p=0.0006). Specifically, both classical and intermediate monocytes were significantly elevated on the first day at high altitude (p=0.0011 and p=0.045, respectively). By the third day at high altitude, classical monocyte populations returned to baseline, while intermediate monocyte population continue to increase (p=0.0004). There was no significant difference in non-classical monocyte populations on day 1 (p=0.89) or on day 3 (p=0.36) of altitude (**Figure 3.1A**). When analyzing based on total monocytes, classical monocyte subpopulation did not change on first day of altitude (p=0.812) but was significantly reduced by the third day (p<0.0001). Intermediate monocytes subpopulations also did not change after one day at altitude (p=0.20) but was significantly higher by the third day (p<0.0001) (**Figure 3.S2**).

T Cell populations were analyzed as a percentage of total WBCs (**Figure 3.1B**). $CD3^+$ T cells of total WBCs were significantly reduced on the first (p=0.0002) and third day of altitude (p=0.0008). Specifically, $CD4^+$ T cells was significantly reduced on first (p<0.0001) and third (p<0.0001) day at high altitude, while there was no change in $CD8^+$

T cells as a percentage of total WBCs on either day (p=0.17 and p=0.93, respectively). DN T cells ($CD3^+$ $CD4^-$ $CD8^-$ $CD16^-$) were significantly increased on both first (p=0.011) and third (p=0.0003) day at high altitude (**Figure 3.1B**). When analyzing B cell populations as a percentage of total WBCs, B cells were significantly elevated following one day (p=0.0093) and three days (p=0.0012) at high altitude (**Figure 3.1C, far left**). Of total CD3+ T cells (% CD3+), there was a significant reduction of CD4+ (p=0.013) on first day at high altitude, which remained reduce by third day at altitude compared to baseline (p=0.0024). There was no change in $CD8^+$ T cells on either first (p=0.43) or third day (p=0.99) at altitude. DN T cells ($CD3^+$ $CD16^+$) were significantly increased on the first (p<0.0001) and third day of altitude (p<0.0001) (**Figure 3.S3**).

NK cell populations were also analyzed as a percentage of total WBCs (**Figure 3.1C**). $CD56^+$ NK Cells frequency of total WBCs did not change following one day at altitude (p=0.88) but was significantly reduced by the third day (p=0.010). $CD16^+$ NK Cells populations were not changed on either first (p=0.74) or third (p=0.45) day at altitude (**Figure 3.1C, middle**). Lastly, while NK T cells are considered to be lymphocytes, they have innate-like features. NK T cells were significantly reduced on the first day at altitude (p=0.016) but recovered to baseline values by day 3 at altitude (p=0.99) (**Figure 3.1C, far right**). These results demonstrate acute high-altitude alters both innate and adaptive immune populations.

TLR4 expression on the surface of live $CD14^+$ PBMCs collected at SL, HA1, and HA3 were quantified via mean fluorescence intensity (MFI) to measure effects of altitude on the TLR4 signaling pathway, which key components were previously found to have significant gene upregulations (Pham *et al*., 2022). MFI of TLR4 was detected via flow

cytometry. MFI of $CD14^{+}TLR4^{+}$ live PBMCs was significantly increased on day 1 at altitude (p=0.022) and was sustained on day 3 at altitude (p=0.025) (**Figure 3.2**).

HIF stability alters CD14 expression and pro-inflammatory cytokine production

CD14 and TLR4 surface expression on PBMCs were quantified to identify if hypoxia and/or HIF stabilization alter key components of the TLR4 signaling pathway. PBMCs were cultured in normoxic versus hypoxic conditions, treated with either control (PBS), HIF activator (DMOG), or a HIF inhibitor (PX478), and stimulated with (n=6) and without LPS (n=10) (100 ng/mL) for 6 hours.

While both DMOG and PX478 treatments alone significantly increased surface CD14 expression in normoxia compared to control (p<0.0001 and p=0.015, respectively), DMOG produced higher CD14 expression compared to all other treatments and conditions (**Figure 3.3A**). Surprisingly, compared to normoxia conditions and within treatment groups, hypoxia alone did not alter CD14 expression in these *in vitro* conditions. However, PX478 treatment + hypoxia + LPS produced significantly higher CD14 than PX478 + hypoxia (p=0.035) (**Figure 3.3D**). This indicates that when HIF was inhibited, LPS stimulation does affect and increase CD14 expression in both normoxic and hypoxic conditions, potentially indicating that HIF activity plays a role in the LPS-induced increase in CD14. These results show that both HIF stabilization and inhibition positively promote CD14 upregulation on PBMCs. However, HIF inhibition did not increase CD14 expression as strongly as HIF activation. Lastly, hypoxic conditions did not affect CD14 expression among treatment groups (**Figure 3.3B-D**). In contrast to CD14, TLR4 expression on PBMCs remain unchanged regardless of treatment or condition. In fact, when stimulated with LPS, there was a consistent decrease in TLR4 across all treatments and conditions (**Figure 3.4**).

TNFα cytokine production was analyzed from both PBMC and whole blood cultures. PBMC cultures were treated with either control (PBS), DMOG, or PX478, stimulated with or without LPS (100 ng/mL), and under normoxic or hypoxic conditions for 6 hours in a 37 °C incubator. While there was a significant increase in TNFα production following LPS stimulation of PBMCs (p=0.012), hypoxic conditions alone did not affect TNFα production. When treated with PX478 or DMOG, TNFα production was significantly reduced despite stimulation with LPS (p=0.76 and p=0.37, respectively) (**Figure 3.5**).

Whole blood cultures were also treated with either control (PBS), DMOG, or PX478, stimulated with or without LPS (10 ug/mL), and under normoxic or hypoxic conditions for 24 hours in a 37 °C incubator. In whole blood cultures, hypoxia did significantly increase TNFα production following LPS stimulation with control treatments (**Figure 3.6A**, p=0.0006). Interestingly, the addition of PX478 eliminated the hypoxia-induced increase in TNFα production by increasing TNFα production in normoxic conditions (**Figure 3.6B**). Lastly, the addition of DMOG completely abrogated TNFα production in response to LPS in both normoxic and hypoxic conditions (**Figure 3.6C**).

Baseline immune cell populations is not a predictor of Acute Mountain Sickness

Immune cell populations (i.e., monocyte subsets, T cell subsets, B cells, NK cells) on first and third day at altitude were tested for associations with self-reported Acute Mountain Sickness (AMS) scores and oxygen saturation (SpO_2). Of all analyzed associations with AMS scores and SpO_2, the following were found to be significant: non-classical monocytes were positively correlated with AMS score on the first day of altitude (p=0.031, r=0.27) (**Figure 3.7A**), $CD16^+$ NK Cells were negatively correlated with SpO_2 on the third day at altitude (p=0.034, r =0.27) (**Figure 3.7B**), and DN T cells were

positively correlated with SpO_2 on third day at altitude (p= 0.0093; r=0.37) (**Figure 3.7C**). All correlation analyses are shown in **Table 3.2.**

When analyzing monocyte total and subset populations on both day 1 and 3 at altitude and separated by AMS severity groups, we found that participants with moderate to severe AMS severity displayed a trend for more total monocytes compared to the mild AMS severity group (p=0.064), but no difference from the group with no AMS (p=0.75) (**Figure 3.8A**). The moderate to severe AMS group also had increased classical monocytes compared to the mild group (p=0.034), but no difference from the no AMS group (p=0.13) (**Figure 3.8B**). All analyses with immune populations and AMS severity groups are shown in **Table 3.3**.

To determine if baseline sea level immune cell populations affected AMS symptom severity at altitude, we analyzed associations between sea level immune populations with AMS scores on day 1 and 3 at altitude. While there was no significance between any of the baseline (sea-level (SL)) immune cell populations and AMS from either day 1 or 3 at altitude, participants with higher baseline B cell levels showed a trend to be more likely to report no AMS compared to the mild AMS group (p=0.058) (**Figure 3.9**). All analyses with sea-level immune populations and AMS severity on day 1 and 3 at altitude are shown in **Table 3.4**.

3.5 DISCUSSION

In this study, we utilized multi-parameter flow cytometry to determine how high-altitude acclimatization may impact the immunological cell balance in healthy lowlander participants. While we previously demonstrated that acute exposure to high-altitude hypoxia triggers significant changes in inflammation-related gene expression (Pham *et al.*, 2022), there is a gap in knowledge regarding how this affects immune cell

development, differentiation, and activity. In this subsequent study, we determined that the changes in the immune cell populations in sojourners to high altitude over time reflect immune adaptation to acute high-altitude hypoxic exposure. In particular, we found shifts in leukocyte populations, such as monocytes, T lymphocytes, and NK cells. In addition, we previously identified a potential hypoxia-induced innate immune system sensitization mechanism through upregulation of TLR4 signaling pathway components at the gene expression level (Pham *et al.*, 2022). Now, we specifically quantified TLR4 expression on the surface immune cells and confirm that acute high-altitude exposure does indeed promote greater TLR4 expression. We also investigated the impact of HIF stabilization on CD14 expression, which is not only a crucial co-receptor for several toll-like receptors (TLRs) (Akashi-Takamura & Miyake, 2008; Baumann *et al.*, 2010; Zanoni *et al.*, 2011; Weber *et al.*, 2012; Ciesielska *et al.*, 2021), but also has a role in host defense, regulating metabolism, and acts as a pattern recognition receptor (PRR) capable of triggering downstream signaling (Wennerås *et al.*, 2001; Granucci *et al.*, 2004; Jersmann, 2005; Wiersinga *et al.*, 2008; Zanoni *et al.*, 2012; Dessing *et al.*, 2012; Zanoni & Granucci, 2013).

Acute high-altitude exposure promotes a pro-inflammatory innate immune phenotype

Monocyte populations

Monocytes are classified as $CD14^{+}CD16^{+}$. Specifically, monocyte subsets have been defined as classical monocytes ($CD14^{+}$ $CD16^{-}$), intermediate ($CD14^{+}$ $CD16^{+}$) and non-classical ($CD14^{dim}$ $CD16^{+}$) (Marimuthu *et al.*, 2018). Monocytes are essential immune cells in the innate immune system that can shape the immune response by playing a role in tissue healing, pathogen clearance, and initiation of the adaptive

immune system. Under steady-state homeostasis, monocyte subsets are maintained in peripheral blood as they differentiate from classical to non-classical phenotypes, constantly monitoring and recruited to tissues to replenish tissue macrophages (Yang *et al.*, 2014). In response to inflammation, monocytes are rapidly mobilized and recruited to sites of injury (Serbina *et al.*, 2003; Ingersoll *et al.*, 2011). As these cells develop from the bone marrow, circulate throughout the blood, and extravasate into tissues, they experience a wide range of oxygen tensions. In particular, sites of inflammation are commonly hypoxic, as the cellular demand for oxygen outstrips the supply, and immune cells can experience low oxygen tensions as low as 2% PO_2 (Sahaf *et al.*, 2008; Bosco *et al.*, 2008; Fangradt *et al.*, 2012; Strehl *et al.*, 2014). While it is well known that hypoxic microenvironments impact immune cell properties, such as cytokine production and surface marker expression (Cramer *et al.*, 2003), we found that acute systemic hypoxemia, or other factors related to acute high-altitude exposure, also alter monocyte subsets.

In the present *in vivo* study, monocyte population subsets significantly favor the classical phenotype on the first day of high-altitude exposure but shifted towards the intermediate monocyte subset by day 3 of acclimatization (**Figure 3.1A**). These data provide support for the theory that acute high-altitude hypoxia exposure does lead to a pro-inflammatory immune phenotype. This agrees with our previous studies, where we identified several inflammatory-related genes that indicate hypoxia-induced sensitization of the innate immune system, including upregulation of TLR4 pathway genes (*TLR4*, *LY96*) and alarmins (*HMGB1*, *HMGB2*, and *S100* protein genes) (Pham *et al.*, 2022). The significant increase in total monocytes and classical monocytes after one day at high altitude supports these previous findings of a pro-inflammatory profile.

To complement this data, research in rats at moderate altitude (1,655m) also found evidence of increase monocytic count as well as greater pro-inflammatory cytokine production in response to LPS at high altitude compared to measures made at sea level, indicating a pro-inflammatory profile at high altitude (Nguyen *et al.*, 2021). In further agreement with our study, previous research on high altitude sojourners also found elevated monocyte counts, particularly in the inflammatory classical monocyte subset (Bhattacharya *et al.*, 2021). Additionally, studies with the Tibetan population, a native high-altitude population that is considered to be one of the most well-adapted groups to the high-altitude environment, reveal that this population has a reduced percentage of total monocytes, of which there are also reduced inflammatory classical monocytes compared to non-Tibetans at high altitude (Bhattacharya *et al.*, 2021). Furthermore, this monocytic suppression in total count and function in the Tibetan population is attributed to a gain-of-function variant in *EGNL1* which encodes for prolyl hydroxylase (PHD), a key enzyme crucial for modulating HIF expression. When comparing the inflammatory profiles between Tibetans with and without the PHD variant, Tibetans with the PHD variant not only had significantly lower total monocyte count and inflammatory monocytic subset, but they also report significant suppression of pro-inflammatory cytokine production, such as TNFα and IL6. This would suggest that through evolutionary selection, a diminished hypoxia-mediated inflammatory response is a beneficial adaptation.

Intermediate monocyte subsets were significantly increased by day 3 of acclimatization to high altitude (**Figure 3.1A; third**). Our monocyte data on day 3 of acclimatization agrees with studies that found elevated intermediate monocytes in HAPH and HAPE patients compared to healthy controls at the same elevation (Bhattacharya *et*

al., 2021; Wu *et al*., 2023b). Although both studies reported that the classical monocyte subsets were not different between healthy controls and patients with HAPH or HAPE, they did find a significant increase in total monocyte count in peripheral whole blood, as well as an increase in both intermediate and non-classical monocyte subsets in the HAPH and HAPE groups. Furthermore, Wu et al. report that while the intermediate monocyte populations were significantly increased in HAPH patients compared to healthy controls at the same elevation, HIF-1a was surprisingly downregulated in this subset, and showed evidence of downregulation in pathways signatures involving phagocytosis, coagulation, and platelet adhesion (Wu *et al*., 2023b). This further suggests the importance of HIF in immune adaptation to high altitude, as HIF inhibition may lead to impaired monocyte function and immunosuppression. Accordingly, long-term chronic high-altitude hypoxia exposure is suspected to result in immune exhaustion, particularly with monocyte populations. This may indicate that, while promotion of intermediate and non-classical monocytes may be beneficial in short term hypoxia or high-altitude exposure, chronic upregulation or excessive quantities of these subsets is likely maladaptive and may result in the development of high-altitude pulmonary hypertension.

Natural Killer (NK) cell populations

Natural Killer (NK) cells are important innate immune cells that are “naturally cytotoxic” and mediate anti-tumor and anti-viral responses. These innate immune cells primarily target physiologically stressed cells that lose surface expression of inhibitory receptors and MHC class l expression, which normally functions to promote self-tolerance. This scenario normally occurs in response to DNA damage, as commonly seen in tumor or viral infection (Paul & Lal, 2017). When activated, NK cells are capable

of producing a wide variety of cytokines, most notably IFN-γ, that can further promote the antitumor properties of NK cells. An important feature of NK cells is that they do not require prior sensitization to kill susceptible targets. NK cells can subdivided based on relative expression of CD16 and CD56. The two major subsets reported in our study are $CD56^{+}$ $CD16^{-}$ and $CD56^{dim}$ $CD16^{+}$.

We found that while there were no significant changes in $CD16^{+}$ NK cells on either day at altitude (p=0.74 and p=0.15, respectively), $CD56^{+}$ NK cells are markedly reduced by day 3 at altitude (p=0.010) (**Figure 1C, middle**). While there is very little research on NK cells at altitude or low oxygen, this conflicts with previous studies that report increased NK cell populations at high altitude and in a decompression chamber that mimics high altitude (Klokker *et al.*, 1993; Facco *et al.*, 2005). This may be due to different conditions in our studies, as the rate of ascent, height of ascent, and exertion of ascent plays a role in the development of high-altitude illnesses (Bärtsch & Swenson, 2013; Luks *et al*., 2017) Regardless, future experiments are necessary to elucidate how high altitude affects NK cell populations, their role in innate immunity at high altitude, and how they influence adaptive immunity.

Overall, our data suggests that acute high-altitude exposure initially promotes a pro-inflammatory phenotype in monocytes that transitions to an anti-inflammatory phenotype over the course of acclimatization. Based on these data, as well as other research, monocyte subsets are suspected to return to baseline values, even if total monocyte count remains elevated, following proper acclimatization to high-altitude. This phenotype has been previously demonstrated in healthy sojourners to high altitude (Bhattacharya *et al.*, 2021), although the duration of high-altitude exposure in these participants is unknown. Other studies with longer hypoxia environment exposure in non-

native sojourners to high altitude also demonstrate immune sensitization to inflammatory stimuli, however total monocyte counts did not peak until 4-7 months at altitude (Feuerecker *et al.*, 2019). These findings warrant further studies to investigate the impact of hypoxia-induced sensitization of immune cells, as both inflammatory and non-inflammatory monocyte subsets are suspected to play a role in hypoxia-induced pathologies.

The role of HIF in hypoxia-induced inflammation and immune sensitization

Our results suggest that HIF stabilization significantly increases CD14 surface expression on PBMCs, regardless of oxygen tension or LPS stimulation (**Figure 3.3**). Because CD14 is an important TLR4 co-receptor, this data suggests that high altitude exposure promotes monocyte induction of trained immunity and sensitization to subsequent inflammatory stimuli (Cheng *et al.*, 2014). The hypoxia response pathway is well established to have significant crosstalk with several other molecular networks, including the regulation of inflammation and immune function. As such, HIF-1α stabilization promotes metabolic reprogramming in immune cells that would in turn promote a pro-inflammatory profile (Corcoran & O'Neill, 2016).

Despite these findings that HIF activation via DMOG increased surface CD14 expression, when analyzing cytokine production from *in vitro* PBMC cultures treated with DMOG, there was significantly lower extracellular TNFα production. This seemingly contradictory result may be explained by off-target effects of DMOG. DMOG activates HIF by inhibiting PHD activity, which under normal conditions, functions to reduce HIF activity. However, PHD also plays a role in regulating the expression of many other proteins, including the IKK complex which modulates NF-κB activity. In support of our findings, multiple previous studies also report that DMOG abrogates TNFα expression in

a mice model, as well as in RAW264.7 macrophages (mouse macrophage cell line) and human gingival fibroblasts (Takeda *et al.*, 2009; Hindryckx *et al.*, 2010; Shang *et al.*, 2019). Furthermore, DMOG has been shown to suppress LPS-induced TNFα expression in a HIF-independent manner (Takeda *et al.*, 2009). Additionally, because DMOG is a PHD inhibitor, and PHD is known to affect NF-κB transcriptional activity, this would suggest that the TNFα suppression via DMOG may be due to PHD inhibition, as opposed to HIF modulation.

Surprisingly, HIF inhibition in PBMCs by PX478 also resulted in significant upregulation of CD14 surface expression, although not to the extent of HIF activation. While it is suspected that HIF plays a role in both promoting and suppressing these key receptors, further studies are required to explore the exact mechanism behind HIF-induced CD14 expression. Further studies regarding the role of HIF in inflammatory responses are necessary, particularly when considering changes in CD14.

Adaptive immune cells are affected by acute high-altitude exposure

The adaptive immune system is comprised of specialized immune cells that mount highly specific responses to pathogens. The two critical components of the adaptive immune system are B lymphocytes, which are responsible for antibody production against antigens and conferring humoral immunity, and T lymphocytes, which mediates cytokine production, trigger cell death in target cells, and are responsible for cellular immunity.

B lymphocytes produce antibodies that mediate protection against pathogens on a long-term scale. However, while it is known that B cells also experience a wide range of oxygen tension throughout development, the impact of hypoxia on B cell function remains controversial (Zhang *et al.*, 2022). Furthermore, there are conflicting reports

regarding how hypoxia regulates B cell populations and function at altitude. Several studies have reported that high altitude does not alter B cell population or function (Meehan, 1987; Facco *et al.*, 2005), while others report increase in both of these parameters (Chohan *et al.*, 1975; Mishra & Ganju, 2010; Feuerecker *et al.*, 2019).

Our research agrees that acute high-altitude exposure does increase B cell populations (**Figure 3.1C**). When considering the time scale response of immune function at high altitude, acute exposure is implicated in promoting protective antibody production against pathogens. Studies have found that hypoxia does favor generation of plasma cells, which are the endpoint of B cell differentiation (Schoenhals *et al.*, 2017). This corresponds with studies that have found increases in antibody production in response to high-altitude (Tengerdy & Kramer, 1968; Chohan *et al.*, 1975). However, long-term chronic exposure may have the opposite effect. Studies on high-altitude native Tibetans and long-term high-altitude residents of Han Chinese ancestry reveal that total B cells were significantly reduced at altitude in both groups compared to a mid-altitude Han Chinese population. (Bai *et al.*, 2022). This may be an effect of the evolutionarily conserved response to hypoxic immunological niches and stage-specific HIF expression throughout B cell development (Burrows *et al.*, 2020; Zhang *et al.*, 2022). In the earliest stages of B cell development, HIF activity is high, but is suppressed at the immature B cell stage. The initial increase in B cells seen in our study may be an effect on B cells already differentiated, but chronic hypoxic exposure prevents the development of new immature B cells, leading to a reduction in circulating B cells in long-term high-altitude residents.

To complement B lymphocytes, T lymphocyte cells play a central role in the adaptive immune response by maintaining homeostasis, immunological memory, and

cell-mediated immunity (McNamee *et al.*, 2013; Tao *et al.*, 2015). Peripheral T cells are comprised of several subsets, such as naïve T cells, $CD4^+$ T cells, and $CD8^+$ T cells. Throughout development, differentiation, or during response to a disease, T cells encounter a wide range of oxygen tensions (McNamee *et al.*, 2013). Changes in oxygen tensions during development not only can affect lymphocyte subsets, but can also impact function and activity, such as their ability to produce cytokines (Caldwell *et al.*, 2001). In agreement with several studies, T lymphocyte balance has been found to be affected by acute high-altitude hypoxia exposure (Klokker *et al.*, 1993; Facco *et al.*, 2005; Mishra & Ganju, 2010; Feuerecker *et al.*, 2019). Likewise, our data has shown that total CD3+ T cells were significantly reduced throughout high-altitude exposure, of which CD4+ T cells were most notably reduced at high altitude, while CD8+ T cells were unaffected (**Figure 3.1B**).

$CD4^+$ T cells are critical in both activating and modulating the adaptive immune response. While our study does not distinguish between specific $CD4^+$ helper subsets (T-helper cells 1 (Th_1) vs T-helper cells 2 (Th_2)), previous research has found that the Th_1/Th_2 immune balance was dysregulated at high altitude, which have implications in long-term immunological alterations (Caldwell *et al.*, 2001; Facco *et al.*, 2005). $CD4^+$ T cell reduction, specifically in the Th_1 subset, has been seen previously in patients who develop hypoxemia, such as in the case of COVID-19 infection (Wu *et al.*, 2020). This has implications regarding an individual's ability to respond to pathogens, as reduced $CD4^+$ T cell capacity in the adaptive immune response has been associated with the disease outcome (Faist *et al.*, 1986; Infante-Duarte, 1999; Roncati *et al.*, 2020; Gil-Etayo *et al.*, 2021). Furthermore, studies have also found that T cells cultured in hypoxic conditions or collected at high altitude had significantly reduced function and proliferative

response when stimulated with mitogen (PHA) (Klokker *et al.*, 1993; Tingate *et al.*, 1997; Facco *et al.*, 2005; Mishra & Ganju, 2010). However, this is controversial, as conflicting studies have found no change or even increased function and cytokine production following stimulation (Caldwell *et al.*, 2001; Feuerecker *et al.*, 2019).

Interestingly, the immune cell changes to day 1 at high altitude match the immunological cell balance in the native Tibetan population (Bai *et al.*, 2022). While the decrease in the $CD4^+$ population is still under investigation, it has been previously found that $CD8^+$ T cells fare better in low oxygen (Meehan *et al.*, 1988; Caldwell *et al.*, 2001; Facco *et al.*, 2005; Bai *et al.*, 2022). This agrees with our data, as we report no changes to the $CD8^+$ T cell population. Because hypoxia is a prominent feature in inflammation, cancer, and tumor microenvironments, studies have found that hypoxia inhibits $CD4^+$ effector function through immunosuppression via regulatory T cells (Karger *et al.*, 2017). Specifically, HIF-1α stability and function was implicated in the exacerbation of the regulatory T cell suppressive capacity in hypoxia (Ben-Shoshan *et al.*, 2008; Lee *et al.*, 2015). Therefore, the decrease in circulating $CD4^+$ T cells may not be due to T cell exhaustion, but suppression of $CD4^+$ population by regulatory T cells.

We also report other subsets of $CD3^+$ T Cells that were significantly altered by hypoxia exposure, such as $CD3^+$ $CD8^+$ $CD16^+$ (NK T Cells) and $CD3^+$ $CD4^-$ $CD8^-$ $CD16^-$ (DN T Cells) subsets. Interestingly, while NK T cells were also significantly reduced on the first day of altitude, the subset recovered to baseline values by third day of altitude. NK T cells are a unique subset of T lymphocytes that also express NK cell surface markers. Although they are T lymphocytes, they have innate immune response-like qualities that play a role in maintenance of self-tolerance and regulation of autoimmunity (Tupin *et al.*, 2007). NK T cells have also been implicated in their role in the host

defense against viruses, bacteria, and parasites. As such, this initial decrease in this specialized subset of T cells population at altitude is interesting, as it would indicate immunosuppression. Decline in circulating NK T cells has been primarily reported in cancer (Molling *et al.*, 2008). Since NK T cells are a subset of T lymphocytes, this initial decrease may be more reflective of suppression of adaptive immunity, further agreeing with the decrease in $CD4^+$ T cells. On the other hand, NK T cell activation can be conferred through other means beyond T cell receptor activation, such as cytokine-mediated activation and endogenous-antigen mediated activation (Mallevaey *et al.*, 2006; Kronenberg & Gapin, 2007). It is possible that while the acute high-altitude hypoxia exposure led to the initial suppression of this unique population, release of cytokines and endogenous antigens from stressed and damaged cells promoted the recovery of NK T cells. Because of their dual role in both innate and adaptive immunity, NK T cells are in a unique position to mediate activation and bridge both sides of the immune system, and therefore may be impacted by both the innate sensitization to further stimuli, as well as the adaptive immunity suppression. The underlying mechanism behind the decrease in NK T cells after acute high-altitude exposure needs to be further studied, as this may provide insight into specific T cell regulation in response to high altitude hypoxia.

DN T cells were the only $CD3^+$ T cell subset that significantly increased on both the first and third day at altitude. This contradicts previous studies that report no changes in DN T cell subpopulations; however, it is important to note that this study was a long-term chronic hypoxia exposure, spanning over 6 months and only on three participants (Tingate *et al.*, 1997). DN T cells are another rare subset of T cells that play an important role in regulating self-tolerance and immune responses (Chen *et al.*, 2004;

Paul *et al.*, 2015). Much like the other T lymphocyte subsets, stimulation through the T cell receptor (TCR) is necessary for function. However, they do not express any other common T cell co-receptors, such as CD4, CD8, or CD28. Similar to NK T cells, DN T cells play a role in bridging the innate and adaptive immune response in homeostasis as well as under inflammatory conditions (Paul *et al.*, 2014; Paul & Lal, 2017). They are capable of producing cytokines that can promote migration of immune cells, induce lysis via granzyme secretion, induce antibody production in B cells, and present antigens to conventional T cells (Manetti *et al.*, 1993; Paul *et al.*, 2014). Recently, research on this novel Treg cell subset has garnered interest due to their ability to regulate the adaptive immune response by suppressing activated $CD8^+$ T cells, $CD4^+$ T cells, and B cells (Zhang *et al.*, 2000; Young & Zhang, 2002). This substantial increase in DN T cells in response to high altitude further highlights how high altitude may lead to adaptive immunosuppression. Overall, we see an increase or at least a recovery in T lymphocytes that are denoted to be innate-like, as well as no change in $CD8^+$ T cells. The only exception is $CD4^+$ T cells, which remains to be significantly reduced throughout stay at altitude. While our current data does not dive into specific subsets of DN T cells, we can appreciate that these unique cells have complex roles that, following acute high-altitude exposure, may affect their functions and potentially contribute to pathologies, as shifts in immunological cell balance can activate the inflammatory response as well as cause immunosuppression. Altogether, this may indicate that, in response to systemic hypoxia exposure, certain immune cell populations and subsets are capable of adapting to the drop in oxygen tension.

Immune cell populations and AMS correlations

Sojourners to high altitude are at risk of developing high-altitude pathologies, such as AMS, HAPE, HAPH, and HACE. While it remains to be determined if, or how, underlying chronic inflammation may play a part in high-altitude pathologies, we hypothesized that increased pro-inflammatory immune cell phenotypes are a driving force for high-altitude pathologies. Baseline monocyte levels (total, classical, intermediate, non-classical) did not predict AMS severity on either the first or third day at altitude. However, we did determine that participants with increased total monocyte, classical monocytes, and non-classical monocyte populations at altitude had higher AMS scores on average. This agrees with other studies that have also reported higher monocytic count in travelers to high altitude, although the timescale of exposure is unknown (Bhattacharya *et al*., 2021; Wu *et al*., 2023b).

Furthermore, Bhattacharya et al. determined that Tibetans carrying an evolutionarily selected variant had reduced monocytic count and function. This would indicate that a suppressed monocyte count and function is a beneficial adaptation to high-altitude hypoxia. Monocytes from Tibetan high-altitude natives with the beneficial *EGLN1* variant, produced less pro-inflammatory cytokines, and have limited migration when cultured in hypoxic conditions (1% O_2). indicating that this variant plays an immunosuppressive role and increases fitness in the high-altitude environment.

In comparison, travelers who develop high-altitude pathologies, such as AMS and HAPE, display both elevated total and inflammatory monocytes, as well as circulating pro-inflammatory cytokines (Bhattacharya *et al.*, 2021). This further serves as evidence that the initial initiation of the inflammatory response to acute high-altitude hypoxia exposure aims to be an adaptive mechanism, however failure to resolve the

inflammatory response may lead to the development or exacerbation of high-altitude pathologies.

There are only a few studies that analyze B cells at high altitude. In our study, we report that participants with no AMS had a higher baseline B cell in total WBCs compared to AMS groups (**Figure 3.9**). This could potentially indicate a protective role of B cells on an acute hypoxia exposure scale. While there are multiple publications that report both enhanced as well as suppressed antibody production in animal and human models at altitude (Tengerdy & Kramer, 1968; Chohan *et al.*, 1975; Singh *et al.*, 1977; Meehan, 1987), this may be indicative of the benefit of functional antibody production. Recently, research on the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) at altitude has found elevated and sustained humoral immune response (Tomas-Grau *et al.*, 2021). This suggests that participants with higher baseline B cells may have a more protective phenotype against the development of high-altitude illness, as well as protection against exacerbated response to subsequent infections at altitude. Further research is necessary to elucidate antibody production and function at high altitude, and if these factors promote or suppress high-altitude pathology development and/or exacerbation.

3.6 CONCLUSION

In conclusion, we demonstrate that acute exposure to high-altitude hypoxia significantly alters both innate and adaptive immune cell populations. Specifically, our analysis reveals evidence of innate immune sensitization, most notably in monocyte subsets, as well as adaptive immune suppression, particularly $CD4^+$ T cells. In addition, we found changes in DN T cells as well as NK T cells, which may impact immune suppression. While our data suggests acute high-altitude exposure initially promotes a

pro-inflammatory phenotype that may transition to an anti-inflammatory phenotype over the course of acclimatization, further studies are necessary to elucidate underlying mechanisms behind hypoxia-induced inflammation and its contribution to high-altitude illnesses. Future studies should expand to encompass analyzing innate and adaptive immune cells in chronic exposure. In particular, comparing native highlanders that are well-adapted, such as the Tibetans, or maladaptive, such as the Andeans, might expand our understanding of the time domain response of hypoxia-induced inflammation. Additionally, characterizing additional immune subsets affected by hypoxemia will provide valuable insights into how immune cell population shifts modulate both the inflammatory and immune response in critical and chronic illnesses, such as acute respiratory disease and COVID19.

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Disclosures

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

Author Contributions

KP and ECH conceived and designed the research. KP, SF, and ECH assisted in sample collection. KP, SS, and ECH analyzed data, interpreted the results of experiments, and prepared figures. KP drafted the manuscript. KP and ECH edited and revised the manuscript.

Figures

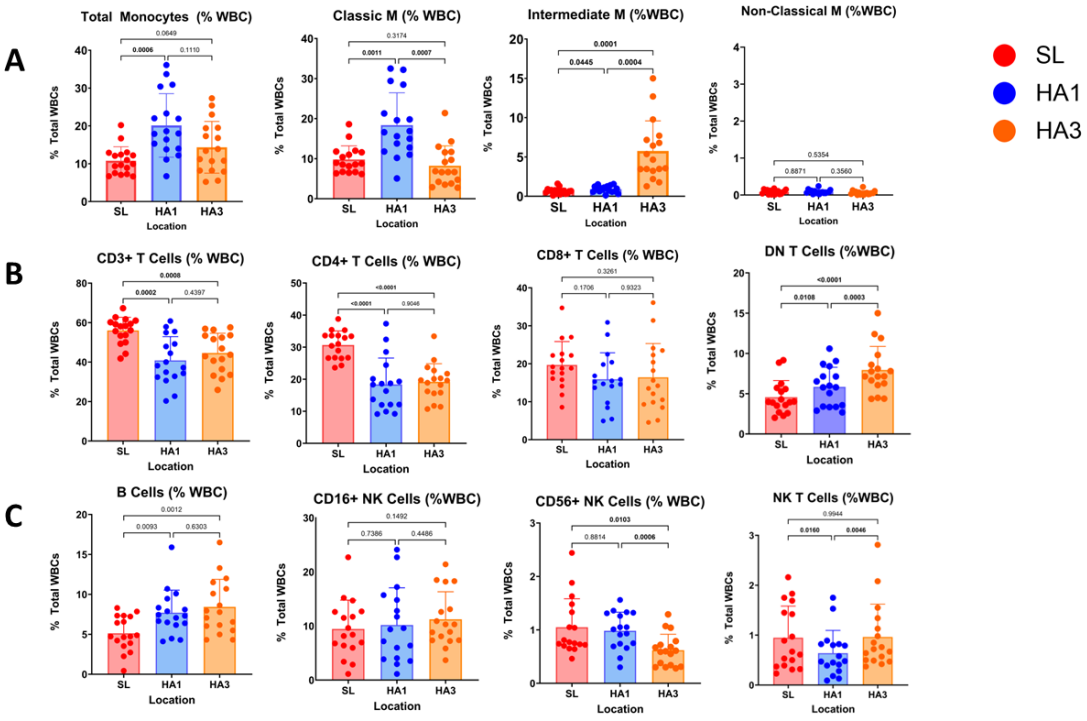

Figure 3.1. Immune population analysis during 3 days of acute high-altitude exposure. Quantification of significantly altered immune populations from analyzed flow cytometry data from total white blood cells (WBCs). PBMCs were collected at sea level (SL), as well as one (HA1) and three days (HA3) at high altitude. **(A)** Total monocyte and monocyte subsets (classical, intermediate, non-classical). **(B)** Total CD3+ T cells and double negative (DN) T cells. **(C)** B cells, CD16+ and CD56+ NK cells, and CD3+ CD16+ (NK) T cells. Graphs are plotted as mean and error bars as standard deviation.

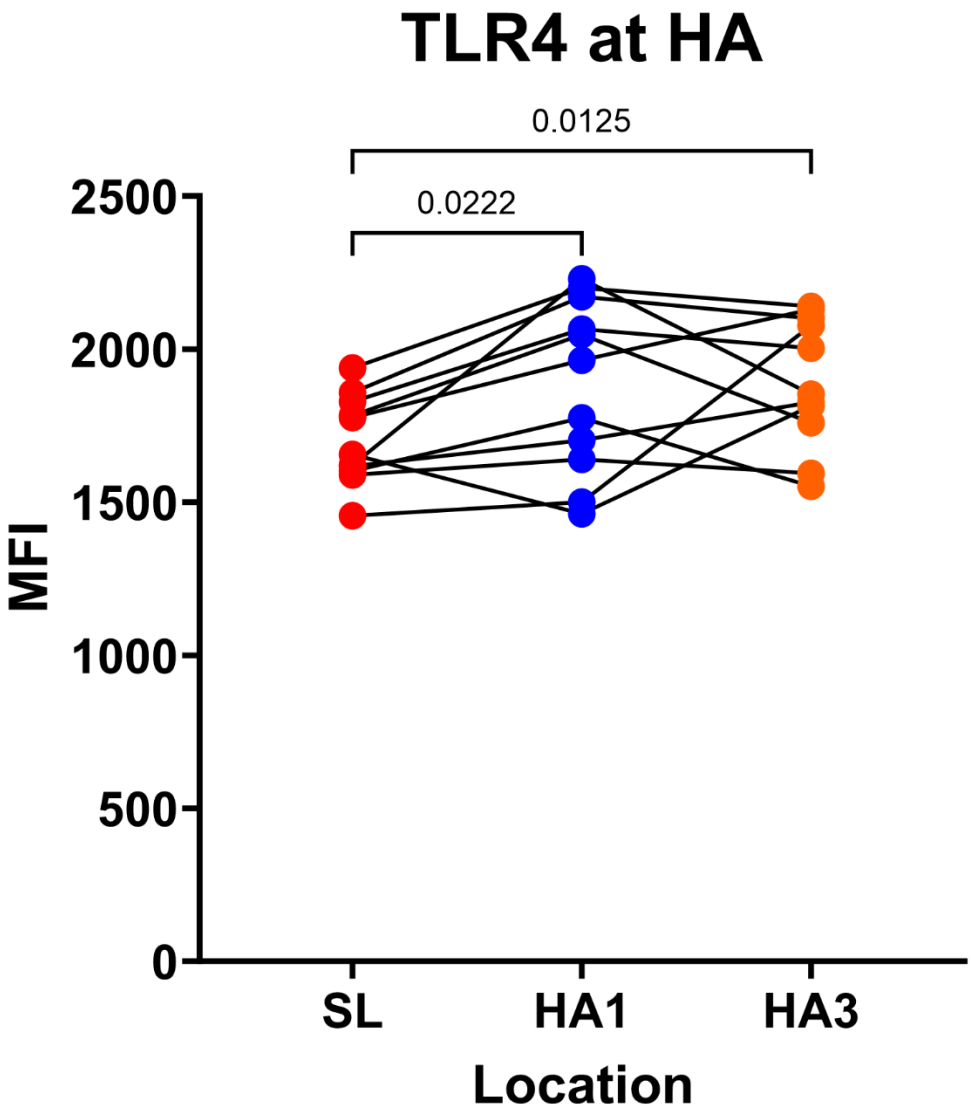

Figure 3.2. TLR4 surface expression on PBMCs throughout 3 days of acute high-altitude exposure. Mean fluorescence intensity (MFI) quantification of TLR4 surface expression from analyzed flow cytometry data from live PBMCs collected at sea level (SL), first day (HA1) and third day (HA3) at altitude.

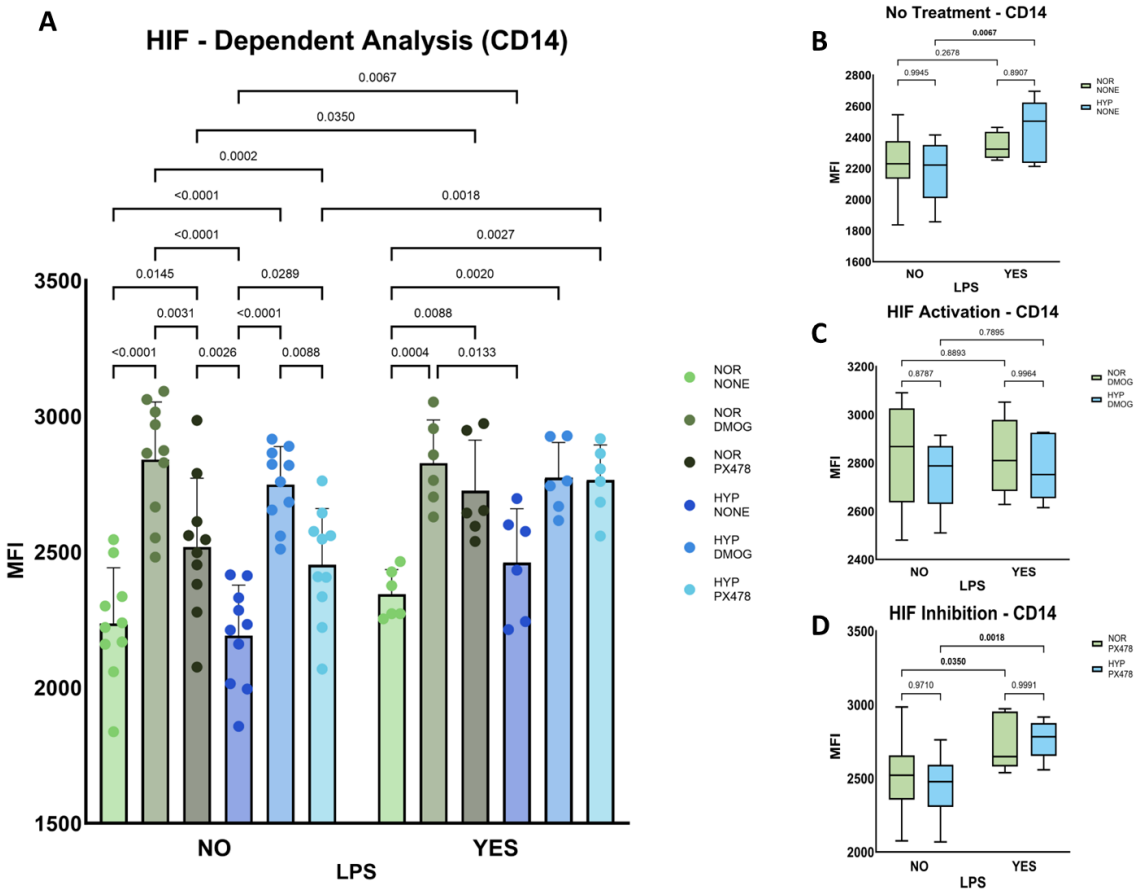

Figure 3.3. HIF stability affects CD14 surface expression on live PBMCs. PBMC cultures were incubated in specific conditions for 6 hours at 37°C. **(A)** Overview of all conditions and treatments. **(B)** Control cultures; normoxia vs hypoxia **(C)** DMOG (HIF activator) cultures; normoxia vs hypoxia **(D)** PX478 (HIF inhibitor) cultures; normoxia vs hypoxia. Graphs are plotted as mean and error bars as standard deviation.

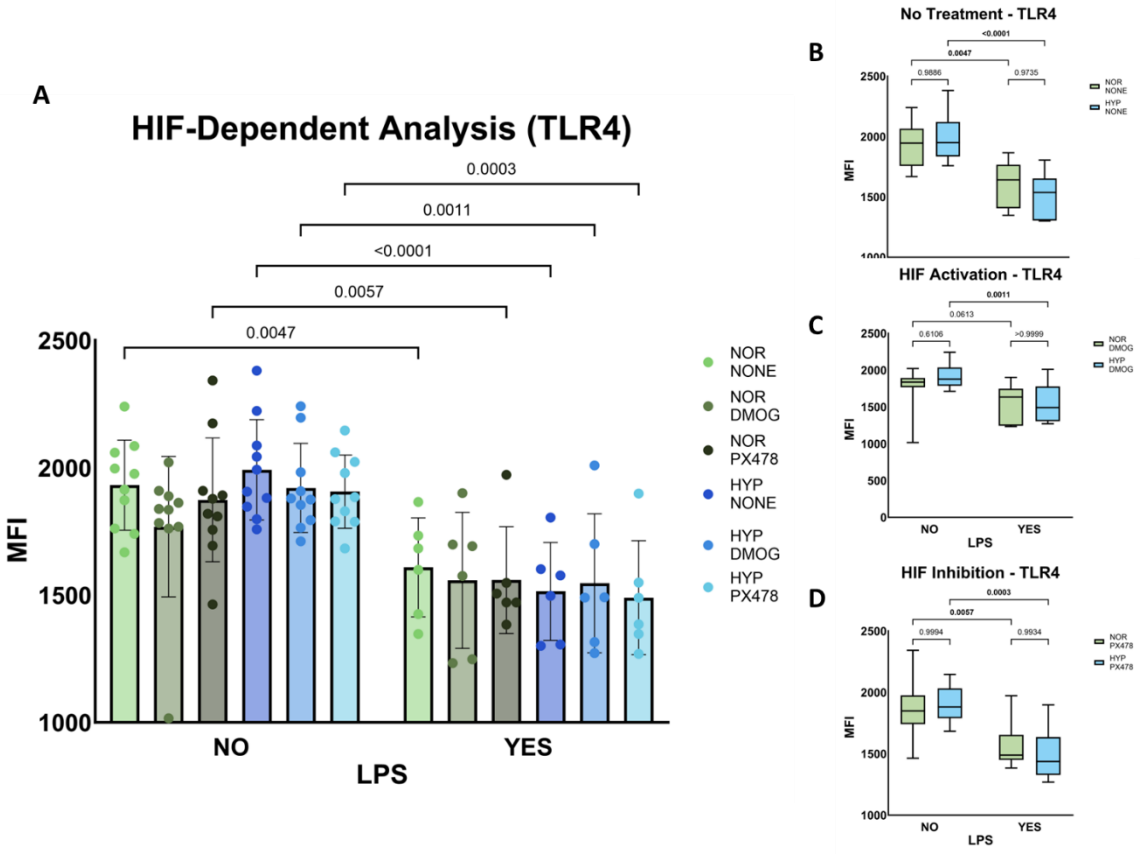

Figure 3.4. HIF stability does not affect TLR4 expression on live PBMCs. PBMC cultures were incubated in specific conditions for 6 hours at 37°C. **(A)** Overview of all conditions and treatments. **(B)** Control cultures; normoxia vs hypoxia **(C)** DMOG (HIF activator) cultures; normoxia vs hypoxia **(D)** PX478 (HIF inhibitor) cultures; normoxia vs hypoxia. Graphs are plotted as mean and error bars as standard deviation.

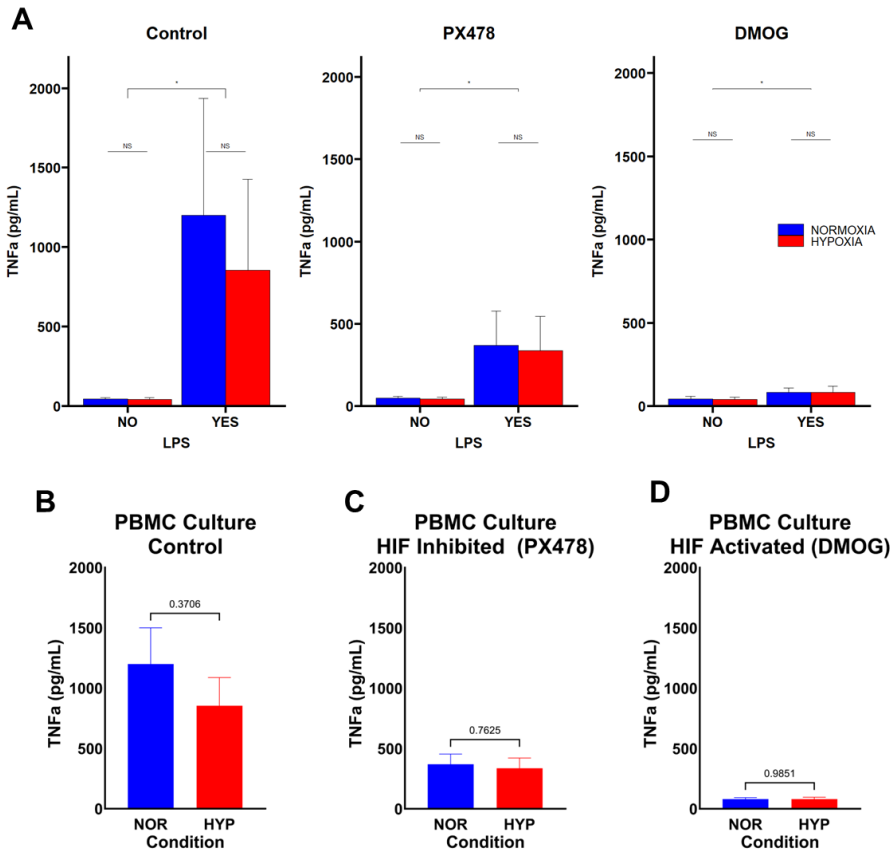

Figure 3.5. TNFα cytokine production from stimulated PBMC cultures. PBMC cultures were cultured in normoxic or hypoxic conditions (1% O_2) for 6 hours. **(A)** PBMCs were cultured with **(B)** Control PBS + LPS, **(C)** PX478 (HIF Inhibitor) + LPS, **(D)** DMOG (HIF Activator) + LPS. Graphs are plotted as mean and error bars as standard deviation.

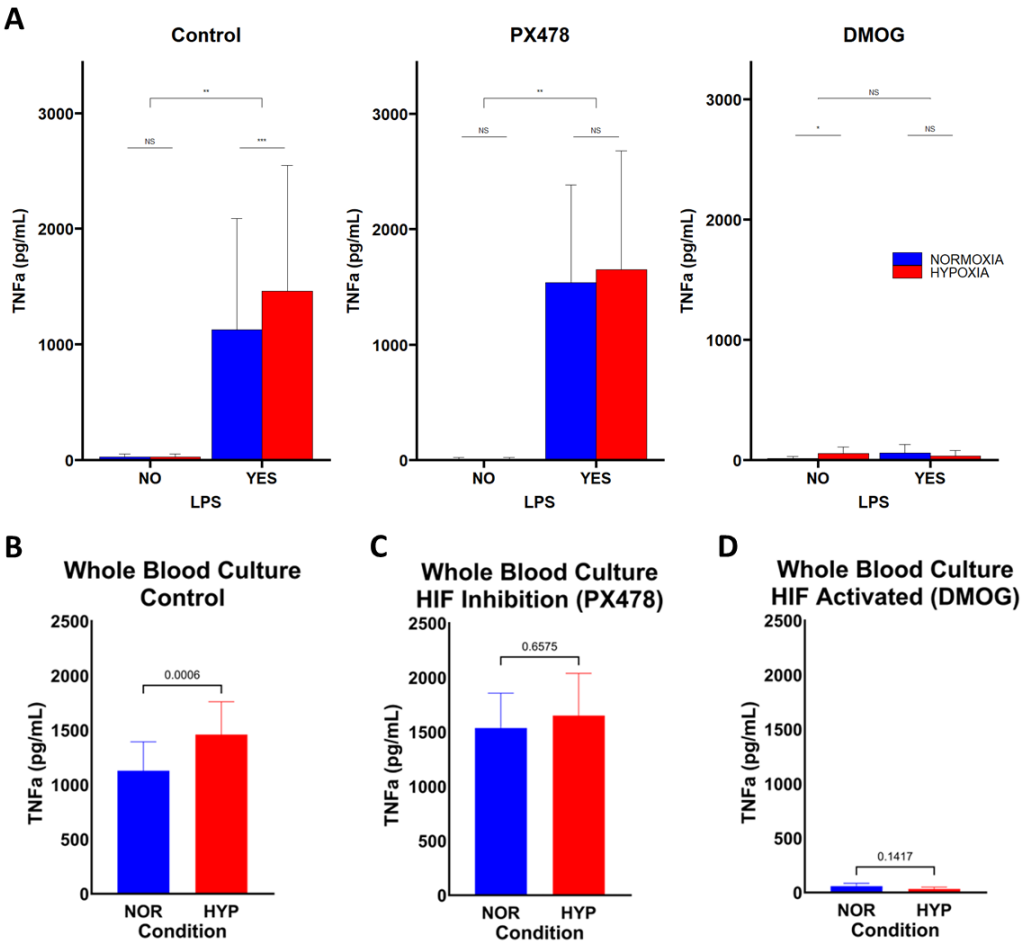

Figure 3.6. TNFα cytokine production from stimulated whole blood cultures. Whole blood cultures were stimulated with 10ug/mL LPS and cultured in either normoxic or hypoxic (1% O_2) conditions for 24 hours. **(A)** Whole blood was cultured with **(B)** Control PBS + LPS, **(C)** PX478 (HIF Inhibitor) + LPS, **(D)** DMOG (HIF Activator) + LPS. Graphs are plotted as mean and error bars as standard deviation.

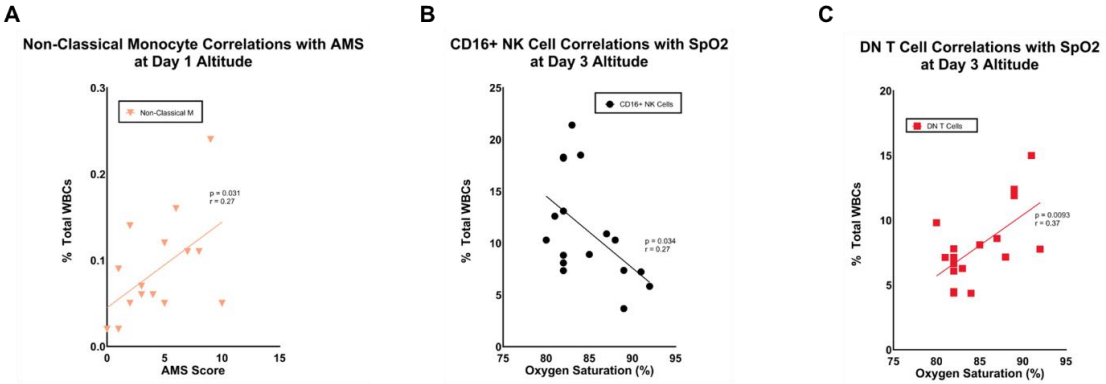

Figure 3.7. Significant correlations with AMS or SpO_2 on either the first or third day at altitude. Correlation analyses between immune cell populations and oxygen saturation (SpO_2) and Acute Mountain Sickness scores (AMS). **(A)** Correlation between non-classical monocyte populations and AMS on day 1 at altitude. **(B)** Correlation between $CD16^+$ NK cell populations and SpO_2 on day 3 at altitude. **(C)** Correlation between DN T cell populations and SpO_2 on day 3 at altitude.

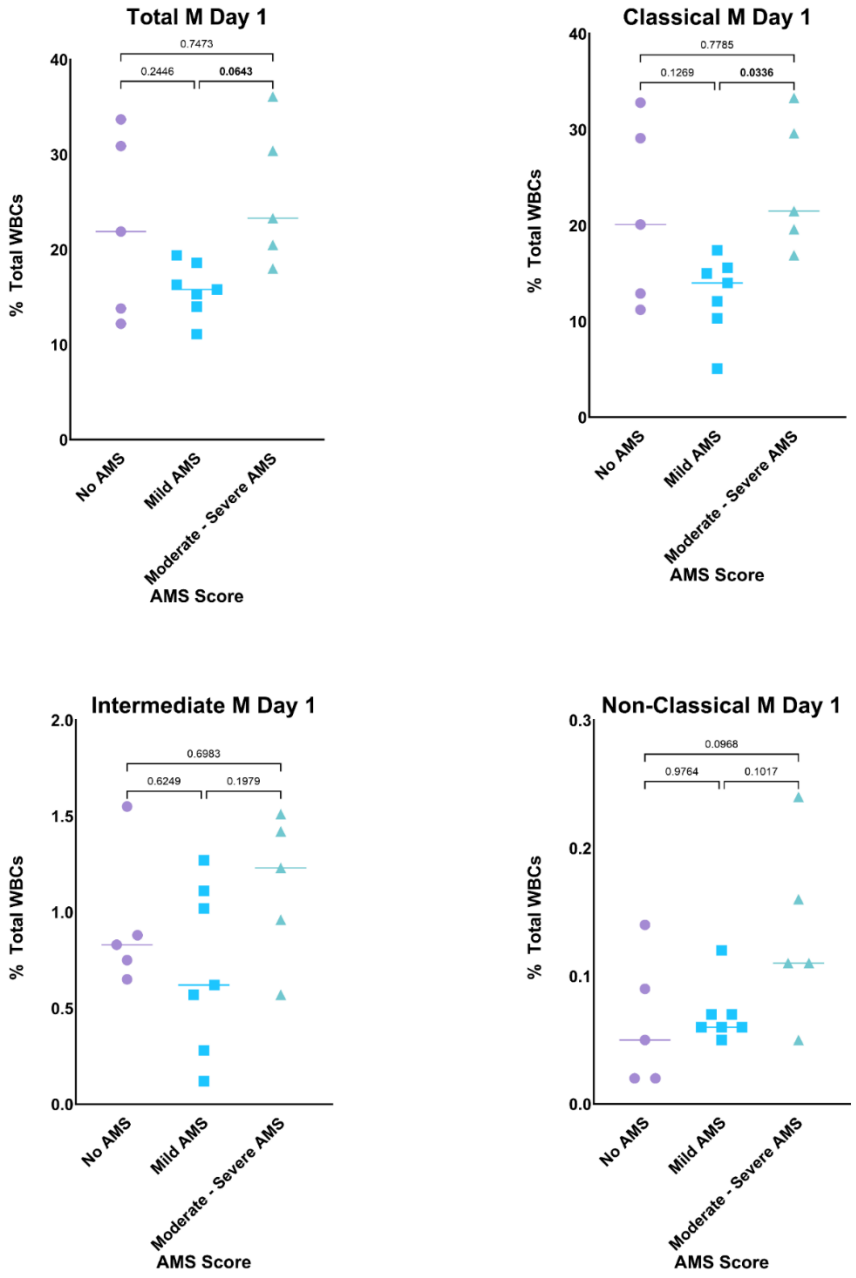

Figure 3.8. Monocyte subpopulations in participants grouped by AMS severity. Monocyte populations grouped by AMS severity. (A) Total monocyte (% WBC). **(B)** Classical monocytes (% WBC). **(C)** Intermediate Monocytes (% WBCs) **(D)** Non-classical monocytes (% WBCs).

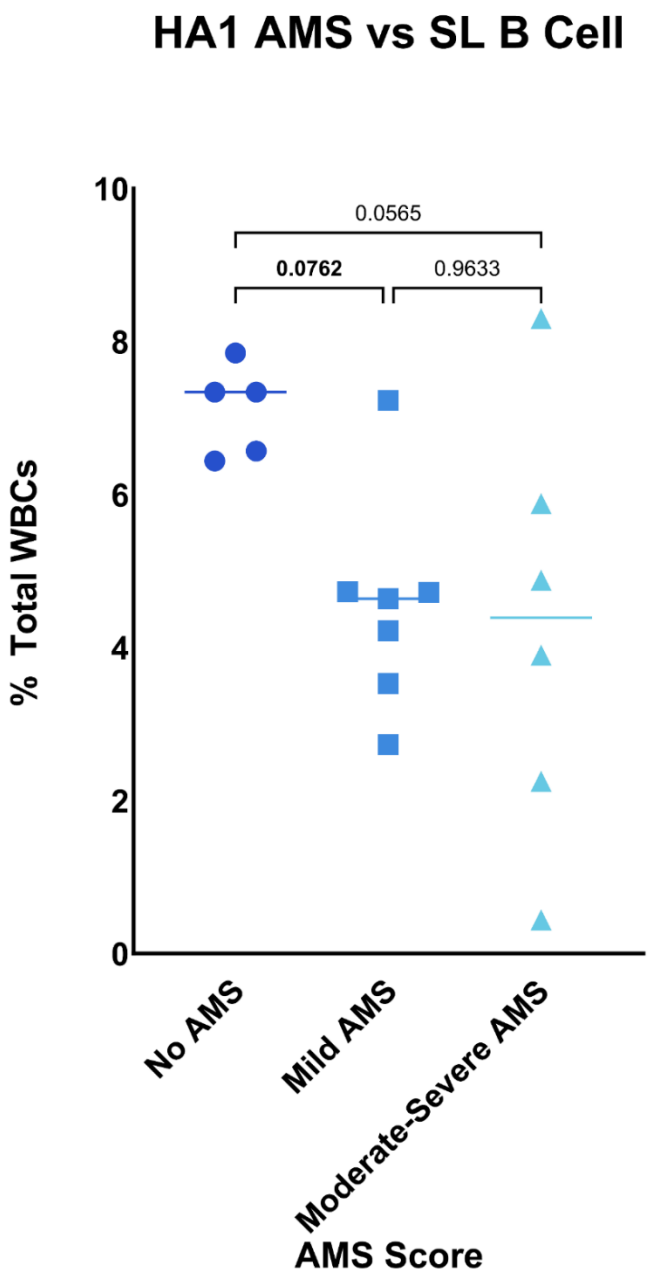

Figure 3.9. Association between baseline B cell population with AMS scores on first day at altitude (HA1). B cell concentration of total WBCs by AMS severity. AMS severity: No AMS = 0-3; Mild = 3-5; Moderate - Severe = 6+.

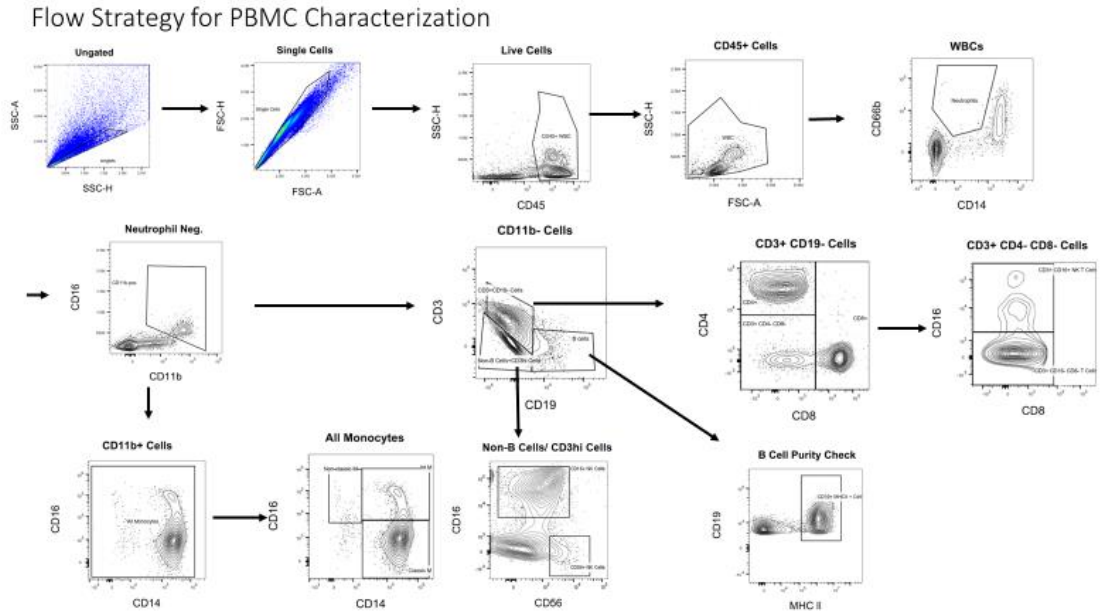

Figure 3.S1. Full gating strategy for high altitude PBMCs. Concatenated sea-level baseline group for flow cytometry PBMC characterization.

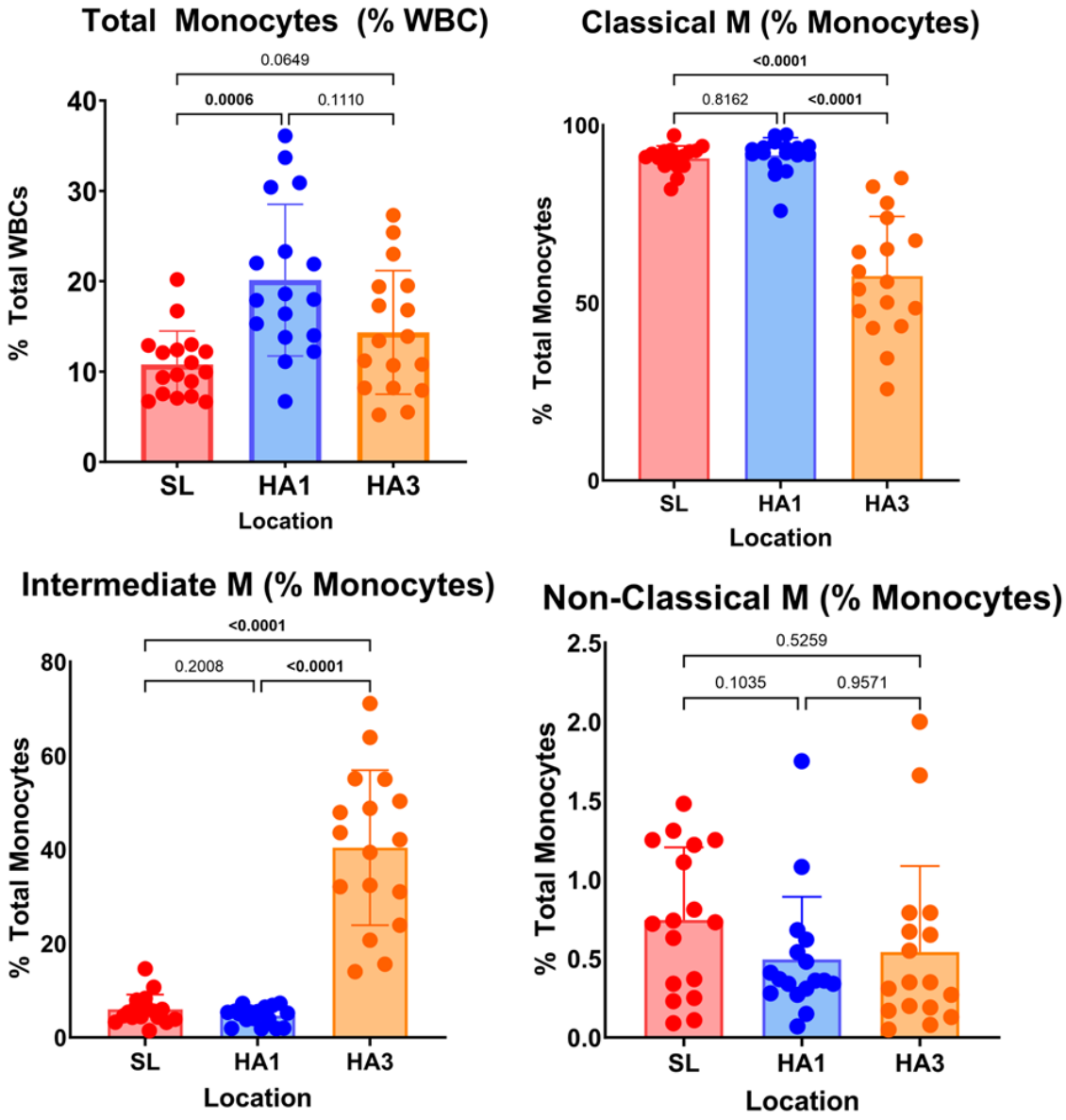

Figure 3.S2. Monocyte subset immune population analysis during 3 days of acute high-altitude exposure. Quantification of monocyte subpopulations from total monocyte (%).

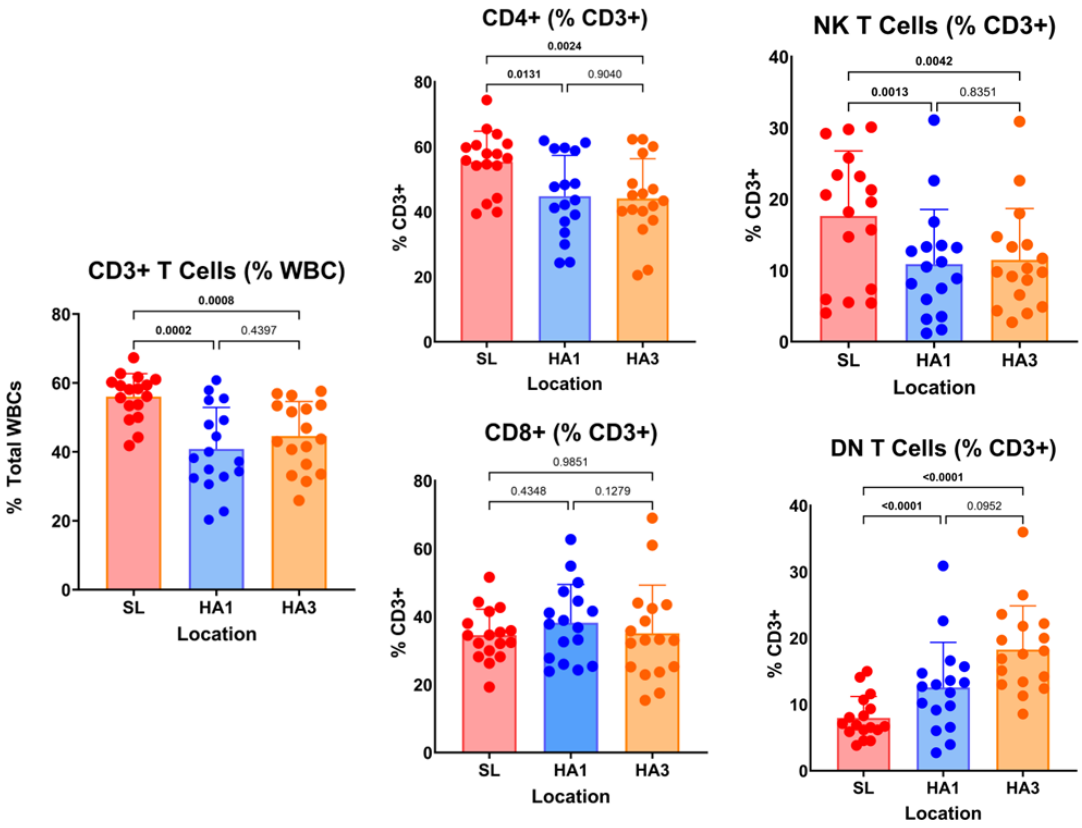

Figure 3.S3. T cell subset immune population analysis during 3 days of acute high-altitude exposure. Quantification of T cell subpopulations from total CD3+ (%).

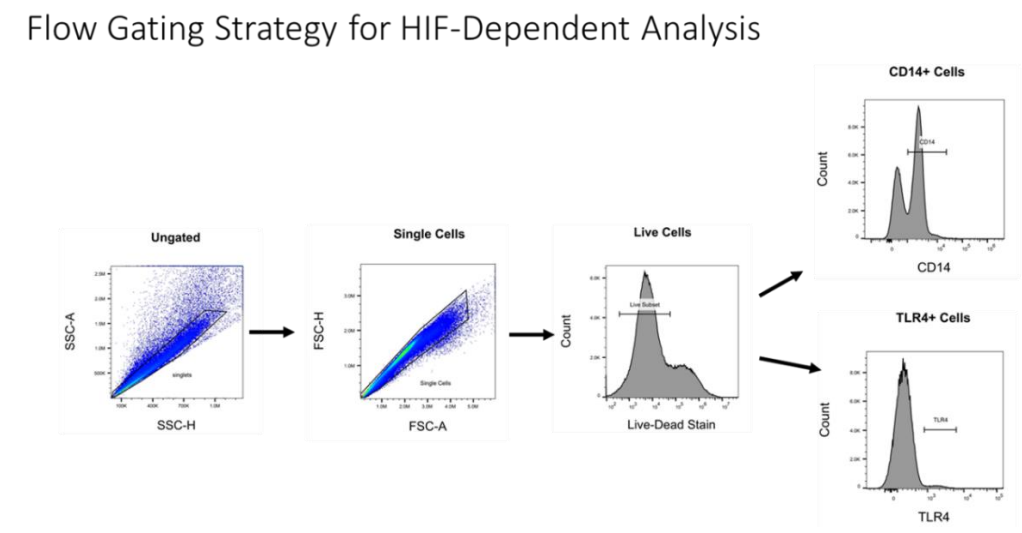

Figure 3.S4. Full gating strategy for HIF-dependent analysis. Concatenated control group used as baseline.

Table 3.1. Physiological measures at baseline and over three days at high altitude.

Variable	SL	HA 1	HA 2	HA 3	ANOVA P
P_{sys}	121 ± 7.1	126 ± 9.5	128 ± 13	126 ± 11	0.177
P_{dia}	79 ± 5.0	85 ± 7.1	85 ± 8.4	85 ± 8.7	0.059
HR	74 ± 8.4	91 ± 13*	90 ± 15*	100 ± 16***	<0.001
SpO_2	95 ± 1.7	84 ± 4.3***	84 ± 3.2***	84 ± 4.1***	<0.001
AMS	0.5 ± 0.6	4.3 ± 2.7***	4.0 ± 2.4**	2.6 ± 2.5*	<0.001

Variable units: P_{sys} and P_{dia} (mmHg); HR (bpm); SpO2 (%). Overall p-values for repeated measures ANOVA are provided. Asterisks indicate significant differences from SL at p<0.05 (*), p<0.01 (**), and p<0.001 (***) levels via post-hoc pairwise comparisons with Bonferroni adjusted p-values.

Table 3.2. Correlation analyses of all immune cell populations and AMS scores and oxygen saturation (SpO_2) on day 1 and 3 at altitude.

Oxygen Saturation (SpO_2) - HA1

Immune Population	r	p-value
Total Monocyte	0.24	0.37
Classical Monocyte	0.30	0.25
Intermediate Monocyte	-0.26	0.33
Non-Classical Monocyte	-0.10	0.72
CD4 T Cells	-0.10	0.71
CD8 T cells	-0.30	0.26
B Cells	-0.13	0.62
CD16+ NK Cells	-0.23	0.38
CD56+ NK Cells	0.04	0.87
DN T Cells	0.10	0.71
NK T Cells	0.32	0.22

Oxygen Saturation (SpO_2) - HA3

Immune Population	r	p-value
Total Monocyte	0.23	0.38
Classical Monocyte	0.05	0.85
Intermediate Monocyte	-0.20	0.44
Non-Classical Monocyte	0.06	0.82
CD4 T Cells	0.27	0.29
CD8 T cells	0.04	0.88
B Cells	0.05	0.85
CD16+ NK Cells	-0.52	0.03
CD56+ NK Cells	0.23	0.37
DN T Cells	0.61	0.0093
NK T Cells	-0.26	0.31

AMS Scores - HA1

Immune Population	r	p-value
Total Monocyte	0.09	0.73
Classical Monocyte	0.06	0.81
Intermediate Monocyte	0.10	0.71
Non-Classical Monocyte	0.52	0.03
CD4 T Cells	0.08	0.77
CD8 T cells	-0.09	0.74
B Cells	-0.08	0.76
CD16+ NK Cells	0.35	0.17
CD56+ NK Cells	0.008	0.98
DN T Cells	-0.40	0.11
NK T Cells	-0.14	0.59

AMS Scores - HA3		
Immune Population	**r**	**p-value**
Total Monocyte	0.05	0.86
Classical Monocyte	0.02	0.94
Intermediate Monocyte	-0.17	0.51
Non-Classical Monocyte	-0.22	0.40
CD4 T Cells	0.16	0.53
CD8 T cells	-0.13	0.62
B Cells	-0.10	0.69
CD16+ NK Cells	-0.20	0.43
CD56+ NK Cells	-0.27	0.29
DN T Cells	0.18	0.50
NK T Cells	-0.035	0.89

Table 3.3. Immune population concentrations (% WBCs) at altitude by AMS severity and altitude (HA1 and HA3).

AMS Score - HA1

Immune Population	No AMS	Mild AMS	Moderate- Severe AMS	ANOVA P
Total Monocyte	23 ± 9.7	16 ± 2.8	26 ± 7.5	0.065
Classical Monocyte	21 ± 9.6	13 ± 4.1	24 ± 7	0.03*
Intermediate Monocyte	0.93 ± 0.36	0.72 ± 0.43	1.1 ± 0.38	0.22
Non-Classical Monocyte	0.06 ± 0.05	0.07 ± 0.02	0.13 ± 0.07	0.069
CD4 T Cells	17 ± 5.9	23 ± 9.8	13 ± 4.8	0.12
CD8 T cells	14 ± 6.5	20 ± 6.5	12 ± 5.9	0.10
B Cells	7.5 ± 2.2	8.7 ± 3.8	6.5 ± 1.3	0.46
CD16+ NK Cells	10 ± 8.3	7.6 ± 5.8	14 ± 7.0	0.36
CD56+ NK Cells	0.99 ± 0.4	1.1 ± 0.36	1.0 ± 0.42	0.90
DN T Cells	6.9 ± 2.9	6.0 ± 2.2	4.5 ± 1.9	0.29
NK T Cells	0.82 ± 0.48	0.47 ± 0.32	0.70 ± 0.59	0.41

AMS Score - HA3

Immune Population	No AMS	Mild AMS	Moderate- Severe AMS	ANOVA P
Total Monocyte	14 ± 6.4	14 ±7.2	15 ± 10	0.99
Classical Monocyte	8.1 ± 3.7	7.5 ± 4.4	10 ± 9.9	0.73
Intermediate Monocyte	5.7 ± 4.3	6.6 ± 4.1	4.2 ± 2.4	0.70
Non-Classical Monocyte	0.06 ± 0.06	0.07 ± 0.04	0.05 ± 0.05	0.83
CD4 T Cells	19 ± 7.0	19 ± 5.5	19 ± 3.1	0.99
CD8 T cells	18 ± 8.8	15 ± 10	14 ± 9.2	0.72
B Cells	8.7 ± 4.0	8.4 ± 3.5	7.9 ± 2.6	0.95
CD16+ NK Cells	11 ± 6.0	12 ± 4.5	8.6 ± 3.5	0.62
CD56+ NK Cells	0.67 ± 0.37	0.59 ± 0.27	0.54 ± 0.19	0.78
DN T Cells	8.0 ± 3.8	7.4 ± 1.7	8.9 ± 2.6	0.78
NK T Cells	0.97 ± 0.78	1.0 ± 0.67	0.85 ± 0.44	0.95

Table 3.4. Baseline (SL) immune populations (% WBCs) grouped by AMS scores on day 1 and 3 at altitude.

SL Immune Populations versus AMS Score - HA1

Immune Population	No AMS	Mild AMS	Moderate-Severe AMS	ANOVA P
Total Monocyte	13 ± 5.2	10 ± 2.6	11 ± 4.1	0.40
Classical Monocyte	12 ± 5.1	9.3 ± 2.3	9.5 ± 2.2	0.24
Intermediate Monocyte	0.72 ± 0.15	0.63 ± 0.34	1.4 ± 1.9	0.47
Non-Classical Monocyte	0.05 ± 0.05	0.1 ± 0.06	0.1 ± 0.06	0.32
CD4 T Cells	29 ± 3.0	30.5 ± 7.0	29 ± 5.1	0.84
CD8 T cells	18 ± 5.0	25 ± 9.5	19 ± 7.3	0.26
B Cells	7.1 ± 0.59	4.5 ± 1.4	4.3 ± 2.8	0.045*
CD16+ NK Cells	9.5 ± 4.2	7.7 ± 4.6	13 ± 6.6	0.28
CD56+ NK Cells	1.2 ± 0.78	0.83 ± 0.15	1.2 ± 0.55	0.31
DN T Cells	5.6 ± 2.1	4.1 ± 1.2	4.7 ± 2.9	0.54
NK T Cells	1.3 ± 0.67	0.81 ± 0.67	1.0 ± 0.70	0.54

SL Immune Populations versus AMS Score - HA3

Immune Population	No AMS	Mild AMS	Moderate-Severe AMS	ANOVA P
Total Monocyte	11 ± 3.9	12 ± 4.2	7.4 ± 0.2	0.34
Classical Monocyte	9.9 ± 3.0	12 ± 3.8	6.9 ± 0.15	0.22
Intermediate Monocyte	1.2 ± 1.6	0.67 ± 0.44	0.28 ± 0.050	0.45
Non-Classical Monocyte	0.10 ± 0.06	0.08 ± 0.06	0.03 ± 0.007	0.25
CD4 T Cells	27 ± 5.3	32 ± 4.2	33 ± 1.5	0.051*
CD8 T cells	23 ± 9.7	19 ± 6.3	18 ± 4.5	0.51
B Cells	5.2 ± 1.6	5.6 ± 2.9	4.1 ± 2.2	0.65
CD16+ NK Cells	12 ± 6.3	7.6 ± 3.0	7.6 ± 4.7	0.23
CD56+ NK Cells	1.0 ± 0.37	1.0 ± 0.54	1.3 ± 1.0	0.80
DN T Cells	4.9 ± 2.2	4.7 ± 2.4	4.3 ± 1.8	0.92
NK T Cells	0.98 ± 0.63	0.82 ± 0.65	1.45 ± 0.86	0.43

Chapter Four

Time domains of inflammatory profiles during high-altitude acclimatization and adaptation

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A version of this chapter will be submitted for publication

4.1 ABSTRACT

High-altitude hypoxia is a physiologically stressful environment due to low oxygen availability, temperatures, humidity, and other factors. These stressors can induce an inflammatory response that can modulate immune metabolism and function. While the molecular pathways that regulate hypoxia and inflammatory responses have significant interplay, the duration of systemic hypoxia exposure may drive altered inflammatory profiles as adaptation to this environment occurs. Whether the inflammatory response to hypoxia is maintained, or blunted over long exposure periods, and if this is an adaptive or maladaptive response, remains unknown. Previous work indicates that acute high-altitude exposure increase expression of several pro-inflammatory cytokines and alarmins, but there is a significant gap in knowledge regarding how the inflammatory profile modulates the immune response, and if chronic hypoxia exposure promotes or impede a pro-inflammatory profile over longer time periods. Furthermore, while native high-altitude populations live in similar hypoxic environments, each have their own distinct adaptations. Therefore, questions remain regarding how the inflammatory profiles in each of these populations differ, and if they contribute to, or protect against, high-altitude illnesses. In the present study, we aim to examine the inflammatory profile across several time domains of high-altitude acclimatization and adaptation. We also examine the inflammatory profile as a potential biomarker of Acute and Chronic Mountain Sickness (AMS, CMS). We examined plasma samples for cytokines essential to the immune response from lowlanders who traveled to high altitude over 3 days (acute time domain), as well as a native Andean population who are known to have a high prevalence of CMS (chronic time domain). The data revealed that on an acute timescale, monocyte chemoattractant protein – 1 (MCP-1) was significantly elevated at

high altitude, whereas on a chronic timescale in the Andeans, IP-10 was found to be significantly elevated. Both of these markers indicate vascular endothelium stress. Furthermore, Andeans classified with excessive erythropoiesis (EE; hematocrit > 63%) had significantly higher IP-10 compared to healthy Andeans, while CMS classification showed no distinction. These results suggest that while both acute and chronic hypoxia exposure promotes a robust inflammatory profile, the differences between these time domains may be part of an underlying mechanism in high-altitude illness pathogenesis and can provide insight into how the immune response may be modulated over long-term hypoxic exposure.

4.2 INTRODUCTION

Maintaining oxygen homeostasis is paramount to survival in high-altitude hypoxic environments. When tissue oxygenation is challenged, rapid physiological mechanisms, such as increased ventilation and red blood cell production, occur over minutes to days to promote increased oxygen delivery (Beall, 2006; Scheinfeldt *et al.*, 2012; Simonson, 2015; Moore, 2017). Over longer time periods of adaptation to high altitude, additional changes occur including shifts in metabolic pathways and increases in capillary density (Yu *et al.*, 2022). However, while the physiological adaptations that occur during acclimatization to high altitude have been well established, questions remain regarding the underlying molecular mechanisms that drive these processes.

The molecular response to hypoxia is primarily controlled by the hypoxia-inducible factor (HIF), the master transcription factor of the hypoxia response pathway. Because oxygen sensing is crucial for survival in all vertebrates, this hypoxia response pathway is highly evolutionary conserved. In addition, it shares significant crosstalk with other molecular pathways to coordinate the response to changes in oxygen availability,

such as the inflammatory pathway controlled by NF-kB. As a result, NF-kB can lead to activation of a wide variety of pro-inflammatory, apoptotic, and oncogenic genes that function to promote metabolic adaptation to hypoxic stress (Pahl, 1999). Together, these pathways play an important role in facilitating and promoting hypoxia adaptation, particularly in the immune system response to insults and injury. In support of this interaction, new evidence indicates that the hypoxemic challenge at high altitude affects the immune system and inflammatory responses. Immune populations of both innate and adaptive immune systems encounter a wide range of oxygen tensions throughout development and circulation, and exposure to systemic acute or chronic hypoxia may alter their specific metabolic function in vastly different ways, such as altered cytokine production and immune phenotype.

While limited, previous studies have examined the impact of high altitude on immune adaptation and tolerance. These works indicates that both the inflammatory profile, as well as several innate and adaptive immune cell populations, are affected (Klokker *et al.*, 1993; Facco *et al.*, 2005; Mishra & Ganju, 2010; Mishra *et al.*, 2018; Feuerecker *et al.*, 2019). For example, studies agree that $CD4^+$ T cell populations are significantly reduced at altitude, while $CD8^+$ T cell concentration remains unaffected. Monocyte subpopulations are also altered following high-altitude exposure, specifically showing increased classical monocytes, as well as subset shifts towards a more anti-inflammatory phenotype with longer hypoxia exposure durations. Hypoxia exposure times in these studies range from hours to months, and as such, this work provides valuable insight into the expected inflammatory response to high altitude over an acute timeframe. More recently, other studies have examined how chronic lifelong hypoxia exposure impacts immune cell populations and function (Bhattacharya *et al*., 2021; Bai

et al., 2022; Mirchandani *et al*., 2022; Wu *et al*., 2023b). However, the current state of the field has focused on characterizing the immunological balance in native highlanders, as these populations have unique evolutionary adaptations populations (Beall, 2006, 2007; Jeong *et al*., 2014; Simonson, 2015). The underlying mechanisms that drive the shift from an acute to a chronic time domain inflammatory profile, as well as determining if these responses provide an adaptive benefit, remain relatively unexplored. When considering if the unique adaptations of each native group are beneficial or maladaptive, potential links between the inflammatory and immune profile with high-altitude illnesses (HAIs) severity may provide insight into HAI pathogenesis. This is particularly important when considering the prevalence of Chronic Mountain Sickness (CMS), a progressive and fatal disease that develops in natives or lifelong altitude residents above 2,500m (Monge, 1943; Simonson, 2015; Villafuerte & Corante, 2016). While lifelong chronic hypoxia exposure increases the risk of CMS, the Andean population has a significantly higher CMS prevalence than any other native highlander group (Monge, 1943; Simonson, 2015). What drives this difference between other highlander groups, as well as what are the differences between those who do and do not develop CMS in Andean highlanders, remains to be a significant gap in our knowledge.

In the current study, we investigate the changes in the inflammatory profile on both an acute and chronic timescale by analyzing circulating cytokine expression in acclimatizing lowlanders at high altitude, as well as in high-altitude native Andeans with and without CMS. Furthermore, as we have previously seen shifts in both the innate and adaptive immune cell populations, we aim to further characterize how immune cells are sensitized to high-altitude hypoxia exposure. We utilized multiplex assays and enzyme-linked immunosorbent assays (ELISA) to characterize circulating cytokines in plasma,

and if peripheral blood mononuclear cells (PBMCs) collected at high altitude have altered immune responses to inflammatory stimuli. We hypothesized that on an acute timescale, PBMCs are sensitized to produce increased pro-inflammatory cytokines in response to toll-like receptor stimulation, but that this response is dampened following acclimatization. When considering the chronic timescale, we hypothesized that Andeans with CMS have an inflammatory profile that resembles the first day of acute high-altitude exposure in lowlanders, while healthy Andeans are more comparable to an acclimatized group and sustain higher levels of systemic inflammation. We predict that maintenance of systemic inflammation as a result of chronic hypoxemia is a contributor to the development of CMS, as well as a potential effect of elevated blood viscosity in these patients.

4.3 METHODS

Sojourners to High Altitude: White Mountain 2022 Field Expedition

Ethical approval

This study was approved by the University of California, Riverside Clinical Institutional Review Board (HS 22-088). All participants were informed of the study's purpose and risks. Participants provided written informed consent in their native language (English). The work was conducted in accordance with the *Declaration of Helsinki*, except for registration in a database.

Participants

The study included 20 healthy participants (N=7 women, 13 men) between 19 and 35 years of age. Participants were recruited by word of mouth and flyers on the UC Riverside campus. All participants reported no known history of cardiopulmonary disease or sleep disturbances, including obstructive sleep apnea, and displayed no

abnormal findings on electrocardiogram (ECG) or pulmonary function testing. Mean age was 25 ± 7 years for women and 26 ± 6 years for men and BMI was 30 ± 5.4 kg/m^2 for women and 31 ± 5.3 kg/m^2 for men. Exclusion criteria included travel above 8,000 feet within one month of the first measurements, a previous history of high-altitude pulmonary or cerebral edema, smoking, and pregnancy.

Experimental design and physiological measures

In the two weeks prior to ascent to high altitude, participants completed initial screening for eligibility at UC Riverside, at approximately 400 m elevation (Riverside, CA, USA). Demographic information including age, height, weight, and blood pressure were collected. Participants also answered questions about their ancestral background (to determine presence of high-altitude ancestry) and medical history including current medications. Participants then completed pulmonary function testing and ECG to verify absence of lung or heart disease.

Participants returned to UC Riverside in the early morning on the day of ascent. Baseline (sea-level, SL) physiological measures were collected at this time, including blood pressure, pulse oximetry (SpO_2), heart rate, and AMS scores via the 2018 Lake Louise scoring criteria with an experimenter asking participants each question (Roach *et al.*, 2018). Fasting blood samples were then collected via standard venipuncture procedures. Breakfast was provided to participants following blood sampling, prior to travel.

The group then traveled by car to Barcroft Station (3800 m elevation) in the White Mountain Research Center (Bishop, CA, USA) over a period of approximately 6.5 hours. At the field station, fasting blood samples and morning measurements were collected each day within 1 hour of waking and before 9 am to keep timing consistent

with sea level measures. Plasma and peripheral blood mononuclear cells (PBMCs) were collected from fasting blood samples. Additional information regarding PBMC processing is found in the 'Stimulation Assay' Method section. Due to logistical constraints, buffy coat was collected from blood at third day at altitude, and then normal PBMC processing was followed at sea level about 24 hours after blood collection.

Physiological measures and fasting blood samples were collected every morning for 3 consecutive days (HA1, HA2, HA3). Pulse oximetry and heart rate values were collected using a Nellcor N-600 pulse oximeter (Medtronic, Minneapolis, MN, USA). Participants sat upright in a chair without their legs crossed and rested, breathing normally, for 3 minutes until values stabilized. Blood pressure measurements were collected in duplicate while participants rested in an upright seated position using a manual sphygmomanometer.

Participants abstained from taking anti-inflammatory medications or other agents that may impact the process of acclimatization, such as acetazolamide (Basaran et al. 2016). Participants were permitted to consume caffeine in moderation (1 cup of coffee or tea) after completing morning measurements but were asked to abstain from caffeine after noon. Three meals per day were provided and participants did not complete any strenuous physical activity. Participants did not consume alcohol, and fluid intake was supervised to ensure participants remained hydrated.

Ventilatory chemoreflex testing

Experimental design, data collection, and data analysis have been previously described in a separate study of the same field expedition (Frost *et al*., in prep). Briefly, ventilatory chemoreflex tests were conducted using Duffin's modified rebreathing protocol (Casey *et al.*, 1987; Duffin & McAvoy, 1988; Mohan & Duffin, 1997; Duffin,

2007). Participants breathed room air for 5 minutes to establish a baseline. They were then instructed to voluntarily hyperventilate until their end tidal PCO_2 reached 23-25 mmHg. Once the threshold was met, the participant was quickly switched to a rebreathing bag. Participants resumed normal breathing as the rebreathing bag allowed their end-tidal PCO_2 to slowly increase to 60 mmHg over a period of about 5-10 minutes. The test was stopped when end tidal PCO_2 reached 60mmHg, total ventilation hit 100L/min, SpO_2 approached 75% (in hypoxic trials), or the participant indicated they were unable to continue. The rebreathing bag contained either a hyperoxic (30% O_2, 6.5 – 7% CO_2, N_2 as needed to balance) or hypoxic (8.5% O_2, 7.5 – 8%CO_2, N_2 as needed to balance) gas mixture during the start of the test. Throughout the test, inspired O_2 was maintained at 30% for the hyperoxic test, and end-tidal PO_2 was maintained at 50 mmHg for the hypoxic test. These two tests were performed with a 15 minute rest interval between each trial. The hyperoxic test was always performed first to prevent any impact of hypoxia exposure on subsequent measures (Katayama et al., 2001; Kiernan et al., 2016; Pamenter & Powell, 2016). Data was recorded by using LabChart (AD Instruments) and analyzed using Rstudio (Rstudio, Boston, MA, USA) with R version 3.6.2. Due to logistical constraints, chemoreflex measures were collected in a subset of participants on the second day at altitude (n=8).

When choosing the data to input to R we did not include room air values or the hyperventilation period. The data selection began when the participant was switched from room air to the rebreathing bag. If there is an equilibration point, this is the point we started from. Data selection included any values from this point to include all points where PCO_2 falls below 60 mmHg. All data was BTPS corrected using the equation ((760-18.7) / (760-47.1)) * ((273 + 37) / (273 + 21)).

The packages mcp and JAGS were used to identify the ventilatory recruitment thresholds and resulting slopes. For files where the function did not choose the correct threshold, the threshold was identified using the raw values in LabChart by viewing the total ventilation channel and identifying the PCO_2 where there is a clear slope increase in ventilation. A range containing that value was entered into the mcp model. Mcp then ran 15 chains to determine the best fit line.

In the event that there were irregular breathing patterns such as severely elevated tidal volume, or irregular frequency, the irregular data was removed from when total ventilation increased above the stable value to where it returned to the stable value. This includes periods at the beginning of the bag breathing period where it is clear that the participant is “coming down” from the hyperventilation period. When choosing data for the hypoxic test only the data where the expired O_2 (bottom of the O_2 curves) is within an acceptable range (50 +/-5 mmHg) was taken. If the participant did not reach a PCO_2 of 45 or 50, while calculating the hypoxic ventilatory response (HVR) at these points, all other data was plotted, and the equation of best fit line was used to give an estimation of SpO_2 at these points.

Statistical analysis for chemoreflex

Respiratory volume data was corrected to BTPS conditions.

Resting ventilatory characteristics were tested twice at sea level and at high altitude at the start of the hyperoxic and hypoxic rebreathing test trials. To determine if there was an effect of test order on resting parameters, we first conducted a two-way repeated measures ANOVA on all parameters and found no effect of treatment (hypoxic, hyperoxic) on resting ventilation parameters, as expected. As a result, we used

measures taken only during the first test period in subsequent analyses of resting parameters.

Each outcome variable was first checked for outliers via the *rstatix* package in R and any points determined to be 3 times above or below the interquartile range were examined to verify no measurement errors. Data were then checked for normality using a Shapiro-Wilks tests via the *rstatix* package in R. If data distributions were normal, we proceeded with paired t-tests to compare resting measurements across sea level and high altitude. In some cases, paired data at high altitude was not available for all participants due to logistical constraints on the number of participants we could test in one day. In these cases, unpaired t-tests were performed. Two-way repeated measures ANOVAs were used to test for effects of altitude and oxygen condition on ventilatory chemoreflex parameters. If a significant two-way interaction or main effect of altitude or oxygen treatment were identified, post-hoc pairwise comparisons with Bonferroni adjustments for multiple comparisons were performed to identify differences across groups. In cases where data distributions were not normal, nonparametric Wilcoxon sign rank tests were performed.

Native Andean Highlanders: Cerro De Pasco, Peru 2019 Field Expedition

Ethics Approval

The study was approved by the Institutional Ethics Committee of Universidad Peruana Cayetano Heredia (Lima, Peru). All participants received a detailed explanation of the study procedures in their native language (Spanish) and signed an informed consent form.

Participants

The study included 62 men between ages 18 and 65 years old. Participants were recruited by word of mouth at Cerro de Pasco, Peru. All participants reported no history of heart or lung disease (besides Chronic Mountain Sickness) and had at least 3 generations of high-altitude ancestry. Exclusion criteria included recent travel below 2,500 meters within one month, smoking, and currently taking anti-inflammatory medications. Participants were grouped based on hematocrit (Hct). Participants with Hct ≥ 63 % were classified as 'EE'. This value is based on suggestions by Villafuerte et al. that a hemoglobin concentration ([Hb]) of 21 g/dL or higher in men classifies as EE, and a conversion of [Hb] = Hct/3 (Villafuerte *et al.*, 2004). The diagnosis of Chronic Mountain Sickness (CMS) is based on a test defined by a consensus statement named "Qinghai CMS score" (León-Velarde *et al.*, 2005). The test includes assessments of 7 signs or symptoms of CMS and the score assigned for EE (score=0 if hemoglobin level is below the cut-off point for EE and score= 3 if hemoglobin level is defined as EE). CMS is diagnosed with a score greater than 5 (León-Velarde *et al.*, 2005).

Experimental Design and Analysis

Cytokine analysis

Peripheral venous blood was collected in a 10mL vacutainer tube containing EDTA (BD, Franklin Lakes, NJ, USA) and processed within 4 hours of collection. Blood samples were spun at 1,000xg for 10 minutes, and the top layer of plasma was collected. As per manufacturer's instructions, plasma samples were assayed for cytokines using LEGENDplex kits and the manufacturer's protocols (BioLegend): "Human Essential Immune Response Panel" (13-plex) at 1:2 dilution. Samples were run

on a Novocyte Quanteon flow cytometer, and data analysis was conducted using BioLegend's LEGENDplex Data Analysis Software.

Isolation of PBMCs and freezing

Peripheral venous blood was diluted 1:1 with PBS. In a separate tube, equal volume (compared to blood volume) of Lymphoprep Density Gradient Medium (1.077 g/mL density) (StemCell, Seattle, WA, USA) was added. Blood was then slowly added to be layered on top of the Lymphoprep. Tubes were centrifuged at 400xg for 30 minutes at room temperature, with slow acceleration and no brakes. PBMCs were carefully collected into a separate 15 mL tube with 8 mL of EasySep media (StemCell, Seattle, WA, USA). PBMCs were centrifuge at 400xg for 5 minutes and resuspended in 5 mL of EasySep media. The wash step was repeated twice. PBMCs were resuspended in 1.5 mL of freezing media (90% FBS, 10% DMSO), and aliquoted in 500 uL volumes. Aliquots were placed in a Mr. Frosty (ThermoFisher, Carlsbad, USA) and into a -80 °C freezer overnight, then transferred to liquid nitrogen the following morning.

At high altitude, the same procedure was followed, with the following exceptions. Due to a lack of a -80 °C freezer at high altitude, the Mr. Frosty containers were placed in a large Styrofoam box filled with dry ice overnight. Aliquots were transferred to a transportable liquid nitrogen dewar for transport to sea level. Due to logistical constraints and timing, PBMCs collected on day 3 at altitude were first collected as buffy coat while at Barcroft Station, and then processed for PBMCs following the same protocol the following day at sea level.

PBMC thawing

PBMCs were first thawed before use. PBMCs were removed from liquid nitrogen and placed in a 37°C water bath for 30-45 seconds, or until a small ice crystal was left. 1 mL of warmed media (RPMI 1640, 10% FBS, 100 U/mL Strep/Penicillin) was added in a dropwise manner to the tube, which was then transferred to a 15 mL falcon tube containing 5 mL of warmed media. The tubes were gently mixed by inverting, and then centrifuged at 330xg for 10 minutes at room temperature. PBMCs were then resuspended in 1 mL of warmed media and counted via hemocytometer for experiments.

Inflammatory stimulation assay

PBMCs collected at sea level, first day and third day at altitude were used for stimulation experiments. 1.0 x 10^5 of PBMCs in a total of 200 uL media at each timepoint was used in the experiment. PBMCs were stimulated with 100 ng/mL LPS (E. coli Serotype (026: B6)) (Sigma-Aldrich, Burlington, Massachusetts, USA) in a 37 °C incubator in normoxic conditions (21% O_2; 5% CO_2) for 6 hours. Following culture, samples were centrifuge at 330xg for 10 minutes, and supernatant was collected and stored at -80 °C for ELISA analysis.

ELISA analysis

According to manufacturer's instructions, the concentration of TNFα and IL6 in cell culture supernatants were analyzed using a TNFα or IL6 Human ELISA kit (Invitrogen, Carlsbad, USA). An automated microplate reader (Synergy Lx Multimode Reader) (Biotek, Seattle, Washington, USA) was used for the measurement of the optical density at 450 nm. The concentrations of each sample were detected based on optical density (OD) and the concentration of the standard.

Statistical analysis

Statistical analyses were conducted in R (version 4.1.0) (R Foundation) and GraphPad Prism v10.0. The statistical significance for inflammatory stimulation assays and cytokine concentration at altitude, we used repeated measures ANOVA and post-hoc Tukey corrections. For the chronic domain cytokine analysis (Andeans), we used unpaired Student's t-test and a p-value <0.05 was considered significantly significant. Outliers were determined using the ROUT method. Three participants did not have PBMCs collected for one or more days at high altitude due to blood collection complications and were excluded from inflammatory stimulation assay analysis.

Correlation analysis

To examine correlations between physiological and immunological parameters in the dataset, pairwise Pearson correlations were conducted using R package *Hmisc* and *corr* function. Correlation analysis results were visualized using the *corrplot* function in Rstudio. Binary variables were converted to 1 (yes) or 0 (no). Using the min-max scaling method, all continuous variables were scaled from 0 to 1 to guarantee that they were all on the same scale. Correlation coefficients with p-values less than 0.05, 0.01, and 0.001 are denoted with "*", "**", and "***" respectively. Correlation matrices were made using unadjusted p-values. To calculate adjusted p-values, p-values were corrected using "BH" (q-value) for multiple comparisons.

4.4 RESULTS

Physiological measures

Table 4.1 provides an overview of physiological measures of the acute time domain (sea level and over three days of acclimatization to high altitude). On the first

morning at high altitude (HA1), 8 of 20 subjects indicated mild AMS (AMS score 3-5 with headache), and 6 of 20 subjects indicated moderate to severe AMS (AMS score 6+ score with headache). By the third day at altitude, 5 of 20 subjects indicated mild AMS, and 3 of 20 subjects indicated moderate to severe AMS. SpO_2 (%) dropped by about 11 percent on the first day at high altitude and remained lower than sea-level values throughout all 3 days at altitude. This was coupled with a 17-point increase in heart rate on the first day at high altitude, which increased a further 9 points by day 3. There was no significant increase in systolic or diastolic blood pressure at high altitude.

Table 4.2 provides an overview of physiological measures of the chronic time domain (native Andean highlanders with or without CMS). Andeans with CMS (n=31; CMS score > 5) had a 4.6-point higher CMS score and 12.7-point higher hematocrit percentage than healthy Andeans. This was coupled with a and a 3.7-point lower SpO_2 percentage in Andeans with CMS. While there was no difference in systolic blood pressure, Andeans with CMS had a 5.2-point higher diastolic blood pressure than healthy Andeans.

PBMCs at high altitude were not sensitized to subsequent inflammatory stimuli

PBMC cultures from sea level, first, and third day at altitude were stimulated with LPS to determine if immune cells at altitude were sensitized to subsequent inflammatory stimuli. ELISA analysis on supernatants collected from each culture was performed to quantify TNFα (n= 17) and IL6 (n= 13) production. TNFα and IL6 production by immune cells after 1 day of high-altitude exposure was not significantly changed compared to cells collected from the same individuals at sea level (p=0.11 and p=0.95, respectively) (**Figure 4.1 and 4.2**). In contrast, TNFα and IL6 production by PBMCs was significantly decreased in cells collected on day 3 at altitude compared to cells collected at sea level

(p=0.0002 and p=0.0082, respectively) (**Figure 4.1 and 4.2)**. Of note, on the first day at altitude, 10 of the 17 participants were reported to have increased TNFα production, despite this not being statistically significant overall (**Figure 4.1B**).

When analyzing for potential correlations between pro-inflammatory cytokine production and AMS scores at altitude, there was no significant correlation between AMS score and high-altitude PBMC production of TNFα nor IL6 on day 1 (TNFα: r= -0.14, p= 0.59; IL6: r= 0.21, p= 0.49) or day 3 at altitude (TNFα: r= 0.29, p= 0.28; IL6: r= 0.053, p= 0.87) (**Figure 4.3A and 4.3B**). Additionally, we also analyzed correlations with oxygen saturation (SpO_2) and pro-inflammatory cytokine production at high altitude, as systemic hypoxemia may impact immune cell function. There were no significant correlations between SpO_2 with TNFα nor IL6 production on first (TNFα: r= -0.075, p= 0.78; IL6: r= -0.042, p= 0.90) or third day (TNFα: r= -0.23, p= 0.39; IL6: r= -0.29, p=0.35) at altitude (**Figure 4.3C and 4.3D**).

Acute high-altitude exposure impacts on cytokine production and physiological parameters

Acute high-altitude exposure impacts MCP-1 cytokine concentration

Plasma samples from sea level and three days at high altitude were analyzed for expression of cytokines essential for the immune response with a bead-assisted multiplex by flow cytometry (LEGENDPlex). Of the 13 cytokines analyzed, MCP-1 was found to be significantly elevated on the first (p= 0.0094) and third day (p= 0.0021) at altitude (**Figure 4.4**).

Phenotype associations

To analyze for underlying mechanisms or effects following acute high-altitude exposure, we looked for significant correlations between phenotypes over three days at altitude with inflammatory cytokine expression in plasma. However, neither AMS score or resting SpO_2 (%) were significantly associated with any cytokine in plasma on any day at altitude ($p>0.05$ for all correlations). Further analysis revealed that age was found to be significantly and positively correlated with AMS scores ($r= 0.76$; $p= 0.00025$) and SpO_2 ($r=-0.55$; $p=0.024$) on the first day at altitude. Age and AMS scores on the first day at altitude remain significant after multiple corrections (adj. $p=0.0059$). (**Figure 4.S1)**. By the second and third day at altitude, Age and AMS scores correlations were no longer significant (day 2: $r=0.40$; $p= 0.082$; day 3: $r= -0.10$, $p= 0.66$) (**Figure 4.2 and 4.S3**).

In a previous study, we analyzed immune cell populations and cell surface markers from total white blood cells (% WBCs). These included T cells ($CD4^+$, $CD8^+$, DN T ($CD3^+$ $CD4^-$ $CD8^-$ $CD16^-$), NK T ($CD3^+$ $CD16^+$), B cells ($CD19^+$), monocyte subpopulations (classical: $CD14^+$ $CD16^-$; intermediate: $CD14^+$ $CD16^+$, non-classical: $CD14^-$ $CD16^+$), and NK cells ($CD16^+$ NK and $CD56^+$ NK). For additional cell surface markers, we analyzed mean fluorescence intensity (MFI) of TLR4 surface expression on all PBMCs, as well as MHC all on total monocytes (Chapter 3: Immune adaptation during acute high-altitude exposure). Below I describe how these findings relate to the phenotypes measured in the same subjects.

Table 4.3 provides a summary of the complete correlation analysis. In brief, on the first day at altitude, we found the following significant associations: Plasma IP-10 levels and the proportion of classical monocytes (% WBC) are positively correlated with TLR4 expression ($r= 0.64$, $p= 0.035$; $r= 0.62$, $p= 0.042$, respectively). Intermediate

monocytes (% WBCs) are positively correlated with IL-1b (r= 0.59; p= 0.013), IL8 (r= 0.60; p= 0.010), TNFα (r= 0.53; p= 0.028), and IFNy (r= 0.59; p= 0.013). $CD4^+$ T cells (% WBCs) are positively correlated with MCP-1 (r= 0.69; p= 0.0021) and negatively correlated with IL12p70 (r= -0.51; p= 0.036). $CD8^+$ T cells (% WBCs) are positively correlated with TNFα (r= 0.49; p= 0.044). DN T cells (% WBCs) are positively correlated with IP-10 (r= 0.74; p= 0.00067). Finally, $CD16^+$ NK cells (% WBCs) are negatively associated with IFNy (r= -0.49; p= 0.043) (**Figure 4.S1, Table 4.3)**. After multiple correction analysis, the following were still significant: $CD4^+$ T cells positively correlated with MCP-1 (adj. p= 0.042) and DN T cells positively correlated with IP-10 (adj. p=0.014) (**Table 4.3**).

On the third day at altitude, we found the following significant associations: DN T cells were positively correlated with IL1b (r= 0.49; p= 0.046), IL2 (r= 0.57; p= 0.017), IL4 (r= 0.59; p= 0.013), IL8 (r= 0.69; p= 0.0022), TNFα (r= 0.56; p= 0.020), and IFNy (r= 0.51; p= 0.038), NK T cells were positively correlated with IL6 (r= 0.53; p= 0.03), IL10 (r= 0.65; p= 0.0050), and IL12p70 (r= 0.76; p= 0.00035), and $CD16^+$ NK cells was positively correlated with IL12p70 (r= 0.55; p= 0.023) (**Figure 4.S2, Table 4.5**). After multiple comparisons, the following were still significant: NK T cells positively correlated with IL12p70 (adj. p=0.0076) and DN T cells positively correlated with IL8 (adj. p= 0.040) (**Table 4.5**). Full correlation analyses of all variables on day 1 and 3 at altitude are found in **Table 4.S1 and 4.S3.**

Associations with ventilatory chemoreflex sensitivity

In a separate study, we determined the HVR, the reflexive increase in breathing in response to hypoxia, as well as the ventilatory recruitment threshold (VRT), the end tidal PCO_2 threshold at which when ventilation is stimulated to increase, after two days

at altitude. Low ventilatory drive has been previously considered as a contributing factor to AMS development (Hohenhaus *et al.*, 1995; Bartsch *et al.*, 2002; Richalet *et al.*, 2012), and systemic inflammation has been linked to modulating ventilatory chemoreflexes (McDeigan *et al.*, 2003; Ladino *et al.*, 2007; Fernández *et al.*, 2008; Huxtable *et al.*, 2011; Hocker *et al.*, 2017). In this study, we sought to determine if the HVR at altitude is correlated with the inflammatory profile or has phenotypic associations in lowlanders traveling to altitude after two days of acclimatization. We found the following to have significant associations: Hypoxic Ventilatory Response at 45mmHg PCO_2 (HVR) was negatively correlated with cytokines on HA2: IL10 (r= -0.86; p= 0.0063), IL6 (r= -0.87; p= 0.0051), TNFα (r= -0.77; p= 0.037), IFNy (r= -0.74; p= 0.035), and IL1b (r= -0.79; p= 0.018). Additionally, correlation with AMS scores was approaching significance (p= 0.064). (**Figure 4.S2, Table 4.4**). After multiple comparisons, the following was still significant: HVR negatively correlated with IL6 (adj. p= 0.049). We also found HVR and IL10 correlation to be approaching significance (adj. p = 0.059) (**Figure 4.10; Table 4.4**). Full correlation analyses of all variables on day 2 at altitude are found in **Table 4.S2**.

Chronic high-altitude exposure impact on cytokine production and phenotype associations

Plasma samples from Andeans with and without CMS were also analyzed with the Essential Immune Response Panel (LEGENDPlex). Of the 13 cytokines analyzed, IP-10 (CXCL10) was significantly elevated in Andeans with EE compared to healthy Andeans (p= 0.0489) (**Figure 4.5**). However, when we classified patients based on CMS status rather than EE status, there was no significant difference in any of the cytokines across CMS and healthy groups (p> 0.999) (**Figure 4.6**). When comparing acute vs

chronic time domains, Andeans with EE had significantly higher IP-10 compared to lowlanders at sea level (p= 0.0085) and on first day at altitude (p= 0.0008). In contrast, healthy Andeans (based off EE values) had significantly less IL4 compared to lowlanders at sea level (p= 0.032), day 1 (p= 0.030), and day 3 (p= 0.033) at altitude, as well as significantly less IL8 compared to day 3 at altitude (p= 0.044) (**Figure 4.7**).

In addition to grouping participants by EE and CMS status, we conducted correlation analyses to account for the wide variation in hematocrit levels and determine if any unique relationships were revealed. These correlation analyses revealed that higher a CMS score was associated with higher hematocrit (r= 0.65, p<0.0001), lower SpO_2 (r= -0.40, p= 0.0029), and positively correlated with EE status (r= 0.71; p<0.0001), as expected based on previous work by others (Heinrich *et al*., 2020). CMS score was also associated with several inflammatory markers (TNFα (r= 0.28, p= 0.032), IL-2 (r= 0.26, p= 0.045), IL6 (r= 0.26, p= 0.048), IL8 (r= 0.33, p= 0.012). When removing one outlier, the results for all but IL8 versus CMS scores were no longer significant. SpO_2 was also found to be significantly positively correlated with IL12p70 (r= 0.30, p= 0.026). While IP-10 was found to be significantly higher in Andeans with CMS, there was no correlation between CMS score and IP-10 concentration (**Figure 4.S4, Table 4.6**). After multiple corrections, the following remained significant: CMS positively correlated with hematocrit (adj. p = <0.0001), negatively correlated with SpO_2 (adj. p= 0.0098), positively correlated with EE status (adj. p< 0.0001), and positively correlated with IL-8 (adj. p = 0.039) (**Table 4.6**). Full correlation analyses of all variables for the Andean population (chronic hypoxia exposure) are found in **Table 4.S4**.

4.5 DISCUSSION

This study examined how acute and chronic high-altitude hypoxia exposure impact cytokine concentration in peripheral blood, and how the cytokine profile differs in these time domains by using a multiplex cytokine array to analyze plasma from lowlanders traveling to high altitude as well as high-altitude native Andeans with or without EE. We also analyze how CMS classification compares to only EE in terms of associations with inflammatory profile, as EE is considered to be the greatest contributing factor to CMS. Our results demonstrate that acute high-altitude exposure impacts cytokine expression and that these impacts have potential associations with AMS and hypoxemia severity. These inflammatory profile changes may be a cause or an effect of changes in immune cell populations at high altitude. In particular, we found significant elevation in MCP-1 on an acute timescale in sojourners to high altitude, while we found significant elevation of IP-10 (CXCL10) on a chronic timescale when comparing Andeans with and without EE. We also investigated relationships between phenotypes, including the ventilatory responses to hypoxia, with the inflammatory profile to determine if there were underlying susceptibilities that can influence AMS development. While both acute and chronic hypoxia exposure are physiologically stressful, each time domain elicits distinct phenotypes that may reflect how an initial beneficial adaptation can turn maladaptive if not properly resolved. Here, we will discuss the impact of the inflammatory profile of both acute and chronic time domains, and how it may play a role in high-altitude illnesses.

Impact of acute versus chronic high altitude hypoxia exposure on cytokine expression and inflammatory sensitivity

In a previous study, we hypothesized that acute high-altitude exposure causes immune sensitization due to upregulation in genes involved in the toll-like receptor 4

(TLR4) signaling pathway, such as *CD14, LY96,* and *TLR4*. Additionally, several alarmins that may sensitize the TLR4 pathway were also upregulated at the gene expression level, such as *HMGB1, S100A8,* and *S100A9* (Pham *et al.*, 2022). When analyzing if immune cells at high altitude were more sensitive to inflammatory stimuli, we did not find any significant elevation in pro-inflammatory TNFα or IL6 cytokine production following LPS stimulation. Despite the non-significance, we did notice that for a subset of the participants, there was an appreciable increase in TNFα production from PBMCs collected on the first day at altitude compared to sea level values, which may indicate a potential role of hypoxia-induced immune sensitization. While we did not see any association between TNFα production and AMS scores on first or third day at altitude, this may have further implications in cases of severe AMS, high-altitude pulmonary edema (HAPE), or high-altitude cerebral edema (HACE). Interestingly, there was a significant reduction in pro-inflammatory cytokine production on the third day at altitude (**Figure 4.1**). However, while we do believe that this could be an effect of immunosuppression, as HIF pathway activation in hypoxia also plays a major role in producing antioxidant properties, due to logistics during sample processing, PBMCs on day 3 at altitude may have lower activity due to their collection as buffy coat and subsequent isolation after return to sea level. Nevertheless, immune cell populations at altitude are altered, and continuous HIF activation and hypoxia exposure do indeed impact surface expression, and potentially cytokine production (Chapter 3: Immune adaptation during acute high-altitude exposure).

Monocyte chemoattractant protein-1 (MCP-1) is a chemotactic cytokine that promotes inflammation by directing the migration and tissue infiltration of several immune cell populations, particularly monocytes and T cells. MCP-1 has been found to

be elevated in several pathological conditions and has potential to be diagnostic biomarker of inflammation in disease (Dalgard *et al.*, 2017; Kobayashi *et al.*, 2018; Chen *et al.*, 2020). Elevated MCP-1 concentration mediated by inflammation and oxidative stress and driven by NF-kB activity have been demonstrated in COPD and diabetes (Chiarelli *et al.*, 2002; Takebayashi *et al.*, 2006; Di Stefano *et al.*, 2018). When considering the synergy between inflammation and oxidative stress interplay in acute high-altitude exposure, previous research has found increased oxidative stress biomarkers following acute high-altitude exposure (Malacrida *et al.*, 2019). Given that MCP-1 protein expression is upregulated in situations of oxidative stress and inflammation, our research agrees with this conclusion, as acute high-altitude hypoxia exposure leads to significantly elevated MCP-1 expression in sojourners to high altitude (**Figure 4.4**). This also agrees with previous studies that investigated pro-inflammatory cytokine profile in patients with a history of high-altitude induced pulmonary edema (HAPE), which have found elevated MCP-1 concentration in HAPE-susceptible travelers (Mishra et al., 2016).

Additionally, we found $CD4^+$ T cells were positively correlated with MCP-1 on the first day at altitude **(Figure 4.8A)**. MCP-1 is also a potent T-lymphocyte chemoattractant (Carr *et al.*, 1994). This suggests T cells are actively recruited to sites of inflammation, however the $CD4^+$ T reduction in response to high-altitude exposure is indicative of immunosuppression. While it is well documented that high-altitude significantly reduces CD4+ T cell concentration in total WBCs, the severity of hypoxemia nor AMS severity was not correlated with $CD4^+$ T cells and was not a driver of $CD4^+$ reduction. Interestingly, we do report that $CD4^+$ T cells were negatively correlated with IL12p70 cytokine expression (**Figure 4.8B**). IL12p70, also known as the natural killer cell

stimulatory factor (NKSF) primarily acts on NK cells and T cells and is a particularly potent stimulator of IFNy (Trinchieri, 2009). In addition to their role in enhancing phagocytic activity in NK and T cells, they also favor differentiation and function of T-helper cell type 1 (Th_1) (Manetti *et al.*, 1993). However, this contradicts previous studies that report a dysregulated Th_1 vs Th_2 balance at altitude, specifically in the Th_1 subset (Klokker *et al.*, 1993; Facco *et al.*, 2005). This also contradicts our finding that $CD16^+$ NK cells are negatively correlated with IFNy, as we expected the opposite to occur. This may have several implications, such as an external factor driving the $CD4^+$ T cell reduction. It is important to note that our data does not find any significant differences in cytokine concentration over 3 days at altitude in IFNy, favored by Th_1, nor in IL4, favored by Th_2, and we also did not find IL12p70 itself to be differentially changed at altitude, yet we see a significant reduction in $CD4^+$ T cells. The $CD4^+$ T cells and IL12p70 relationship also fell out of significance following multiple corrections, although this may warrant further studies to determine if there is a true relationship between these variables.

Furthermore, because these are otherwise healthy individuals in a stressful environment, and although we do expect that the inflammatory and immune profile to shift in response to the high-altitude hypoxia exposure, these correlations may have other underlying factors that contribute to these results. Previous research found that *FASLG* was positively correlated with SpO_2 (Pham *et al.*, 2022). *FASLG*, which encodes for FasL, regulates immune system activation particularly in T cells (Mogi *et al*., 2001). We suspected that upon high-altitude exposure, a higher SpO_2 with elevated *FASLG* expression may be indicative that the physiological response to hypoxia quickly adapts and appropriately blunts the immune response. This could also suggest that the

suppression of $CD4^+$ T cells would impact IFNy production, as we also do not see any significant changes in IFNy cytokine concentration at altitude. However, additional studies are needed to elucidate what causes $CD4^+$ T cell reduction, why the Th_1 vs Th_2 balance is dysregulated, and if the FAS pathway plays a role in high-altitude immune modulation.

IP-10 is an inflammatory chemotactic cytokine that is produced by leukocytes, neutrophils, monocytes, and epithelial cells in response to IFNy (Luster & Ravetch, 1987; Dyer *et al.*, 2009; Liu *et al.*, 2011). IP-10 promotes chemotactic activity of CXCR3+ cells, such as macrophages, NK cells, and $CD4^+$ and $CD8^+$ T cells, while also inducing apoptosis, regulating cell growth and proliferation, and anti-angiogenesis (Liu *et al.*, 2011). We identified elevated IP-10 plasma concentration in Andeans with EE. Interestingly, when grouping by CMS score instead of EE classification, IP-10 was no longer significant compared to healthy Andeans (**Figure 4.6**). Furthermore, we found correlations between CMS score and higher hematocrit concentration, lower oxygen saturation, as well as several inflammatory cytokines (IL2, IL6, IL8, and TNFα) (**Figure 4.9**). However, when removing outliers and taking into account multiple corrections, only IL8 remained significant. Despite the strong associations with CMS, these relationships are not seen as a function of EE status or hematocrit. Therefore, this suggests that while EE is known as the main mechanism responsible for CMS development and is one of the major contributors to determining CMS classification, there are most likely several other contributing factors that lead to CMS, as seen by the correlations with several pro-inflammatory cytokines. This agrees with studies indicating that CMS and EE classifications are not interchangeable, and that other underlying mechanisms, in addition to EE, may drive CMS development (Gonzales *et al.*, 2013; Hancco *et al.*, 2020;

Oberholzer *et al.*, 2020). Having said that, excessive red blood cell concentration increases blood viscosity and can cause shear stress on endothelial cells and impact cardiovascular function. Indeed, Andean highlanders with EE have increased cardiovascular risk compared to healthy Andeans (Corante *et al.*, 2018). To complement the EE vs CMS classification, IL8, a chemotactic cytokine crucial for neutrophil chemotaxis, was significantly correlated with CMS scores, which would indicate an immunological response to injury. Andeans with CMS may have other underlying risk factors that, coupled with EE, may trigger CMS pathogenesis. Together, this suggests that in addition to EE, Andeans with CMS have a more robust inflammatory profile that may be a driving factor in CMS development.

Cytokine profiles of high-altitude acclimatization time domains

In contrast to the acute time domain, chronic hypoxia exposure elicits different effects on the cytokine profile. MCP-1 plasma concentration was not significantly elevated in the Andean population, a surprisingly conclusion, as we expected that chronic hypoxia exposure would stimulate significantly higher levels of MCP-1 in all native Andeans, and an even greater concentration in Andeans with EE. Furthermore, this data contradicts previous research that have found elevated MCP-1 levels in Han Chinese patients with high-altitude polycythemia (HAPC) (Yi *et al.*, 2021). However, while both Andeans with EE and Han Chinese with HAPC have elevated erythrocytosis, the Andean population may be subjected to other evolutionary adaptations, as they have migrated to the Andean Altiplano more than 10,000 years ago and have survived in this hypoxic environment for several generations, whereas the Han Chinese are relatively new immigrants to high altitude in comparison (Bigham *et al.*, 2013; Moore, 2017). This may suggest that MCP-1 has underlying impacts when considering a time scale of days

to years, but the native Andean highlanders who have survived for generations at high altitude may have evolutionary variants that mitigate MCP-1 concentration or function.

While there were no changes in MCP-1, IP-10 was significantly elevated in Andeans with EE compared to lowlanders at sea level and on first day at altitude. This significant elevation in IP-10 in only Andeans with EE also has several implications. Elevated IP-10 has been found in a rat model of LPS-induced HACE, which may suggest that IP-10 may play a role in the development or exacerbation of severe high-altitude illnesses (Shi *et al.*, 2023). Additionally, HACE is considered to be the end-stage of AMS, which may explain why in our acute timescale study, we did not find significantly higher IP-10, as no participant developed HACE during their stay at altitude. This may indicate that Andeans with EE are more susceptible to developing high-altitude pathologies, specifically CMS, however other factors are suspected to work concurrently with the pro-inflammatory cytokines to develop high-altitude pathologies.

In several pathological conditions, most notably in cases of severe COVID-19 infection, and severe acute respiratory syndrome (SARS), MCP-1 and IP-10 are elevated (Uguccioni *et al.*, 1999; Jiang *et al.*, 2012; Chen *et al.*, 2020; Mulla *et al.*, 2022; Eichhorn *et al.*, 2023; Tiwari *et al.*, 2023). Furthermore, these cytokines were associated with disease severity. These pathologies are clinically characterized with hypoxemia, and while there are other mechanisms to consider, there is a strong synergistic role between the hypoxia and inflammatory response that can provide insight into how these biomarkers can be indicative of CMS development. However, further research is necessary to determine if MCP-1 and/or IP-10 are potential biomarkers of CMS in highlanders, and additional investigations on MCP-1 and IP-10 in other native

highlanders will provide invaluable insight into how inflammatory adaptations may confer or exacerbate high-altitude illnesses.

High hypoxic ventilatory response is correlated with AMS severity and a robust inflammatory profile

In sea-level sojourners, the immediate increase in ventilation in response to acute high-altitude hypoxia exposure is one of the most crucial aspects of high-altitude acclimatization, as this significantly increases the level of oxygen in alveolar air. However, this adaptation is not sustained throughout their stay at high altitude. HVR, the reflexive increase in breathing in response to reduced arterial oxygen pressure, follows the same trend, where HVR is significantly elevated in lowlanders acutely exposed to high altitude but seems to become reduced in amplitude over prolonged exposure (Sato *et al*., 1994; Powell *et al.*, 1998; Hupperets *et al*., 2004; Brutsaert *et al*., 2005; Brutsaert, 2007; Dempsey *et al*., 2014). While it is well known that sojourners to high altitude have an increased HVR at high altitude, there is still significant individual variation in HVR sensitivity. In addition to the HVR variability in sea-level populations, lower HVR values are significantly more common (Oeung *et al.*, 2023). This has implications in an individual's susceptibility to hypoxia-related illnesses. Finally, studies have reported that failure to increase HVR at high altitude may be a contributing factor in AMS development and severity (Hohenhaus *et al.*, 1995; Bartsch *et al.*, 2002).

With the same participants, we investigated the HVR and the ventilatory recruitment threshold (VRT) at sea level and after 2 days at high altitude to determine if these factors are associated with the inflammatory profile**.** We report that the isocapnic HVR measured at 45mmHg end-tidal PCO_2 (HVR) was approaching a significant correlation with AMS scores ($p=0.063$). This would indicate that participants with higher

HVR fare better at altitude in terms of AMS symptom severity (Matsuzawa *et al*., 1989; Hohenhaus *et al*., 1995; Bartsch *et al*., 2002). Furthermore, HVR at altitude is significantly negatively correlated with IL10, IL6, TNFα, IFNy, and IL1b on the second day at altitude (HA2) (**Figure 4.10**). This would suggest that individuals who have low HVR also have a more prominent inflammatory profile, which could contribute to or exacerbate high-altitude illnesses (Huxtable *et al.*, 2011; Hocker *et al.*, 2017).

When comparing these results to a chronic high-altitude time domain profile, specifically of native Tibetan and Andean highlanders, we see these trends as well. Despite living in similar high-altitude hypoxic environments, these two populations followed two very different routes of high-altitude adaptation that ultimately achieved the same goal of increased oxygen delivery (Beall, 2006, 2007b). However, the native Andean highlanders are considered to have less beneficial adaptations to high altitude compared to Tibetans. The Andean population has the highest prevalence of Chronic Mountain Sickness (CMS), a clinical syndrome that occurs in native and life-long residents at altitude and is characterized by excessive erythropoiesis and severe hypoxemia (Monge, 1943; Monge *et al.*, 1989; Villafuerte & Corante, 2016). When comparing key adaptations in these native populations, HVR is significantly higher in Tibetans than Andean highlanders. As a matter of fact, the Tibetans express a normal HVR more comparable to sea-level populations, while the Andeans have a blunted HVR (Zhuang *et al*., 1993; Beall *et al*., 1997; Brutsaert *et al*., 2005). Additionally, Andeans have significantly elevated hematocrit levels, which can contribute to increased blood viscosity and thus are at higher risk of vascular damage via shear stress, which can trigger the release of inflammatory cytokines (Beall *et al.*, 1990, 1998; Huertas *et al.*, 2013; Corante *et al.*, 2018). In comparison, the Tibetans have markedly lower hematocrit levels (Beall,

2007). To further complement these adaptive benefits, the Tibetan population have an evolutionary selected variant that has been found to promote less circulating pro-inflammatory cytokines as well as pro-inflammatory immune cells (Bhattacharya *et al.*, 2021). Together, this suggests that in sojourners to high altitude, the combination of high HVR without an exacerbated inflammatory profile is protective against AMS.

4.6 CONCLUSION

In conclusion, we demonstrate significant differences in the acute and chronic time domains of high-altitude acclimatization. Specifically, our analysis has determined relationships between AMS severity, ventilatory responses, and immune cell populations with inflammatory cytokines on an acute timescale, as well as CMS or EE severity with inflammatory cytokines on a chronic timescale. While it is clear that high-altitude induced hypoxemia has significant impacts on the inflammatory profile, further studies are essential to elucidate what benefits, if any, come from a robust inflammatory response. Additionally, because we see associations with the physiological response to hypoxia, we suspect that individuals with chronic inflammation as well as having a low HVR in response to hypoxia may be at greater risk of developing high-altitude illnesses. With the rising cases of co-morbidities that have underlying chronic inflammation coupled with the commonly low HVR in lowlanders, this may provide insights into who are susceptible to severe high-altitude illnesses. Lastly, IP-10 is a potential biomarker for EE severity in Andeans and may be implicated in CMS pathogenesis. By characterizing the inflammatory profile in time domains of high-altitude acclimatization, this work provides insight into how the inflammatory response, alongside the immune and physiological response, may play a role in high-altitude illnesses, and how differences in native

highlander profiles may reflect specific evolutionary adaptations that promotes survival in high-altitude hypoxic environments.

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Disclosures

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

Author Contributions

KP and ECH conceived and designed the research. KP, SF, and ECH assisted in sample collection. KP, SS, and ECH analyzed data, interpreted the results of experiments, and prepared figures. KP drafted the manuscript. KP and ECH edited and revised the manuscript.

Figures

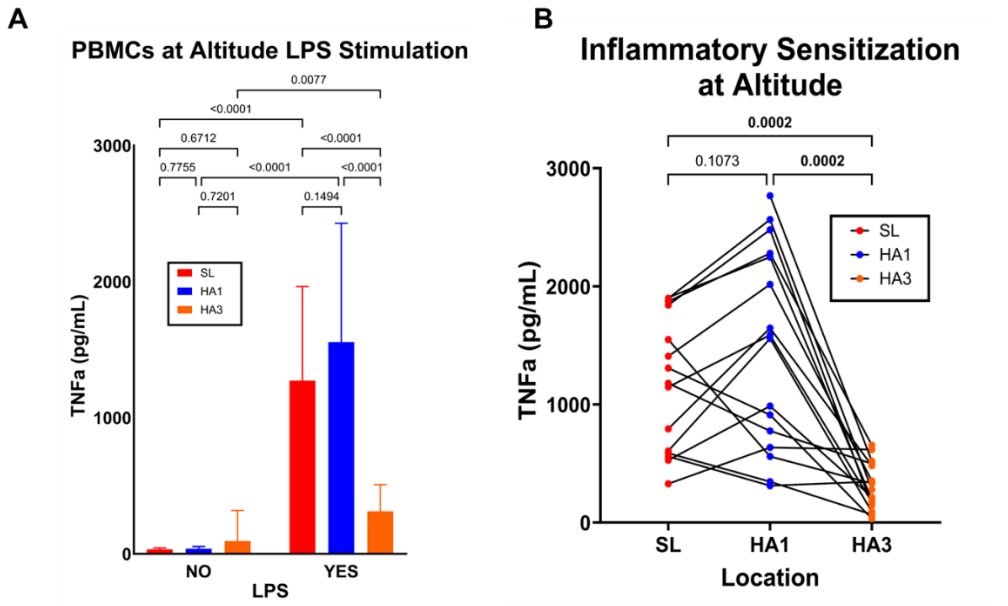

Figure 4.1. **TNFα cytokine production in PBMC collected at altitude (day 1 and 3) and stimulated with LPS. (A)** Overall change in TNFα production following inflammatory stimulus (LPS) over 3 days at altitude. **(B)** Changes in TNFα production following inflammatory stimulus (LPS) over 3 days at altitude. Outliers were removed from data. Graphs are plotted as mean and error bars as standard deviation.

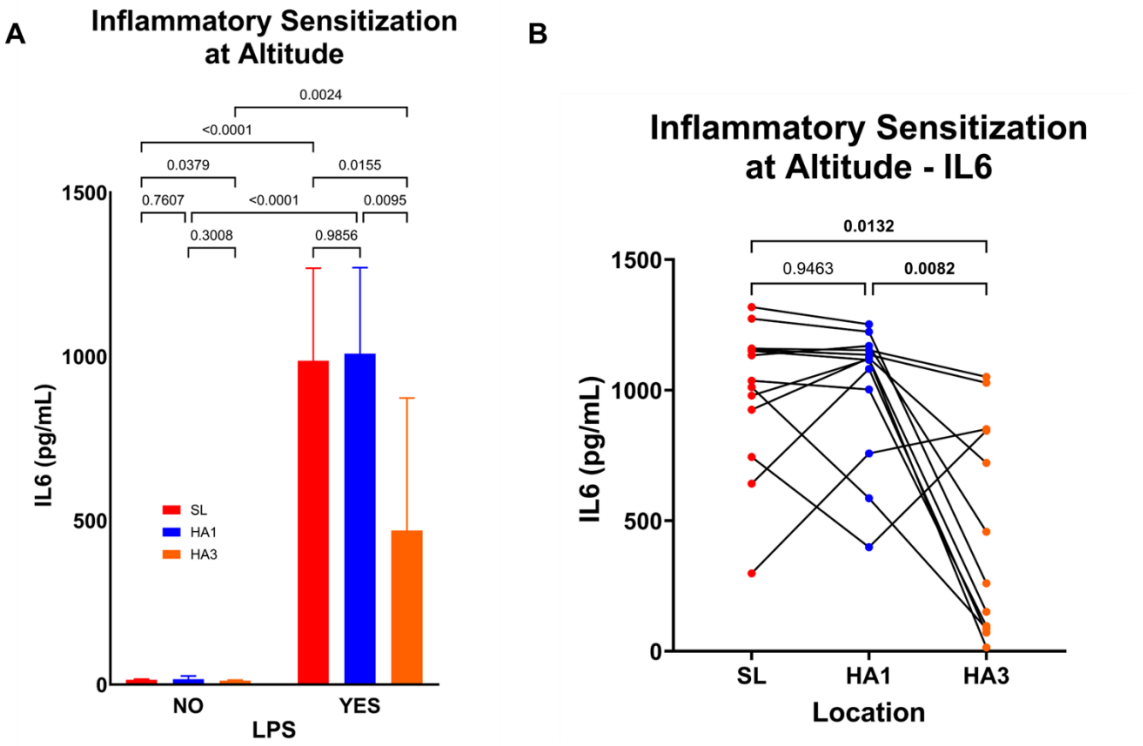

Figure 4.2. IL6 cytokine production in PBMC collected at altitude (day 1 and 3) and stimulated with LPS. (A) Overall change in IL6 production following inflammatory stimulus (LPS) over 3 days at altitude. **(B)** Individual changes in IL6 production following inflammatory stimulus (LPS) over 3 days at altitude. Graphs are plotted as mean and error bars as standard deviation.

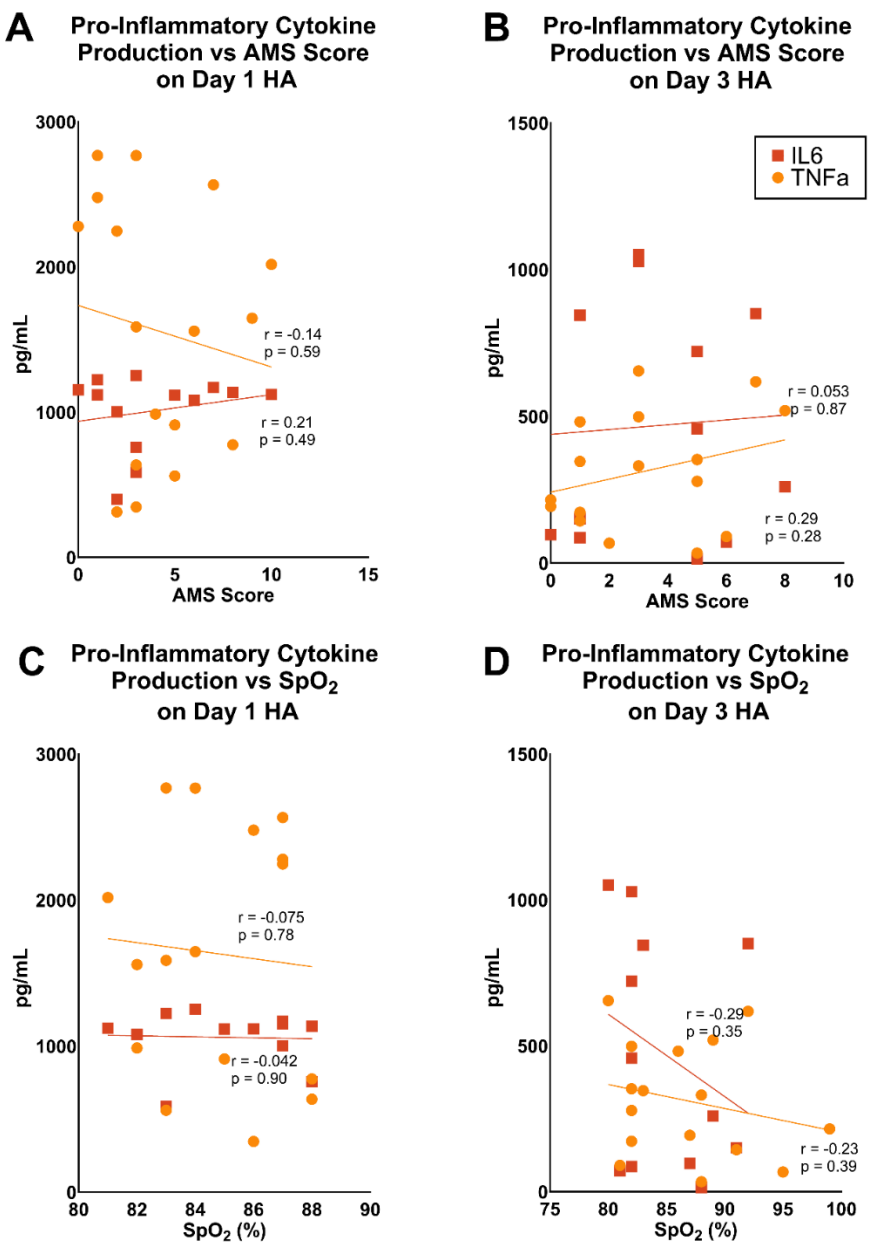

Figure 4.3. Associations between pro-inflammatory cytokine production, Acute Mountain Sickness (AMS) scores, and oxygen saturation (SpO_2). TNFα and IL6 associations with AMS scores on **(A)** day 1 and **(B)** day 3 at altitude; TNFα and IL6 associations with SpO_2 on **(C)** day 1 and **(D)** day 3 at altitude.

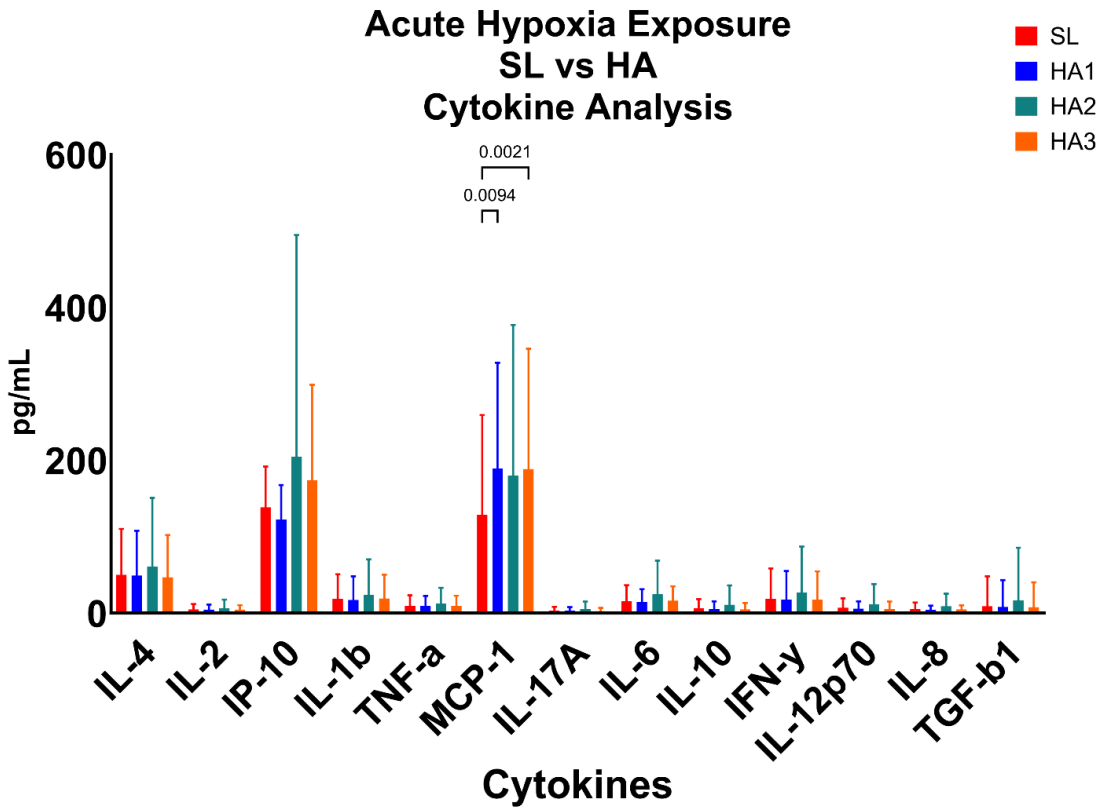

Figure 4.4. Cytokine expression across 3 days at altitude compared to sea level (baseline). Sea-level (SL); High altitude day 1, 2, and 3 (HA1, HA2, HA3). Only significant p-values shown.

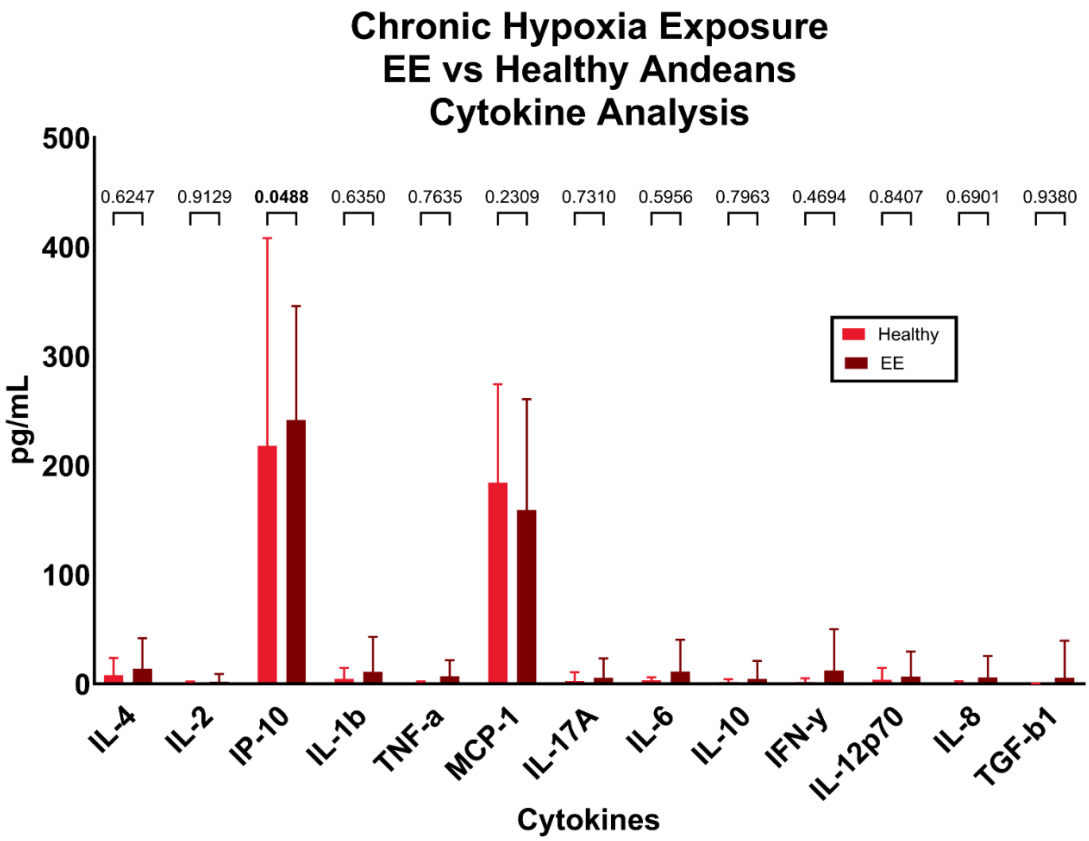

Figure 4.5. Cytokine expression in native Andean highlanders with or without excessive erythrocytosis (EE).

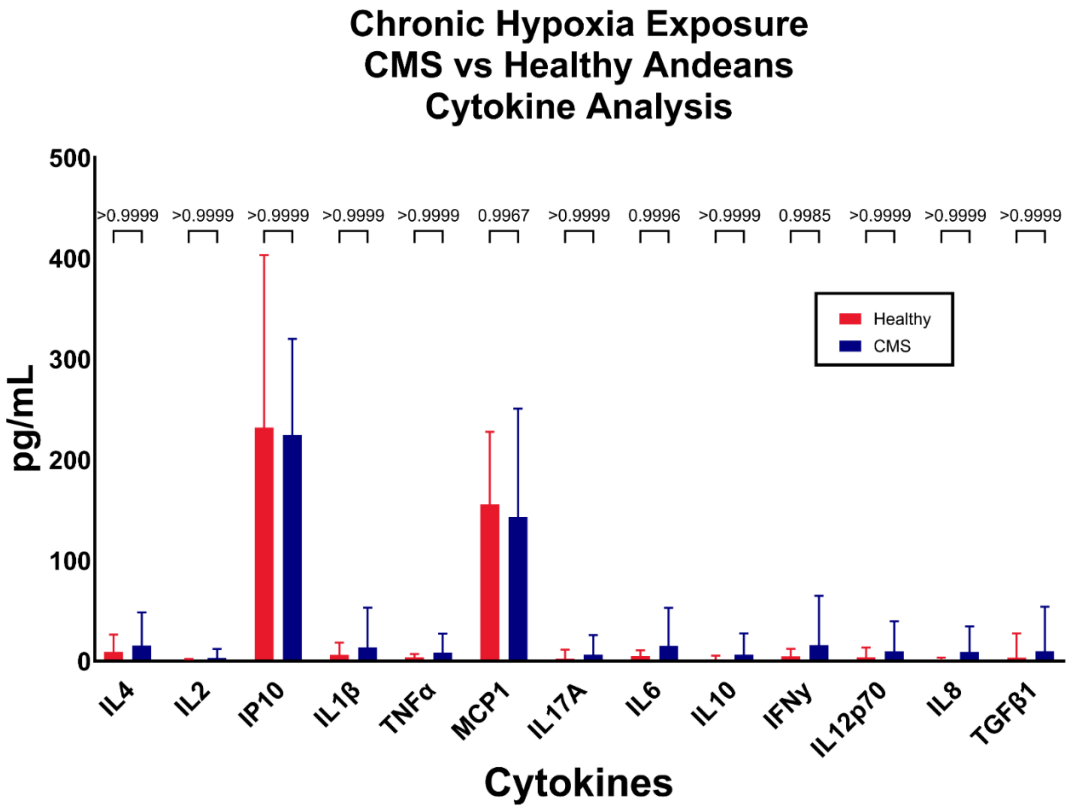

Figure 4.6. Cytokine expression in native Andean highlanders with or without Chronic Mountain Sickness (CMS).

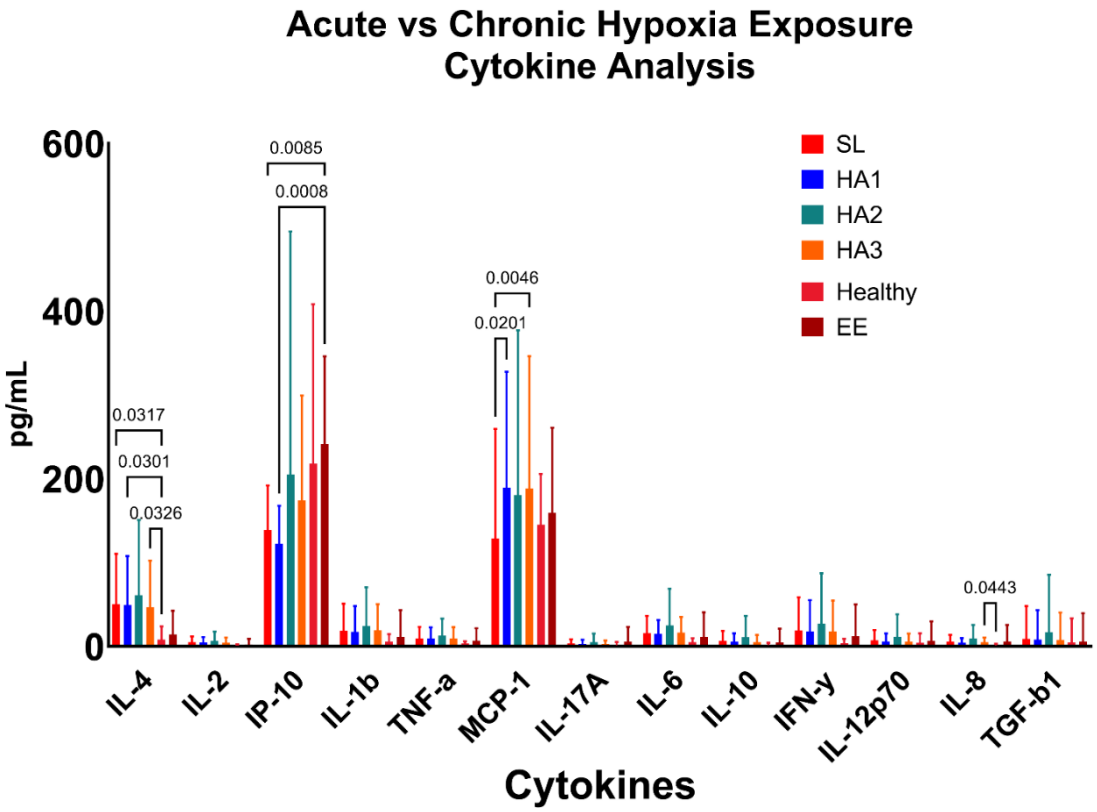

Figure 4.7. Cytokine expression during acute exposure (Sea-level (SL); High altitude day 1, 2, and 3 (HA1, HA2, HA3)) and during chronic exposure (native Andeans with excessive erythrocytosis (EE) or without (healthy).

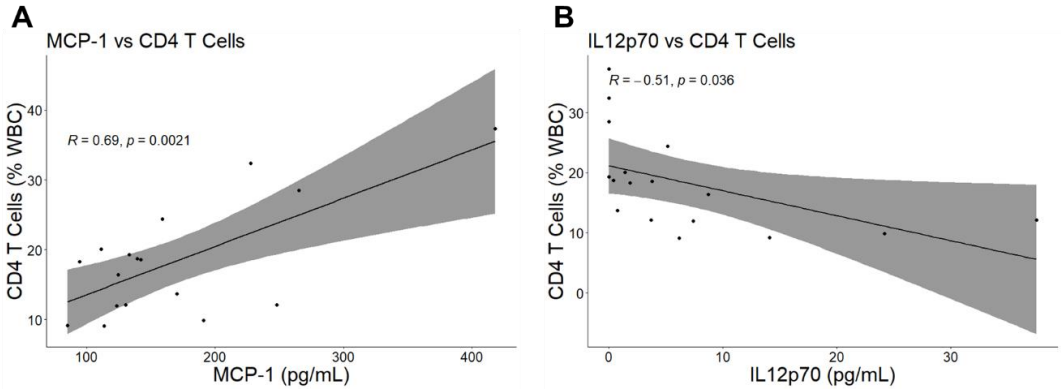

Figure 4.8. Correlation plots between cytokine expression and immune populations on day 1 at altitude. (A) MCP-1 and CD4 T Cells and **(B)** IL12p70 vs CD4T Cells after 1 day at high altitude.

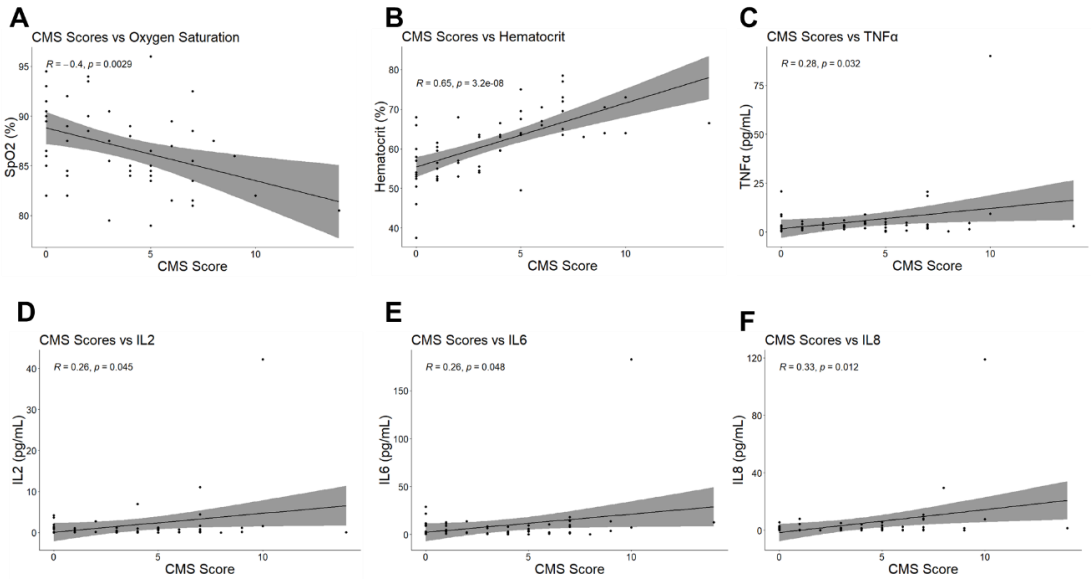

Figure 4.9. Relationships in phenotypes and cytokines in native Andean highlanders. (A) Oxygen saturation (SpO_2) and Chronic Mountain Sickness (CMS) Scores; **(B)** Hematocrit and CMS Scores; **(C)** TNFα and CMS Scores; **(D)** IL2 and CMS Scores; **(E)** IL6 and CMS Scores; **(F)** IL8 and CMS Scores. After removing outliers and multiple corrections, only IL8 versus CMS remain significant.

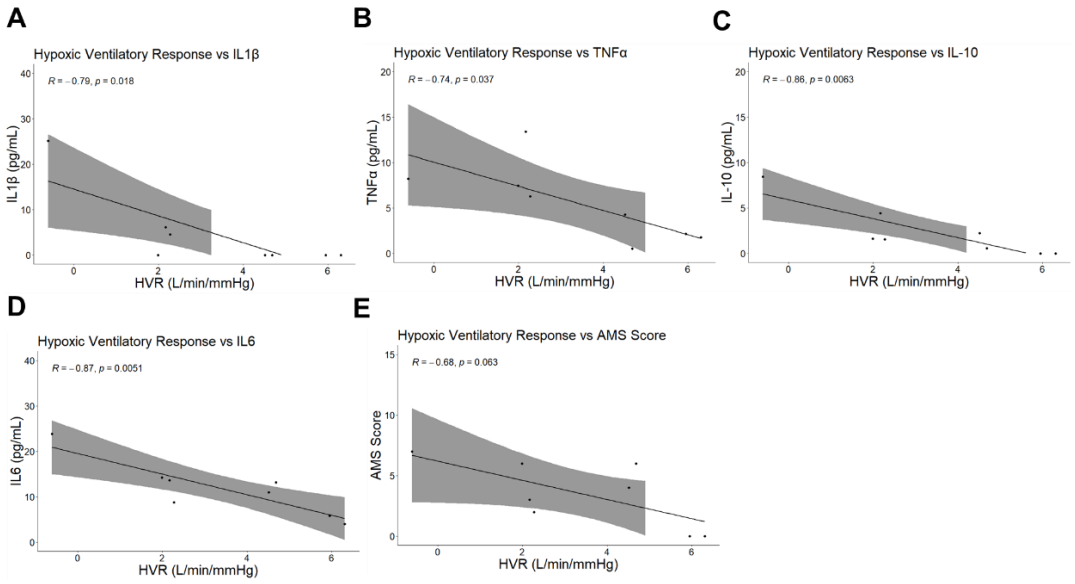

Figure 4.10. Correlation plots of high-altitude day 2. (A) IL1b and Hypoxic Ventilatory Response (HVR) **(B)** TNFα and HVR; **(C)** IL10 and HVR; **(D)** IL6 and HVR; **(E)** AMS Scores and HVR. After multiple corrections, only IL6 and HVR remain significant.

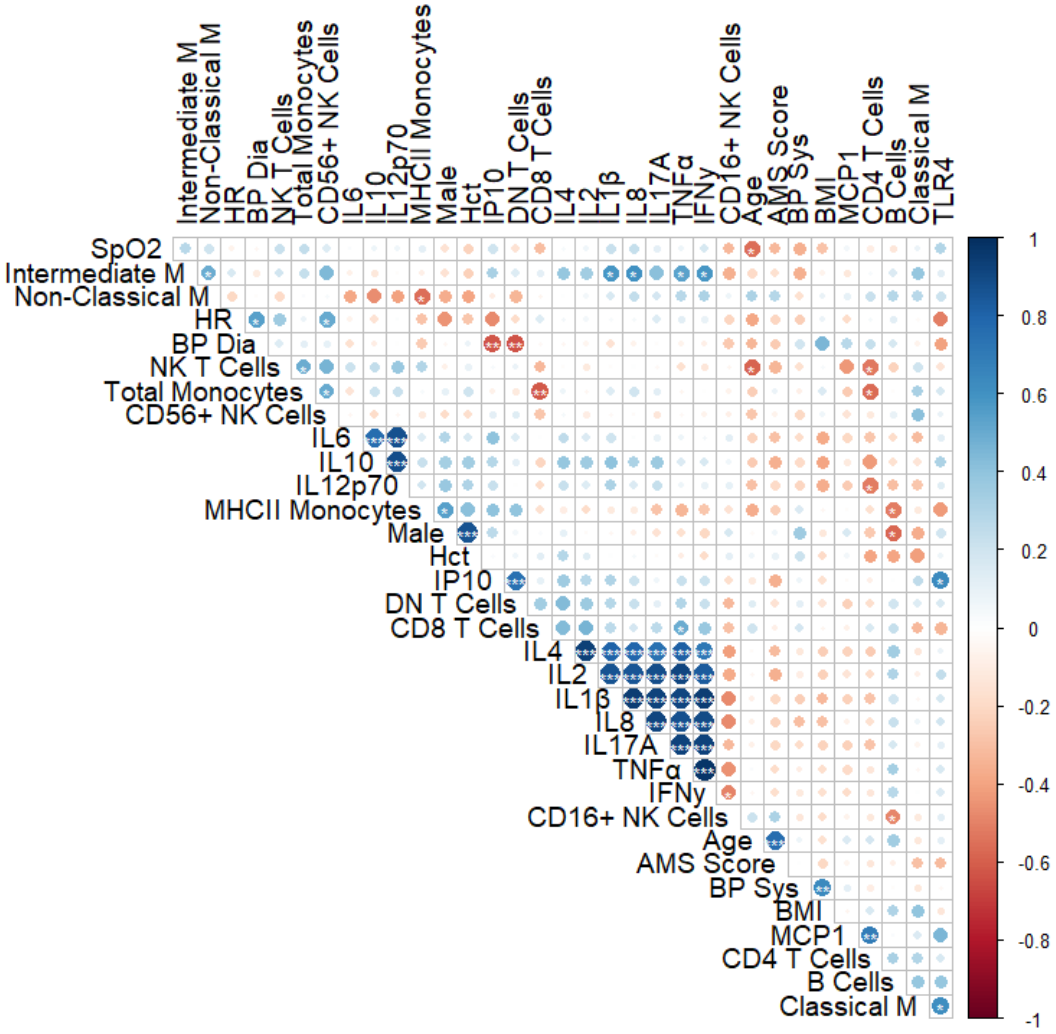

Figure 4.S1. Correlation Matrix of demographic, immune, and cytokine parameters on High-Altitude Day 1.

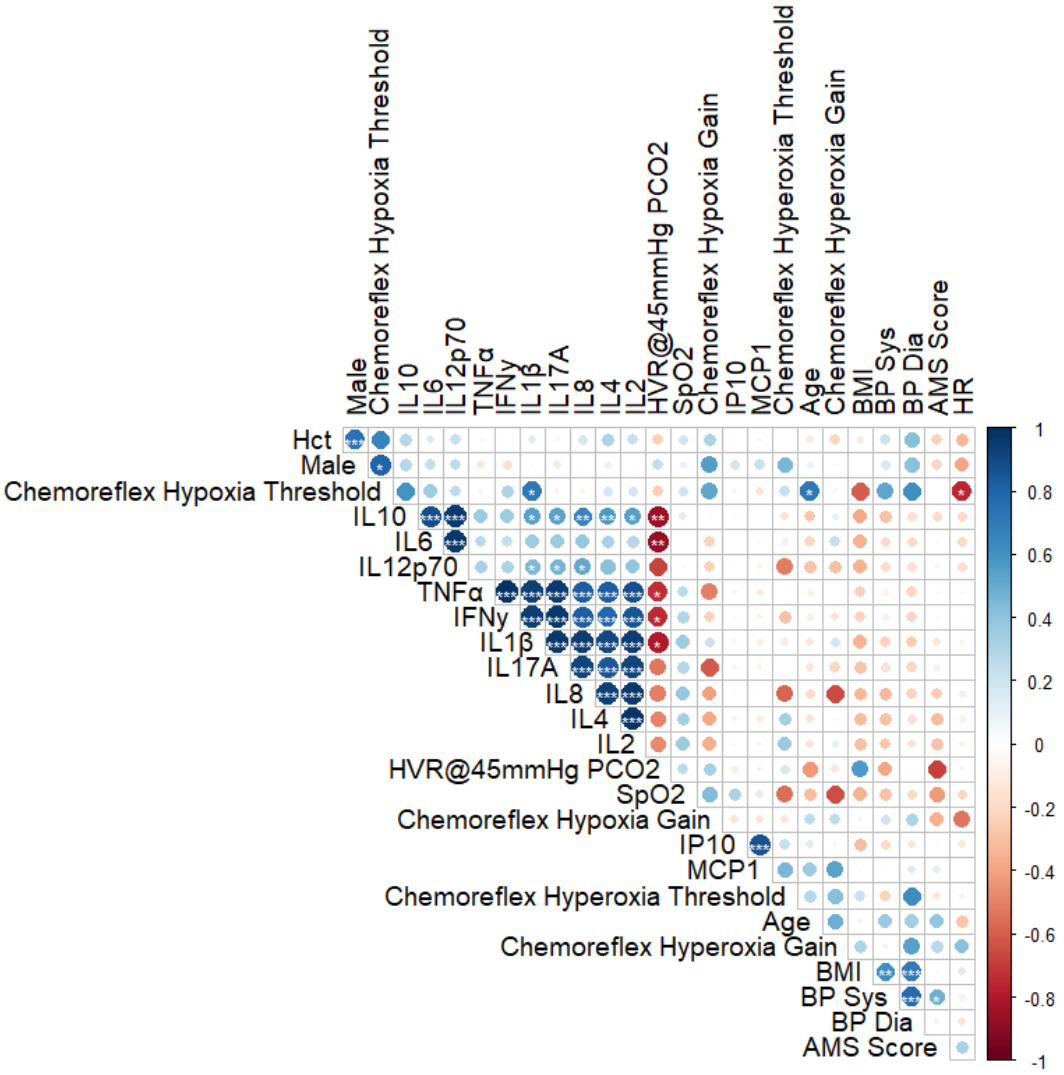

Figure 4.S2. Correlation Matrix of demographic, chemoreflex, and cytokine parameters on High-Altitude Day 2.

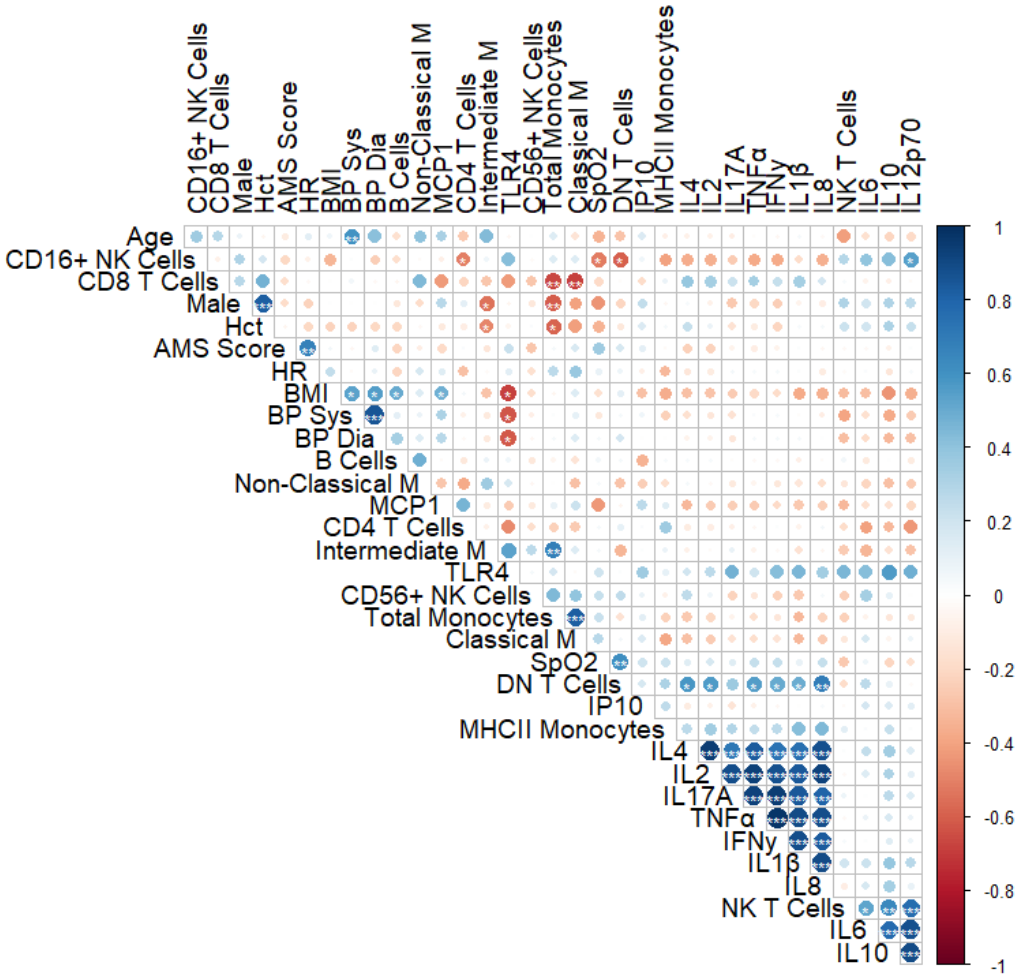

Figure 4.S3. Correlation Matrix of demographic, immune, and cytokine parameters on High-Altitude Day 3.

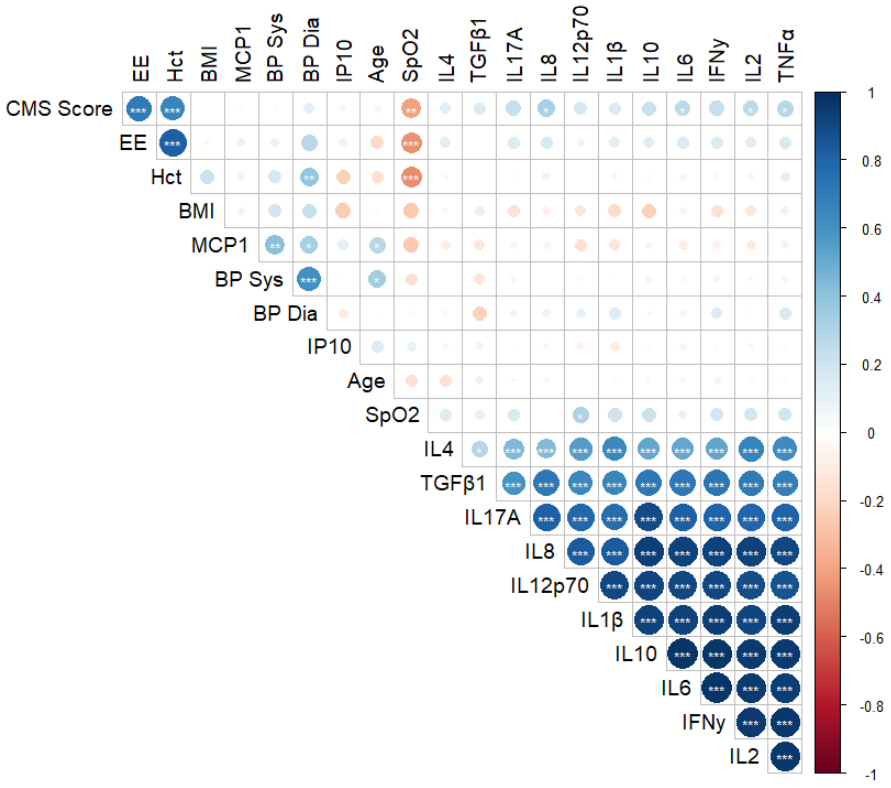

Figure 4.S4. Correlation Matrix of native Andeans with or without CMS or EE.

Table 4.1. Physiological measures at baseline and over three days at altitude

Acute Time Domain (Sojourners to Altitude)					
Variable	**SL**	**HA 1**	**HA 2**	**HA 3**	**ANOVA P**
P_{sys}	121 ± 7.1	126 ± 9.5	128 ± 13	126 ± 11.	0.177
P_{dia}	79 ± 5	85 ± 7.1	85 ± 8.4	85 ± 8.7	0.059
HR	74 ± 8.4	91 ± 13	90 ± 15*	100 ± 16***	<0.001
SpO_2	95 ± 1.7	84 ± 4.3***	84 ± 3.2***	84 ± 4.1***	<0.001
AMS	0.5 ± 0.6	4.3 ± 2.7***	4 ± 2.4**	2.6 ± 2.5*	<0.001
BMI	30.5 ± 5.2	-	-	-	-
Age	25.6 ± 6.1	-	-	-	-

Variable units: P_{sys} and P_{dia} (mmHg); HR (bpm); SpO_2 (%); BMI (kg/m^2). Overall p-values for repeated measures ANOVA are provided. Asterisks indicate significant differences from SL at p<0.05 (*), p<0.01 (**), and p<0.001 (***) levels via post-hoc pairwise comparisons with Bonferroni adjusted p-values.

Table 4.2. Physiological measures in native Andean highlanders with or without Chronic Mountain Sickness (CMS).

Chronic Time Domain (Native Andean Highlanders)			
Variable	**Healthy**	**CMS**	**P-value**
P_{sys}	111 ± 18.9	113 ± 9.3	0.252
P_{dia}	70.3 ± 10.1	75.5 ± 8.7	**0.026***
SpO_2	88.7 ± 3.8	85.0 ± 3.5	**<0.001*****
Hct	55.0 ± 5.0	67.7 ± 4.3	**<0.001*****
CMS	1.3 ± 1.5	5.9 ± 2.9	**<0.001*****
BMI	26.5 ± 4.5	26.8 ± 3.1	0.39
Age	41.4 ± 11.5	37.1 ±10.4	0.067

Variable units: P_{sys} and P_{dia} (mmHg); SpO_2 (%); Hct (%); BMI (kg/m^2). Overall p-values for unpaired t-tests are provided. Asterisks indicate significant differences from "Healthy" at p<0.05 (*), p<0.01 (**), and p<0.001 (***) levels.

Table 4.3. Relationships between cytokines, phenotypes, and immune populations on first day at altitude (HA1).

Variable 1	Variable 2	Correlation (r)	p-value	adj. p
Male	**Hct**	0.87	3.41E-06	1.20E-04
Age	**AMS Score**	0.76	2.50E-04	0.0059
IP10	**DN Tcells**	0.74	6.70E-04	0.014
MCP1	**CD4+ T cells**	0.69	0.0021	0.042
BP (Sys)	**BMI**	0.64	0.0061	0.12
IP10	**BP (Dia)**	-0.63	0.0072	0.13
BP (Dia)	**DN Tcells**	-0.63	0.0083	0.15
IL8	**Intermediate Monocytes**	0.60	0.01	0.18
IFNy	**Intermediate Monocytes**	0.59	0.013	0.21
IL1b	**Intermediate Monocytes**	0.59	0.013	0.22
Age	**NK T cells**	-0.58	0.014	0.22
Male	**B Cells**	-0.57	0.016	0.25
Age	**SpO_2**	-0.55	0.021	0.30
Male	**MHC II Monocytes**	0.54	0.024	0.33
Heart Rate	**BP (Dia)**	0.54	0.024	0.33
TNFα	**Intermediate Monocytes**	0.53	0.028	0.36
IP10	**TLR4**	0.64	0.035	0.43
IL12p70	**CD4+T Cells**	-0.51	0.036	0.43
IFNy	**CD16+ NK cells**	-0.49	0.043	0.47
TNFα	**CD8 T cells**	0.49	0.044	0.47
Heart Rate	**CD56+ NK Cells**	0.51	0.045	0.47
Heart Rate	**CD16+ NK cells**	-0.48	0.052	0.52
IP10	**HR**	-0.47	0.055	0.54
IL1b	**CD16+ NK cells**	-0.47	0.056	0.54
IL10	**Non-Classical Monocytes**	-0.47	0.059	0.56
BP (Dia)	**BMI**	0.46	0.064	0.58
TNFα	**CD16+ NK cells**	-0.45	0.069	0.61

Table 4.4. Relationships between cytokines, phenotypes, and chemoreflex parameters on second day at altitude (HA2).

Variable 1	Variable 2	Correlation (r)	p-value	adj. p
BP (Sys)	**BP (Dia)**	0.78	5.30E-05	6.10E-04
Male	**Hct**	0.73	2.30E-04	0.0026
BP (Dia)	**BMI**	0.70	5.20E-04	0.0056
BP (Sys)	**BMI**	0.61	0.0043	0.043
IL6	**HVR**	-0.87	0.0051	0.049
IL10	**HVR**	-0.86	0.0063	0.059
Male	**Chemoreflex Hypoxia Threshold**	0.81	0.015	0.12
IL1b	**HVR**	-0.79	0.018	0.15
AMS Score	**BP (Sys)**	0.49	0.029	0.22
Heart Rate	**Chemoreflex Hypoxia Threshold**	-0.76	0.030	0.22
IFNy	**HVR**	-0.74	0.035	0.25
TNFα	**HVR**	-0.74	0.037	0.26
IL1b	**Chemoreflex Hypoxia Threshold**	0.72	0.045	0.30
Hematocrit	**BP (Dia)**	0.42	0.062	0.39
AMS Score	**HVR**	-0.68	0.063	0.39
SpO_2	**AMS**	-0.42	0.064	0.39
IL12p70	**HVR**	-0.67	0.067	0.39
Male	**BP (Dia)**	0.42	0.068	0.39
Hematocrit	**Chemoreflex Hypoxia Threshold**	0.67	0.069	0.39

Table 4.5. Relationships between cytokines, phenotypes, and immune populations on third day at altitude (HA3).

Variable 1	Variable 2	Correlation (r)	p-value	adj. p
BP (Sys)	**BP (Dia)**	0.86	1.43E-06	5.00E-05
Male	**Hct**	0.83	1.30E-05	3.50E-04
IL12p70	**DN T Cells**	-0.03	3.50E-04	0.98
AMS Score	**Heart Rate**	0.68	0.0011	0.02
IL8	**DN T Cells**	0.69	0.0022	0.04
IL10	**NK T cells**	0.65	0.005	0.08
Age	**BP (Sys)**	0.59	0.0061	0.10
SpO_2	**DN T Cells**	0.61	0.0093	0.14
Male	**Total Monocytes**	-0.61	0.0093	0.14
BP (Dia)	**BMI**	0.56	0.011	0.16
IL4	**DNT cells**	0.59	0.013	0.18
Hematocrit	**Total Monocytes**	-0.58	0.014	0.19
BP (Sys)	**BMI**	0.54	0.015	0.20
IL2	**DNT cells**	0.57	0.017	0.23
BMI	**TLR4**	-0.69	0.02	0.25
TNFα	**DNT cells**	0.56	0.02	0.25
IL12p70	**NK T cells**	0.76	0.023	0.0076
Male	**Intermediate Monocytes**	-0.54	0.024	0.29
IL6	**NK T cells**	0.53	0.03	0.35
SpO_2	**CD16+ NK Cells**	-0.52	0.034	0.39
MCP1	**BMI**	0.49	0.035	0.39
BP (Sys)	**TLR4**	-0.63	0.038	0.41
IFNy	**DN T Cells**	0.51	0.038	0.41
BMI	**B Cells**	0.50	0.04	0.42
BP (Dia)	**TLR4**	-0.61	0.045	0.46
IL1b	**DNT cells**	0.49	0.046	0.46
Hematocrit	**Intermediate Monocytes**	-0.48	0.049	0.48
Male	**SpO_2**	-0.44	0.051	0.48
Hematocrit	**CD8+ T cells**	0.47	0.057	0.53
IL10	**BMI**	-0.44	0.058	0.53
MCP1	**CD4T Cells**	0.47	0.059	0.53
MCP1	**SpO_2**	-0.44	0.062	0.55
Age	**BP (Dia)**	0.42	0.067	0.59

Table 4.6. Relationships between cytokines and phenotypes in native Andean highlanders. Excessive erythrocytosis (EE).

Variable 1	Variable 2	Correlation (r)	p-value	adj. p
EE	**Hematocrit**	0.81	1.28E-15	7.48E-15
EE	**CMS Score**	0.71	5.03E-10	2.20E-09
Hematocrit	**CMS Score**	0.65	3.19E-08	1.32E-07
BP Sys	**BP Dia**	0.61	2.30E-06	8.63E-06
Hematocrit	**SpO_2**	-0.47	3.78E-04	0.0013
EE	**SpO_2**	-0.46	5.06E-04	0.0018
MCP1	**BP (Sys)**	0.41	0.0025	0.0086
CMS Score	**SpO_2**	-0.40	0.0029	0.0098
Hematocrit	**BP Dia**	0.39	0.0043	0.014
IL8	**CMS Score**	0.33	0.012	0.039
BP Sys	**Age**	0.34	0.013	0.04
MCP1	**BP(Dia)**	0.34	0.015	0.046
IL12p70	**SpO_2**	0.30	0.026	0.081
MCP1	**Age**	0.28	0.031	0.095
TNFα	**CMS Score**	0.28	0.032	0.095
IL2	**CMS Score**	0.26	0.045	0.13
IL6	**CMS Score**	0.26	0.048	0.14
EE	**BP Dia**	0.27	0.053	0.15
MCP1	**SpO_2**	-0.26	0.054	0.15
SpO_2	**BMI**	-0.27	0.059	0.93
IP10	**Hct**	-0.23	0.07	0.19

Table 4.S1. All relationships between cytokines, phenotypes, and immune populations on first day at altitude (HA1).

Variable 1	Variable 2	Correlation (r)	p-value	adj. p
TNFα	**IFNy**	0.97	1.52E-11	8.52E-09
IL1b	**IL8**	0.95	2.44E-09	6.83E-07
IL1b	**IFNy**	0.94	5.58E-09	1.04E-06
IL4	**IL2**	0.93	1.71E-08	2.39E-06
IL1b	**IL17A**	0.93	3.06E-08	2.93E-06
IL17A	**IFNy**	0.93	3.14E-08	2.93E-06
TNFα	**IL17A**	0.92	4.97E-08	3.98E-06
IL1b	**TNFα**	0.92	8.70E-08	6.10E-06
IL2	**TNFα**	0.92	9.97E-08	6.22E-06
IL17A	**IL8**	0.91	1.59E-07	8.90E-06
IFNy	**IL8**	0.89	6.99E-07	3.56E-05
IL10	**IL12p70**	0.89	9.07E-07	3.92E-05
IL2	**IL17A**	0.89	9.09E-07	3.92E-05
IL6	**IL12p70**	0.88	1.87E-06	7.49E-05
TNFα	**IL8**	0.87	2.44E-06	9.11E-05
Male	**Hct**	0.87	3.41E-06	1.20E-04
IL2	**IL1b**	0.86	3.94E-06	1.30E-04
IL2	**IL8**	0.86	4.94E-06	1.54E-04
IL2	**IFNy**	0.84	1.37E-05	4.04E-04
IL4	**TNFα**	0.82	3.47E-05	9.74E-04
IL4	**IL1b**	0.81	5.44E-05	1.45E-03
IL4	**IL8**	0.79	8.51E-05	2.17E-03
IL6	**IL10**	0.76	2.24E-04	5.48E-03
Age	**AMS Score**	0.76	2.50E-04	5.85E-03
IL4	**IL17A**	0.75	3.67E-04	8.24E-03
IP10	**DN Tcells**	0.74	6.68E-04	1.44E-02
IL4	**IFNy**	0.71	8.86E-04	1.84E-02
MCP1	**CD4+ T cells**	0.69	2.10E-03	4.20E-02
BP(Sys)	**BMI**	0.64	6.12E-03	1.18E-01
IP10	**BP (Dia)**	-0.63	7.18E-03	1.34E-01
BP(Dia)	**DN Tcells**	-0.63	8.26E-03	1.49E-01
IL8	**Intermediate Monocytes**	0.60	1.05E-02	1.78E-01
IFNy	**Intermediate Monocytes**	0.59	1.27E-02	2.10E-01
IL1b	**Intermediate Monocytes**	0.59	1.35E-02	2.16E-01
Age	**NK T cells**	-0.58	1.39E-02	2.17E-01
Male	**B Cells**	-0.57	1.62E-02	2.46E-01
Age	**SpO_2**	-0.55	2.11E-02	2.96E-01
Male	**MHC II Monocytes**	0.54	2.44E-02	3.26E-01
HR	**BP (Dia)**	0.54	2.44E-02	3.26E-01
TNFα	**Intermediate Monocytes**	0.53	2.78E-02	3.63E-01
IP10	**TLR4**	0.64	3.46E-02	4.32E-01

IL12p70	**CD4+T Cells**	-0.51	3.60E-02	4.34E-01
IFNy	**CD16+ NK cells**	-0.49	4.34E-02	4.68E-01
TNFα	**CD8+ T cells**	0.49	4.44E-02	4.70E-01
HR	**CD56+ NK Cells**	0.51	4.52E-02	4.70E-01
HR	**CD16+ NK cells**	-0.48	5.24E-02	5.25E-01
IP10	**HR**	-0.47	5.46E-02	5.38E-01
IL1b	**CD16+ NK cells**	-0.47	5.57E-02	5.39E-01
IL10	**Non-Classical Monocytes**	-0.47	5.89E-02	5.59E-01
BP(Dia)	**BMI**	0.46	6.42E-02	5.81E-01
TNFα	**CD16+ NK cells**	-0.45	6.92E-02	6.13E-01

Table 4.S2. All relationships between cytokines, phenotypes, and chemoreflex parameters on second day at altitude (HA2).

Variable 1	Variable 2	Correlation (r)	p-value	adj. p
TNFα	IFNy	0.99	1.27E-16	3.81E-14
IL4	IL2	0.98	6.07E-14	9.10E-12
IL6	IL12p70	0.97	2.86E-12	2.86E-10
IL2	IL8	0.96	1.18E-11	8.53E-10
IL17A	IFNy	0.96	1.42E-11	8.53E-10
IL1b	IL17A	0.96	2.13E-11	1.07E-09
IL10	IL12p70	0.96	4.43E-11	1.90E-09
TNFα	IL17A	0.95	6.86E-11	2.57E-09
IL1b	IL8	0.95	3.19E-10	1.06E-08
IL2	IL1b	0.94	5.38E-10	1.61E-08
IL1b	IFNy	0.94	5.95E-10	1.62E-08
IL1b	TNFα	0.94	1.03E-09	2.58E-08
IL4	IL8	0.93	2.83E-09	6.54E-08
IL17A	IL8	0.92	1.25E-08	2.69E-07
IL2	IL17A	0.92	1.35E-08	2.69E-07
IL4	IL1b	0.91	4.20E-08	7.88E-07
IL6	IL10	0.89	2.17E-07	3.84E-06
IL2	TNFα	0.88	3.91E-07	6.52E-06
IP10	MCP1	0.86	1.02E-06	1.61E-05
IL2	IFNy	0.86	1.30E-06	1.95E-05
IL4	IL17A	0.85	2.53E-06	3.61E-05
IFNy	IL8	0.83	6.58E-06	8.98E-05
IL4	TNFα	0.82	7.99E-06	1.04E-04
TNFα	IL8	0.82	8.29E-06	1.04E-04
IL4	IFNy	0.79	3.79E-05	4.55E-04
BP (Sys)	BP (Dia)	0.78	5.26E-05	6.07E-04
Male	Hct	0.73	2.33E-04	2.59E-03
BP (Dia)	BMI	0.70	5.23E-04	5.60E-03
IL10	IL8	0.67	1.26E-03	1.30E-02
BP (Sys)	BMI	0.61	4.25E-03	4.25E-02
IL6	HVR	-0.87	5.06E-03	4.90E-02
IL10	HVR	-0.86	6.26E-03	5.87E-02
IL4	IL10	0.57	8.48E-03	7.71E-02
IL1b	IL10	0.55	1.25E-02	1.08E-01
IL2	IL10	0.55	1.26E-02	1.08E-01
Male	Chemoreflex Hypoxia Threshold	0.81	1.50E-02	1.24E-01
IL17A	IL10	0.53	1.53E-02	1.24E-01
IL1b	HVR45	-0.79	1.84E-02	1.45E-01
IL12p70	IL8	0.51	2.03E-02	1.56E-01
AMS Score	BP (Sys)	0.49	2.92E-02	2.19E-01
HR	Chemoreflex Hypoxia Threshold	-0.76	3.02E-02	2.21E-01
IFNy	HVR45	-0.74	3.47E-02	2.48E-01

TNFα	**HVR45**	-0.74	3.69E-02	2.58E-01
IL17A	**IL12p70**	0.46	3.93E-02	2.68E-01
IL1b	**Chemoreflex Hypoxia Threshold**	0.72	4.47E-02	2.98E-01
IL1b	**IL12p70**	0.45	4.82E-02	3.15E-01
Hct	**BP (Dia)**	0.42	6.23E-02	3.91E-01
AMS Score	**HVR45**	-0.68	6.25E-02	3.91E-01
SpO_2	**AMS**	-0.42	6.43E-02	3.92E-01
IL4	**IL12p70**	0.42	6.64E-02	3.92E-01
IL12p70	**HVR45**	-0.67	6.73E-02	3.92E-01
Male	**BP (Dia)**	0.42	6.79E-02	3.92E-01
Hct	**Chemoreflex Hypoxia Threshold**	0.67	6.94E-02	3.93E-01

Table 4.S3. All relationships between cytokines, phenotypes, and immune populations on third day at altitude (HA3).

Variable 1	Variable 2	Correlation (r)	p-value	adj. p
Age	BP (Sys)	0.59	6.09E-03	9.76E-02
Age	BP (Dia)	0.42	6.68E-02	5.86E-01
Male	SpO_2	-0.44	5.06E-02	4.81E-01
Male	Hct	0.83	1.30E-05	3.48E-04
Male	Total Monocytes	-0.61	9.34E-03	1.42E-01
Male	Intermediate Monocytes	-0.54	2.39E-02	2.86E-01
IL4	IL2	0.94	1.64E-09	3.96E-07
IL4	Il1b	0.76	1.81E-04	4.06E-03
IL4	TNFα	0.83	1.08E-05	3.03E-04
IL4	IL17A	0.72	5.78E-04	1.16E-02
IL4	IFNy	0.73	3.78E-04	7.86E-03
IL4	IL8	0.87	9.73E-07	4.10E-05
IL4	DNT cells	0.59	1.28E-02	1.80E-01
IL2	Il1b	0.84	5.44E-06	1.69E-04
IL2	TNFα	0.93	5.55E-09	7.78E-07
IL2	IL17A	0.88	5.22E-07	2.66E-05
IL2	IFNy	0.87	1.02E-06	4.10E-05
IL2	IL8	0.93	1.00E-08	1.12E-06
IL2	DNT cells	0.57	1.73E-02	2.26E-01
IL1b	TNFα	0.89	3.10E-07	1.93E-05
IL1b	IL17A	0.85	5.03E-06	1.66E-04
IL1b	IFNy	0.89	4.72E-07	2.65E-05
IL1b	IL8	0.89	2.95E-07	1.93E-05
IL1b	DNT cells	0.49	4.58E-02	4.58E-01
TNFα	IL17A	0.92	1.54E-08	1.44E-06
TNFα	IFNy	0.98	7.65E-13	4.29E-10
TNFα	IL8	0.88	6.12E-07	2.86E-05
TNFα	DNT cells	0.56	1.97E-02	2.46E-01
MCP1	SpO_2	-0.44	6.17E-02	5.50E-01
MCP1	BMI	0.49	3.47E-02	3.89E-01
MCP1	CD4+ T Cells	0.47	5.87E-02	5.31E-01
IL17A	IFNy	0.94	2.12E-09	3.96E-07
IL17A	IL8	0.81	3.04E-05	7.75E-04
IL6	IL10	0.77	1.07E-04	2.50E-03
IL6	IL12p70	0.87	1.28E-06	4.80E-05
IL6	NK T cells	0.53	2.99E-02	3.50E-01
IL10	IL12p70	0.89	2.72E-07	1.93E-05
IL10	BMI	-0.44	5.75E-02	5.29E-01
IL10	NK T cells	0.65	5.05E-03	8.33E-02
IFNy	IL8	0.83	1.01E-05	2.97E-04
IFNy	DN T Cells	0.51	3.80E-02	4.10E-01
IL12p70	NK T cells	0.76	2.26E-02	7.61E-03
IL12p70	DN T Cells	-0.03	3.53E-04	9.81E-01

IL8	**DN T Cells**	0.69	2.16E-03	4.03E-02
SpO_2	**CD16+ NK Cells**	-0.52	3.43E-02	3.89E-01
SpO_2	**DN T Cells**	0.61	9.29E-03	1.42E-01
Hct	**Total Monocytes**	-0.58	1.38E-02	1.89E-01
Hct	**CD8+ T cells**	0.47	5.68E-02	5.29E-01
Hct	**Intermediate Monocytes**	-0.48	4.95E-02	4.81E-01
AMS Score	**HR**	0.68	1.06E-03	2.05E-02
BP (Sys)	**BP (Dia)**	0.86	1.43E-06	5.00E-05
BP (Sys)	**BMI**	0.54	1.50E-02	2.00E-01
BP (Sys)	**TLR4**	-0.63	3.79E-02	4.10E-01
BP (Dia)	**BMI**	0.56	1.08E-02	1.59E-01
BP (Dia)	**TLR4**	-0.61	4.54E-02	4.58E-01
BMI	**B Cells**	0.50	3.98E-02	4.17E-01
BMI	**TLR4**	-0.69	1.95E-02	2.46E-01

Table 4.S4. All relationships between cytokines and phenotypes in native Andean highlanders. Excessive erythrocytosis (EE).

Variable 1	Variable 2	Correlation (r)	p-value	adj. p
EE	**Hct**	0.81	1.28E-15	7.48E-15
EE	**CMS Score**	0.71	5.03E-10	2.20E-09
EE	**BP Dia**	0.27	5.27E-02	1.52E-01
EE	**SpO_2**	-0.46	5.06E-04	1.77E-03
IL4	**IL2**	0.67	4.95E-11	2.36E-10
IL4	**IL1b**	0.64	3.89E-10	1.74E-09
IL4	**TNFα**	0.63	1.31E-09	5.61E-09
IL4	**IL17A**	0.45	4.97E-05	1.83E-04
IL4	**IL6**	0.52	1.89E-06	7.27E-06
IL4	**IL10**	0.52	1.90E-06	7.27E-06
IL4	**IFNy**	0.53	9.12E-07	3.61E-06
IL4	**IL12p70**	0.56	1.47E-07	5.93E-07
IL4	**IL8**	0.44	7.47E-05	2.71E-04
IL4	**TGFb**	0.29	1.21E-02	3.91E-02
IL2	**IL1b**	0.91	3.08E-30	3.23E-29
IL2	**TNFα**	0.96	9.01E-43	3.78E-41
IL2	**IL17A**	0.80	8.63E-18	5.33E-17
IL2	**IL6**	0.95	1.52E-39	4.57E-38
IL2	**IL10**	0.95	3.43E-39	8.00E-38
IL2	**IFNy**	0.95	2.30E-39	6.03E-38
IL2	**IL12p70**	0.89	8.76E-27	7.08E-26
IL2	**IL8**	0.92	1.55E-32	1.81E-31
IL2	**TGFb**	0.71	1.08E-12	5.40E-12
IL2	**CMS Score**	0.26	4.50E-02	1.33E-01
IP10	**Hct**	-0.23	7.00E-02	1.93E-01
IL1b	**TNFα**	0.94	2.43E-36	4.26E-35
IL1b	**IL17A**	0.76	1.62E-15	9.17E-15
IL1b	**IL6**	0.93	7.54E-33	9.89E-32
IL1b	**IL10**	0.92	1.02E-32	1.26E-31
IL1b	**IFNy**	0.95	2.65E-38	5.57E-37
IL1b	**IL12p70**	0.90	3.52E-29	3.21E-28
IL1b	**IL8**	0.84	3.10E-21	2.33E-20
IL1b	**TGFb**	0.65	1.78E-10	8.30E-10
TNFα	**IL17A**	0.81	8.49E-19	5.57E-18
TNFα	**IL6**	0.95	2.92E-38	5.58E-37
TNFα	**IL10**	0.95	1.42E-39	4.57E-38
TNFα	**IFNy**	0.96	3.98E-44	2.09E-42
TNFα	**IL12p70**	0.87	2.07E-24	1.61E-23
TNFα	**IL8**	0.90	8.19E-29	7.17E-28
TNFα	**TGFb**	0.69	6.68E-12	3.26E-11
TNFα	**CMS Score**	0.28	3.15E-02	9.45E-02
MCP1	**BP (Sys)**	0.41	2.51E-03	8.63E-03
MCP1	**BP(Dia)**	0.34	1.48E-02	4.63E-02
MCP1	**SpO_2**	-0.26	5.38E-02	1.53E-01

MCP1	**Age**	0.28	3.14E-02	9.45E-02
IL17A	**IL6**	0.81	5.45E-19	3.69E-18
IL17A	**IL10**	0.90	9.75E-28	8.19E-27
IL17A	**IFNy**	0.81	1.04E-18	6.65E-18
IL17A	**IL12p70**	0.78	4.98E-17	2.99E-16
IL17A	**IL8**	0.81	4.53E-19	3.17E-18
IL17A	**TGFb**	0.59	1.81E-08	7.62E-08
IL6	**IL10**	0.98	3.45E-51	3.63E-49
IL6	**IFNy**	0.98	1.10E-52	2.31E-50
IL6	**IL12p70**	0.91	1.84E-29	1.84E-28
IL6	**IL8**	0.93	5.61E-33	7.86E-32
IL6	**TGFb**	0.72	3.14E-13	1.66E-12
IL6	**CMS Score**	0.26	4.81E-02	1.40E-01
IL10	**IFNy**	0.91	6.32E-50	4.43E-48
IL10	**IL12p70**	0.94	9.57E-31	1.06E-29
IL10	**IL8**	0.72	1.75E-35	2.82E-34
IL10	**TGFb**	0.22	3.17E-13	1.66E-12
IFNy	**IL12p70**	0.91	3.09E-29	2.95E-28
IFNy	**IL8**	0.93	5.07E-33	7.60E-32
IFNy	**TGFb**	0.72	1.56E-13	8.60E-13
IL12p70	**IL8**	0.83	1.73E-20	1.25E-19
IL12p70	**TGFb**	0.65	2.98E-10	1.36E-09
IL12p70	**SpO_2**	0.30	2.63E-02	8.12E-02
IL8	**TGFb**	0.71	4.17E-13	2.14E-12
IL8	**CMS Score**	0.33	1.18E-02	3.87E-02
Hct	**CMS Score**	0.65	3.19E-08	1.32E-07
Hct	**BP Dia**	0.39	4.32E-03	1.44E-02
Hct	**SpO_2**	-0.47	3.78E-04	1.35E-03
CMS Score	**SpO_2**	-0.40	2.91E-03	9.84E-03
BP Sys	**BP Dia**	0.61	2.30E-06	8.63E-06
BP Sys	**Age**	0.34	1.27E-02	4.04E-02
SpO_2	**BMI**	-0.27	5.92E-02	9.27E-01

Chapter 5

Related Projects

Chapter 5.2 - The normal distribution of the hypoxic ventilatory response and methodological impacts: a meta-analysis and computational investigation

Chapter 5.3 - Health disparities in COVID-19: immune and vascular changes are linked to disease severity and persist in a high-risk population in Riverside County, California

5.1 INTRODUCTION

In addition to the role of inflammation and immune function at high altitude, there are several decades of research investigating the hypoxic ventilatory response (HVR), the reflexive increase in breathing in response to reduced arterial oxygen pressure. However, it is necessary to determine the overall distribution of this reflex, as variation may not only occur per individual, but across different populations. Furthermore, by characterizing the normal distribution of the HVR, this will assist to guide clinical decisions in situations where arterial oxygen pressure is affected, such as if patients are hypoxemic. For example, COVID-19 infection can cause profound hypoxemia in patients, but when coupled with low HVR, this may manifest as “silent hypoxemia”, where patients, despite low oxygen saturation, do not experience breathing discomfort (Tobin, 2020; Xie et al., 2020; Tobin et al., 2020b, 2020a; Dhont et al., 2020; Chandra et al., 2020; Wilkerson et al., 2020; Allali et al., 2020; Bickler et al., 2021a; Simonson et al., 2021). This is a dangerous phenomenon, as this may mask underlying symptoms and increase the risk of mortality. Additionally, COVID-19 may have long-lasting impact on the inflammatory and immune profile, which also have physiological consequences (Xie et al., 2020; Ackermann et al., 2020; Westmeier et al., 2020; Lopez et al., 2021; Cheon et al., 2021; Ekstedt et al., 2022). This chapter investigates two components: the normal distribution of HVR in populations and the immune and vascular changes linked to COVID-19 disease severity.

Chapter 5.2

The normal distribution of the hypoxic ventilatory response and methodological impacts: a meta-analysis and computational investigation.

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KEY POINTS

- The hypoxic ventilatory response (HVR) plays a crucial role in determining an individual's predisposition to hypoxia-related pathologies.
- There is notable variability in HVR sensitivity across individuals as well as significant population-level differences.
- We report that the normal distribution of the HVR is positively skewed, with a significant prevalence of low HVR values amongst the general healthy population. We also find no significant impact of the experimental protocol used to induce hypoxia, although HVR is greater with isocapnic versus poikilocapnic methods.
- These results provide insight into the normal distribution of the HVR, which could be useful in clinical decisions of diseases related to hypoxemia.
- Additionally, the low HVR values found within the general population provides insight into the genetic adaptations found in populations residing in high altitudes.

5.2.1 ABSTRACT

The hypoxic ventilatory response (HVR) is the increase in breathing in response to reduced arterial oxygen pressure. Over several decades, studies have revealed substantial population-level differences in the magnitude of the HVR as well as significant inter-individual variation. In particular, low HVRs occur frequently in Andean high-altitude native populations. However, our group conducted hundreds of HVR measures over several years and commonly observed low responses in sea-level populations as well. As a result, we aimed to determine the normal HVR distribution, whether low responses were common, and to what extent variation in study protocols influence these findings. We conducted a comprehensive search of the literature and examined the distributions of HVR values across 78 studies that utilized step-down/steady-state or progressive hypoxia methods in untreated, healthy human subjects. Of these studies, 35 (59.3%) were moderately positively skewed (skew>0.5), and 21 (35.6%) were significantly positively skewed (skew>1), indicating that lower HVR values are common. The skewness of HVR distributions does not appear to be an artifact of methodology or the unit with which the HVR is reported. Further analysis demonstrated that the use of step-down hypoxia versus progressive hypoxia methods did not have a significant impact on average HVR values, but that isocapnic protocols produced higher HVRs than poikilocapnic protocols. This work provides a reference for expected HVR values and illustrates substantial inter-individual variation in this key reflex. Finally, the prevalence of low HVRs in the general population provides insight into our understanding of blunted HVRs in high-altitude adapted groups.

5.2.2 INTRODUCTION

The hypoxic ventilatory response (HVR) is the increase in breathing in response to reductions in arterial oxygen partial pressure (Pa_{O2}). This reflex is the body's first defense against oxygen limitation. During an acute hypoxic stimulus (seconds to minutes), changes in arterial P_{O2} are detected by the peripheral (carotid body) chemoreceptors. This leads to increased afferent input to the respiratory centers and reflex activation of respiratory muscles which increase tidal volume (V_T) and frequency (f_R) of breathing (Pamenter & Powell, 2016). The amplitude of this response is dependent on the arterial P_{CO2} (Pa_{CO2}), with larger increases in total ventilation occurring for the same drop in Pa_{O2} when Pa_{CO2} is higher.

There is significant individual variation in HVR sensitivity which may drive susceptibility to, or protection against, hypoxia-related pathologies. In the context of high-altitude physiology and medicine, studies have linked HVR to exercise performance at altitude (Schoene *et al.*, 1984; Masuyama *et al.*, 1986), susceptibility to high altitude pulmonary edema (HAPE) (Hackett et al., 1988; Matsuzawa et al., 1989), and development of acute mountain sickness (AMS) (Moore *et al.*, 1986*b*). In clinical cases, high respiratory drive in response to hypoxemia may result in patient self-inflicted lung injury in acute respiratory distress syndrome (ARDS) (Vassilakopoulos *et al.*, 2004; Wang *et al.*, 2005; Spinelli *et al.*, 2020*a*), and high or low hypoxic ventilatory responses may exacerbate central and obstructive sleep apnea syndromes, respectively (Solin *et al.*, 2000; Ainslie *et al.*, 2013). Finally, low HVRs may have contributed to "silent hypoxemia" in COVID-19, in which patients presented with very low arterial oxygen levels but minimal dyspnea (Nouri-Vaskeh *et al*., 2020; Tobin *et al*., 2020a; Bickler *et al*., 2021b; Swenson *et al*., 2021).

Given the critical role of the HVR in determining the physiological consequences of hypoxic episodes, it is important to understand the natural variation in this reflex, how it differs across populations, and how this trait can be used to predict susceptibility to hypoxia-promoted diseases. Furthermore, understanding the normal distribution of this trait among the general population will provide valuable information to guide clinical decisions. This will also inform our understanding of possible genetic determinants of the HVR. Previous work has demonstrated that this ventilatory reflex is low, or “blunted”, in high-altitude Andean native populations compared to other high-altitude native groups, as well as sea-level residents. This blunted HVR has been described as a unique adaptation, or maladaptation, to the high-altitude environment. However, it is possible that this phenotype is also common in lowlanders, and that these individuals may share genetic variants associated with lower HVR. Therefore, the goal of this meta-analysis is to quantitatively examine the available literature on the HVR to determine the normal distribution of this reflex and how methodology impacts the measured ventilatory sensitivity to hypoxia. Throughout several years of collecting HVR measurements in diverse populations, we observed that high HVRs appear to be uncommon in healthy sea-level populations. We therefore aimed to test the hypothesis that low HVR values are more common in the general population and that this result is not dependent on the specific method used. To test this hypothesis, we collected and analyzed HVR values, methodological details, and population demographics from all published studies examining the HVR which include raw data.

5.2.3 METHODS

Literature summary and data extraction

We conducted a literature search following the PRISMA guidelines for systematic reviews and meta-analyses to identify peer-reviewed studies reporting individual HVR measurements in healthy populations (Page *et al*., 2021). The search was conducted on August 12, 2021. Papers were located via PubMed searches for “hypoxic ventilatory response” or “hypoxic chemosensitivity” across any time period. Studies were then screened for titles which indicated that HVR measurements were made or that respiratory reflexes or chemoreflexes were measured in humans, and that the paper was not a review. Studies were then screened for abstracts that indicated the appropriate HVR measures were made and included at least one group of healthy subjects receiving no additional experimental intervention. Finally, a complete review of each remaining paper for methodological details was conducted. Published studies that included mean or raw individual HVR data in table or scatterplot form, as well as sufficient participant demographics and methodological information to determine the type of HVR test conducted, were included in our analyses. At each step of the filtering process, titles, abstracts, or whole studies were evaluated by two reviewers and disagreements were settled by a third reviewer.

Raw data was extracted from eligible studies. Individual HVR measures for each participant had to be provided in a table, as a scatterplot with individual points, or in supplementary data. For scatterplot data, values were extracted from graphs with the Image Calibration tool in ImageJ (National Institutes of Health, MD, USA). In one study, hundreds of HVR measures were provided in scatterplot form. This may have led to the exclusion of some individual data points from this study due to overplotting. Studies

reporting measurements in distinct populations (i.e., high-altitude versus sea-level populations) were separated into subgroups by population to ensure that across-population differences in HVR characteristics were accounted for.

Methodological details for each study were collected as categorical variables. Eligible studies were classified as using step-down/steady-state or progressive hypoxia administration methods. Step-down or steady-state HVR tests involve measuring total ventilation after several minutes of equilibration to two or more constant fractions of inspired oxygen (F_IO_2), arterial oxygen pressures (P_aO_2), or arterial oxygen saturation (SpO_2) targets and calculating the change in ventilation across two O_2 levels. Progressive tests involve rebreathing or other methods which produce a continuous decrease in inspired PO_2 over time. In this case, the HVR is calculated as the curvilinear relationship between total ventilation and PO_2 (Weil *et al.*, 1970). CO_2 status during the protocol was classified as "isocapnic" if end-tidal PCO_2 was maintained at a constant level while O_2 was manipulated, or "poikilocapnic" if end-tidal PCO_2 was allowed to decrease freely with hyperventilation. The elevation at which measurements were made was also recorded. When the altitude of measurement was not provided, it was determined from the location of measurements (if provided). If neither elevation or exact location of measurements were provided, the measurement elevation was assumed to be the elevation of the corresponding author's home institution or institution providing IRB approval. Age was included in the dataset only if individual ages were provided for each participant. Participants were assumed to be of sea-level ancestry if data was collected at sea level and there was no other indication of participant ancestry. Units of measurement (A or L/min/%SpO_2) and target hypoxic values were also recorded (as SpO_2, F_IO_2, P_IO_2, P_AO_2, or P_aO_2).

Collected measurements were limited to cases in which HVR measurements were made under resting conditions at any altitude. Measurements made under additional experimental conditions (e.g., exercise, hypercapnia, head-down tilt angles, sleep) were not included. However, if a study utilizing these treatments also included baseline measures under no treatment condition, these baseline measures were included. In one case, measurements taken with a head up tilt of 85° were utilized (Hildebrandt *et al.*, 2000*a*) since this angle is often utilized in seated participants during HVR measures. If several HVR measures were conducted in short succession (Basaran et al. 2016), only the first measure was included in our dataset, when provided, to allow comparison across studies and prevent the inclusion of HVR values elevated by prior intermittent hypoxia exposure. Of note, several studies conducted HVR measures in duplicate or triplicate and reported means.

For studies targeting a specific inspired oxygen pressure or fraction, an estimated corresponding saturation value was determined to facilitate comparisons of hypoxia targets across studies. These calculations were based on the Severinghaus equations for human blood O_2 dissociation computations at a pH of 7.4 and temperature of 37°C (Severinghaus, 1979). Arterial PO_2 was estimated from inspired PO_2 using the alveolar gas equation with the A-a gradient calculated as (age + 10) / 4 (Sharma *et al.*, 2019; Hantzidiamantis & Amaro, 2022). An alveolar-arterial oxygen gradient of 10 was used if participant age was not provided. This value was determined by calculating the normal A-a gradient for a healthy individual aged 33 years, since this was the average age of all participants in the total dataset.

Comparison with within-lab controlled datasets

In a separate analysis, we gathered HVR values collected in a single laboratory using the same methodology to determine how mean and skew values compared to the larger literature. Each of these studies used isocapnic, step-down hypoxia protocols. 7 datasets were included in this separate analysis, 4 of which are published and 3 unpublished. Of the four published studies, three were included in the larger literature review (Garcia *et al*., 2000b; Hupperets *et al*., 2004; Basaran *et al*., 2016) and one was excluded due to the inclusion of intravenous infusions during testing (Weinger *et al.*, 1998). Three additional unpublished datasets were included which utilized the same protocol. For each dataset, only HVR measures collected at sea level under no treatment condition were included.

Data simulation

Data recovered in the meta-analysis included data reported in two unit types: "A" or "L/min/SpO_2". The unit A is the mathematical parameter which determines the curvilinear shape of the ventilatory response to changes in PO_2, with higher A values representing higher HVRs. Alternatively, the L/min/SpO_2 unit represents the linear change in ventilation as a function of SpO_2 or SaO_2. Since a primary goal of this study is to determine the distributions of HVR values across populations and methodologies, we aimed to determine if HVRs reported in different units were comparably distributed, or if the use of the L/min/SpO_2 unit preferentially produced the skewed distributions we observed.

Similarly, it was unclear if the degree of hypoxia administered during the test impacted the skewness of the data. In particular, we hypothesized that higher positive

skewness would be observed for the same dataset if values were reported in L/min/SpO_2 and utilized a modest SpO_2 target. However, since no dataset included both A values and L/min/SpO_2 values within the same individuals, we prepared a simulated dataset to address this question.

We first simulated a set of 500 random HVR curves, and their corresponding A and V_E^0 values, constrained by the mean and standard deviation of HVR values reported in Weil et al. (1970) (means: A = 180.2 ± 14.5; V_E^0 = 4.8 ± 0.3). For each curve, four P_AO_2 values were chosen (120, 50, 40, 37 mmHg) to represent normoxic as well as mild, moderate, and severe hypoxic targets respectively, based on commonly used HVR protocols. Ventilation values corresponding to each P_AO_2 value were then calculated for each curve based on the following equation:

$$V_E = V_{E0} + \frac{A}{P_{AO2} - 32}$$

To convert these simulated HVR values from A to L/min/%SpO_2 units, we first calculated arterial oxygen saturations at the four chosen P_AO_2 values directly from the standard oxygen dissociation curve using an A-a gradient of 10 as described above. These calculations were conducted using the Kelman strategy which allows the calculation of SO_2 or PO_2 at various temperatures, carbon dioxide levels, and pH levels based on data from Severinghaus (1966) (Kelman 1966). As such, P_AO_2 values of 100, 70, 55, and 48 mmHg correspond to arterial saturations of 96.8, 91.0, 80.4, and 71.1%, respectively, assuming a PCO2 of 40, pH of 7.4, and temperature of 37°C.

HVR values were determined for each of the 500 ventilatory response curves based on the calculated increase in ventilation from baseline (P_AO_2 = 120 mmHg) to each hypoxic target (P_AO_2 = 50, 40, and 37 mmHg) as

$$HVR = \frac{\Delta V_E}{\Delta SaO_2}$$

Statistical analysis

All statistical analyses and data simulations were conducted in R Studio (Version 1.4.1717). Summary statistics were calculated in R using the *psych* package. To determine if data were normally distributed or skewed, Kolmogorov-Smirnov statistics are provided for datasets of N ≥ 50, and Shapiro-Wilks statistics are provided for datasets of N < 50. Comparisons of HVR values across methods were conducted with unpaired t-tests after removal of extreme outliers (see results). Additional general linear models were used to determine if significant relationships were upheld after adjusting for cofactors such as PO_2 or PCO_2 method, sex, and study population. To test for significant differences in mean HVR values across studies with the lab-controlled dataset, one-way ANOVAs and post-hoc Tukey HSD tests were used. For correlation analyses, Shapiro-Wilk normality tests were first conducted, and Spearman rank correlations were utilized for non-normally distributed datasets. Data are reported throughout the paper as means ± standard deviation and error bars represent 95% confidence intervals.

5.2.4 RESULTS

Study filtering

The initial literature search revealed 861 records. Of these, 630 were removed during title filtering and 71 were removed during abstract filtering. A final subset of 160 studies received complete review. After this review, 78 studies remained in the final dataset and provided either mean or raw HVR values and sufficient methodological detail to include in this analysis (**Figure 5.2.1**) (Doekel *et al*., 1976; Zwillich *et al*., 1977; Riley *et al*., 1977; Scoggin *et al*., 1978; Hackett *et al*., 1980, 1988b; Stanley *et al*., 1983;

White *et al*., 1983, 1987; Ward, 1984; Moore *et al*., 1984, 1986c, 1986a; TANAKA *et al*., 1986; Aitken *et al*., 1986; OKITA *et al*., 1987; Regensteiner *et al*., 1988, 1989, 1990; Milledge *et al*., 1988, 1991; Matsuzawa *et al*., 1989; LEVINE *et al*., 1992; Gold *et al*., 1993; Selland *et al*., 1993; Zhuang *et al*., 1993; Reeves *et al*., 1993; Chowdhury *et al*., 1993; Amin *et al*., 1994; Kikuchi *et al*., 1994; Feiner *et al*., 1995; Swenson *et al*., 1995; Harms & Stager, 1995; Markov *et al*., 1996; Sato *et al*., 1996; REDLINE *et al*., 1997; Beall *et al*., 1997; Katayama *et al*., 1999, 2000, 2001, 2002; Garcia *et al*., 2000a, 2000c; Hildebrandt *et al*., 2000a, 2000b; Warren *et al*., 2000; Prisk *et al*., 2000; Zhang & Robbins, 2000; Jokic *et al*., 2000; Muza *et al*., 2001; Teppema *et al*., 2002, 2005, 2006; Bärtsch *et al*., 2002; Pokorski & Marczak, 2003b, 2003a; Bhaumik *et al*., 2003; Drumm *et al*., 2004; Hupperets *et al*., 2004; Koehle *et al*., 2005; Spicuzza *et al*., 2005; Teichtahl *et al*., 2005; Terblanche *et al*., 2005; Brutsaert *et al*., 2005; Karan *et al*., 2005; Foster *et al*., 2005; Lusina *et al*., 2006; Steinback & Poulin, 2007; Faulhaber *et al*., 2012; Kovtun & Voevoda, 2013; Albert & Swenson, 2014; Caravita *et al*., 2015; Basaran *et al*. 2016; Pfoh *et al*., 2016, 2017; Goldberg *et al*., 2017; Smith *et al*., 2017; Broens *et al*., 2019). The complete dataset with additional details including study populations and location of measurements is located in **Table 5.2.S1**. Several individual studies included multiple datasets which we evaluated separately due to differences in methodology, treatment (such as measures made at sea level versus high altitude), or study population. This yielded 132 separate data sets for analysis. Of these 132 datasets, 31 reported HVR units as A values (23.5%) and 101 reported L/min/SpO_2 units (76.5%). 119 datasets utilized isocapnic protocols (90.2%) and 13 used poikilocapnic protocols (9.8%). 93 used progressive hypoxia or rebreathing methods (70.5%) and 39 used step-down or step-down methods (29.5%).

*Su*mmary of HVR measurements across all studies reporting raw data

In the final analysis, 72 datasets reported mean HVR values and 60 datasets provided raw HVR datasets. Across all studies, the mean HVR was 126.0 ± 69.2 for reported A units, and 0.98 ± 0.89 for reported L/min/SpO_2 units. Density plots demonstrating the distribution of measurements in each study that included raw HVR values are provided in **Figure 5.2.2**. We calculated the skewness of HVR distributions for each of these datasets. Of the 60 datasets reporting raw HVR values, 35 (58.3%) were at least moderately positively skewed (skew>0.5), and 21 (35%) were significantly positively skewed (skew>1). Notably, no studies showed moderate or significant negative skew. Therefore, HVR distributions tend to be at least moderately positively skewed in nearly half of all studies, indicating that lower HVR values are more common. Skewness for all studies that included raw HVR measurements was determined via Shapiro-Wilks tests for normality. 24 out of 72 datasets (33.3%) were significantly different from a normal distribution (**Table 5.2.S2**).

Impact of methodology on the HVR and its distribution

Since reported HVR values display significant positive skewness, we aimed to determine if specific HVR methodologies contributed to this result, or to the mean HVR value. For this analysis, we included additional tests with only studies conducted at sea-level since HVR increases with high-altitude acclimatization and differs across native high-altitude populations. The initial analysis revealed two extreme within-study outliers, in study 6 and 24, who were reported to have HVR values of 11 and 7.5 L/min/SpO_2, respectively (**Figure 5.2.S1A**). These outliers were removed in the subsequent analyses. Additionally, datasets 55b-k were removed due to extreme outlier means within these high-altitude acclimatized group of datasets (**Figure 5.2.S1B**). These

studies are later examined independently to explore the impact of high-altitude acclimatization on the HVR.

After removing outliers, the mean HVR across all populations was 126.0 ± 69.2 for studies reporting A units, and 0.70 ± 0.38 L/min/SpO_2. For studies conducted in sea-level residents at sea-level, the mean HVR was 121.0 ± 56.6 for studies reporting A units and 0.65 ± 0.34 L/min/SpO_2. Within studies reporting HVR in L/min/SpO_2 units, both step-down and progressive methods were used to manipulate inspired oxygen. There was no significant impact of progressive versus step-down methods on average HVR across all studies ($t(53.9) = 0.72$, $p=0.469$, **Figure 5.2.3A**), or within sea-level studies only ($t(44.0)=-0.49$, $p=0.626$, **Figure 5.2.3D**). HVRs were higher on average when measured with isocapnic protocols (all studies: $t(13.4)=3.4$, $p=0.004$, **Figure 5.2.3B**; SL only: $t(11.4)=3.0$, $p=0.01$, **Figure 5.2.3E**). This relationship was also observed in studies reporting A units (all studies: $t(28.6)=4.9$, $p<0.001$, **Figure 5.2.3C**; SL only: $t(24.5)=5.3$, $p<0.001$, **Figure 5.2.3F**), however only 2 datasets included A units with poikilocapnic methods. The significant effect of CO_2 method on HVR remained after adjusting for O_2 method and study population in studies reporting HVR in L/min/SpO_2 units ($p=0.002$). This relationship was not upheld in studies reporting A values ($p=0.188$), although there were only 2 datasets in this group utilizing poikilocapnic methods, so we are underpowered to make this comparison in this group. Furthermore, among step-down methods using isocapnic protocols, there was a significant positive association between the end-tidal PCO_2 target and the mean HVR across studies via a Spearman rank correlation analysis ($rho = 0.55$, $p=0.017$) (**Figure 5.2.4**).

Across all studies, the unit used to report HVR did not significantly impact the skew of the data distribution (A: skew = 0.86 ± 0.59, L/min/SpO_2: skew=0.71 ± 0.66,

t(13.8)=0.68, p=0.507, **Figure 5.2.5A**). This remained true after adjusting for methodologies and population in linear models (p=0.741). There was also no impact of step-down versus progressive (t(27.2)=1.3, p=0.208) or isocapnic versus poikilocapnic methods (t(13.4)=-2.1, p=0.059) on skewness (**Figure 5.2.5B-C**), although there was a nonsignificant trend for higher skewness in studies using progressive hypoxia administration and poikilocapnia. Interestingly, linear models examining the impact of unit, methodology, and population on skewness indicated a significant, but modest, impact of CO_2 method on skew in studies reporting HVR in L/min/SpO_2 units (p=0.007, adj. model R^2=0.10), but no impact of CO_2 method in studies reporting A units (p=0.575).

Studies used a wide range of hypoxia targets (**Figure 5.2.6**). The mean SpO_2 hypoxia target was 77.2 ± 7.2%. Since step-down methods allow the participant to stabilize at the hypoxia target for a longer period of time, the targets for studies using this method were typically higher (85.4 ± 6.9%). The mean end-tidal PO_2 hypoxia target was 41.7 ± 3.4 mmHg for all studies and 46.9 ± 2.4 mmHg for step-down methods. The mean F_IO_2 hypoxia target was 9.4 ± 3.4% for all studies, and 10.8 ± 2.5% for step-down methods. Two studies utilized quite low SpO_2 hypoxia targets of 55% and 45% (Hackett *et al.*, 1988; Gold *et al.*, 1993). The study utilizing a 45% target applied a rebreathing technique and this was the threshold at which the test was terminated unless the participant became distressed. The study using a 55% target replicated the Rebuck and Campbell rebreathing technique (Read, 1967). Therefore, both studies with the lowest hypoxia targets would not have maintained these low SpO_2 levels for prolonged periods as is typically done with step-down methods.

To determine if the hypoxia target had a significant impact on HVR, we first conducted Spearman rank correlations on mean HVR values collected at sea-level as a

function of author-reported SpO_2, end-tidal PCO_2, and F_IO_2 hypoxia targets. In the overall dataset, for studies reporting HVR in L/min/SpO_2 units, there was no relationship between reported SpO_2 or end-tidal PO_2 hypoxia target and mean HVR across studies (SpO_2: p=0.99, rho=-0.002; $ETPO_2$: p=0.99, rho=0.003; **Figure 5.2.7A-B**). This remained true after adjusting for study source, sex, and O_2 and CO_2 methods (Adj. model R^2 = 0.35, p=0.445). A trend emerged for higher HVR values at lower F_IO_2 targets (F_IO_2: p=0.05, rho=-0.71; **Figure 5.2.7C**), however this also did not remain after adjusting for study source, sex, and O_2 and CO_2 methods in linear models (Adj. model R^2=-0.46, p=0.733). For studies reporting A units, multiple hypoxia targets were provided only for studies reporting SpO_2 units and there was no significant relationship (p=0.23, rho=0.32; **Figure 5.2.7D**). We then calculated the estimated SpO_2 level for each study using the reported end-tidal PO_2 or inspired PO_2 hypoxia target levels. With this expanded dataset, there was still no significant relationship between SpO_2 hypoxia target and mean HVR (p=0.51), and this result remained after splitting groups into isocapnic and poikilocapnic methods (isocapnic: p=0.38, poikilocapnic: p=0.69). Notably, when investigating these relationships using all available individual HVR values collected at sea-level (N=2303), there were significant increases in HVR at lower target SpO_2 and F_IO_2 targets (SpO_2: R = -0.23, p<0.001; F_IO_2: R=-0.50, p<0.001). This relationship remained for studies reporting F_IO_2 targets, but not for studies reporting SpO_2 after adjusting for study source, sex, and O_2 and CO_2 methods in linear models.

Comparison with within-lab controlled datasets

The mean and distribution of HVR values collected over 7 studies completed in the same laboratory are provided in **Figure 5.2.8**. Within this group, there were some significant differences across datasets (F(6, 9.8 = 5.3, p<0.001)). Dataset B had

significantly higher mean HVR than dataset A and D (adj. $p<0.05$ for all). Dataset C had significantly higher mean HVR values than dataset A, D, and E (adj. $p<0.05$ for all). This difference across studies seems to be explained by the chosen isocapnic P_{CO2} targets. Of the published studies, dataset B utilized an isocapnic target 3.8 mmHg above the eupneic PCO_2 level and dataset C utilized an isocapnic target of 4 mmHg above the normoxic baseline PCO_2. In contrast, studies A and D maintained isocapnia at the eupneic level, resulting in a comparatively lower HVR value. Overall, there was no significant difference in the mean HVR measured across this dataset and the dataset from our literature review ($t(6.6) = -0.76$, $p=0.47$). There was also no significant difference in skewness ($t(7.1) = 1.0$, $p=0.34$) and overall these datasets demonstrated a mean positive skew of 0.35.

Impact of the duration of high-altitude acclimatization on HVR

21 datasets across 7 studies reported HVR values at high altitude over various periods of acclimatization ranging from 1 to 56 days (Muza *et al*., 2001.; White *et al*., 1983, 1987; Hackett *et al*., 1988; Bärtsch *et al*., 2002; Hupperets *et al*., 2004; Basaran *et al.*, 2016; Smith *et al*., 2017). Only one dataset reported A values and was therefore excluded (Muza *et al.*, 2001). Another dataset did not specify the exact time period of acclimatization and was also excluded (Hackett *et al.*, 1988). This left 19 datasets across 6 studies. Among the remaining datasets, the altitude of measurements ranged only from 3800 to 4559 m elevation. Across this relatively narrow elevation range, there was a significant increase in HVR measured across studies as a function of time spent at high altitude ($p<0.001$, $Rho=0.7$) (**Figure 5.2.9A**). Since only one study had reported HVR values after more than 7 days of acclimatization and this study tended to report higher overall HVR values, we investigated if this relationship held after removing these

datapoints at greater than 7 days of acclimatization. While a positive relationship between HVR and time of acclimatization remained in this data subset, it was no longer significant, likely due to limited datasets for analysis and across-study variability ($p=0.344$) (**Figure 5.2.9B**). Finally, the predicted increase in HVR at high altitude compared to sea-level measures was observed ($t(19.2)=6.10$, $p<0.0001$; SL: 0.66 ± 0.37 L/min/SpO_2; HA: 2.28 ± 1.17 L/min/SpO_2) (**Figure 5.2.9C**). There was no significant difference in the skewness of HVR values in datasets collected at high altitude versus sea level ($t(6.8)=-0.61$, $p=0.563$), although future work may be required to address this question since limited datasets were available for comparison at high altitude.

HVR values across sex

Of the 69 datasets including mean data only, 20 datasets across 14 studies included only men, 15 datasets across 5 studies included only women, and 34 datasets across 17 studies included both men and women in their participant cohorts. Of the 60 datasets across 44 studies including raw data, 38 datasets across 24 studies included men, and 10 datasets across 9 studies included women. Only 47% of datasets included individual participant sex data for raw HVR values out of a total of 2442 individual HVR measures. Within these studies including individual participant HVR and sex data, there were 872 measures from men across 24 studies, and 439 measures from women across 9 studies. Notably, a majority of the measures in women were provided in Beall et al. (1997) who report an impressive 418 men and 420 women of high-altitude ancestry. Due to some overplotting in the figures from this paper, we were able to extract 360 of these HVR values in men and 347 of these HVR values in women. This means that a small subset of the remaining measures from other studies were from women (women: 92 measures from 8 studies, men: 512 measures from 23 studies).

To determine if significant differences in HVR were observed across men and women, we first examined data from studies reporting raw HVR values collected in sea-level residents at sea-level, using L/min/SpO_2 units. In linear models adjusted for O_2 and CO_2 method, as well as study source, there was no significant effect of sex on the HVR ($p = 0.196$, Adj $R^2 = 0.271$).

Data simulations

The experimental data from studies included in this meta-analysis demonstrated a potential trend for higher HVR values when lower F_IO_2 hypoxia targets were chosen. Therefore, we also conducted additional tests using simulated data to determine if lower hypoxia targets would be more likely to result in higher HVR values when using step-down methods. 500 complete HVR curves were simulated as described above. **Figure 5.2.10A-B** demonstrates a subset of 10 of these random HVR curves. We then calculated the HVR value for each individual curve at three different levels of hypoxia (PO_2 = 50, 40, and 37 mmHg). There was no impact of the chosen hypoxia target on the skewness of the HVR distribution. However, lower hypoxia targets result in higher mean HVR values and more variation in measurements (**Figure 5.2.11**). Notably, lower PO_2 levels gave higher mean HVR values when calculated as the linear relationship between ventilation rate and corresponding estimated SpO_2.

5.2.5 DISCUSSION

Summary of findings

- Across all studies, the mean HVR is 126.0 ± 69.2 A or 0.98 ± 0.89 L/min/SpO_2.
- HVR distributions are positively skewed, indicating that lower HVR values are more common.

- There is no significant impact of progressive versus step-down hypoxia methods on average HVR.
- Isocapnic methods produce higher HVR measures.
- HVR is elevated at high altitude compared to sea-level and increases as a function of time spent at high altitude.
- This analysis identified no significant differences in HVR across men and women, although women were largely underrepresented in available datasets.

The primary aim of this meta-analysis was to determine if low hypoxic ventilatory responses are common in the general population and how methodology impacts the magnitude and variation in HVR measurements. We examined 118 datasets from 78 separate studies which reported mean or raw HVR values in healthy adults using typical steady-state/step-down or progressive/rebreathing methods. We found that a majority of studies (58.3%) reported at least moderately positively skewed datasets (skewness > 0.5), with over one third (35%) being significantly positively skewed (skewness > 1). Notably, no studies were negatively skewed. Therefore, this result indicates that lower HVR values are typical and high HVR values are relatively uncommon.

The average amplitude of the HVR is further reduced, as expected, in studies using poikilocapnic methods, compared to isocapnic methods in which end-tidal CO_2 is not allowed to fall with hyperventilation, in both step-down/steady-state and progressive hypoxia methodologies (**Figure 5.2.3**). Moreover, methods using higher end-tidal PCO_2 targets for isocapnia produce, on average, higher HVR values (**Figure 5.2.4**). While isocapnic protocols may not represent the natural ventilatory response during environmental hypoxia exposures, in which hypoxia-induced increases in ventilation

reduce arterial PCO_2, isocapnic protocols are essential for quantifying the independent peripheral chemoreflex sensitivity to hypoxia without constantly changing contributions from CO_2 and pH-sensitive peripheral and central chemoreceptors. Isocapnic protocols also allow comparisons of HVR values across individuals and studies.

This comprehensive retrospective analysis of published data clearly documents the general observation made by investigators in this field that there is a wide range of normal hypoxic ventilatory responses. Previously, there has been much discussion about blunted hypoxic ventilatory responses in high-altitude Andean native populations (Moore, 2000; Brutsaert *et al*., 2005; Heinrich *et al*., 2020). It has been argued that this is a unique adaptation observed in this group, particularly when comparing these phenotypes to those observed in Tibetan high-altitude native groups (Beall, 2007). However, we found that low HVR values similar to those observed in Andean groups are also seen in sea-level residents using similar methodologies and that these low responses seem to be more common than previously appreciated. From an evolutionary perspective, the high prevalence of relatively low poikilocapnic HVRs among the general population is perhaps expected due to the fact that humans have evolved under conditions of fairly high oxygen availability with few scenarios in which chronic sustained hypoxia would be experienced. With the exceptions of native high-altitude groups, humans have inhabited primarily lower elevation areas with sufficient atmospheric oxygen conditions. Therefore, there is little selective pressure to drive natural selection on higher hypoxic ventilatory responses.

This meta-analysis also revealed a disparity in data available on the HVR in women compared to men. Of the studies including raw data, only about a quarter of them included women. With this available data, we did not find significant differences in

HVR across sex groups. This is consistent with other studies reporting no differences in HVR across men and women (Bhaumik *et al*., 2003). However, other studies have demonstrated higher (Aitken et al. 1986) and lower (White *et al*., 1983; Goldberg *et al*., 2017) HVRs in women also. It is known that the impact of female horomones, particularly progesterone which is a respiratory stimulant, on hypoxic chemosenstivity may lead to variable results and contribute to this confusion (Regensteiner et al. 1990; Schoene et al. 1981). Hence, this important question requires further study that should include women with HVRs measured in the context of the menstrual cycle phase, using rigorous measures of progesterone levels or menopausal state.

These results also provide insight into the prevalence of silent hypoxemia observed clinically, particularly throughout the COVID-19 pandemic. Based on the studies evaluated here, if an average patient's saturation falls 10 points, (e.g., from 95% to 85%), this would result in only a 2.8 L/min increase in breathing if not coupled with hypercapnia since the average poikilocapnic HVR is 0.28 L/min/SpO_2. Often, moderate hypoxic stimuli are not detected by the participant and these slight increases in total ventilation are achieved without dyspnea when not coupled with hypercapnia or changes in airflow resistance or lung compliance (Simonson et al., 2021). This is illustrated by the significantly lower HVR observed in studies utilizing poikilocapnic methods compared to isocapnic methods. Therefore, if CO_2 retention does not occur, it is unlikely that a substantial 10-point desaturation would cause significant hyperventilation or dyspnea. However, in more advanced cases, hypercapnia or prolonged hypoxemia resulting in ventilatory acclimatization would further increase total ventilation and more effectively stimulate dyspnea (Powell *et al*., 1998; Pamenter & Powell, 2016).

The increase in total ventilation as a function of arterial oxygen saturation has been described as, effectively, a linear function (Edelman *et al.*, 1970; Weil *et al.*, 1970). Based on the analyses conducted here, this does seem to apply when tests are performed with saturations between a 100-80% window. However, after this point, assuming PO_2 and SpO_2 fall along the normal hemoglobin-oxygen dissociation curve, total ventilation begins to increase at a faster rate as saturation continues to fall. This is observed in **Figure 5.2.10 (A-B)** by the increased slope of the ventilation versus PO_2 or SpO_2 curves at lower PO_2 and SpO_2 levels. Based on our data simulations, the degree to which ventilation deviates from this linear relationship below approximately 80% SpO_2 is variable across individuals (**Figure 5.2.10 A-B**). Nonetheless, it appears that HVR tests using two SpO_2 levels as targets can assume a linear relationship between ventilation and SpO_2 as long as the two chosen points are above 80% SpO_2. This is supported by pooled HVR data across studies using several different SpO_2 targets (**Figure 5.2.7**). We saw that the slope of the relationship between mean HVR and SpO_2 target was near zero, indicating that, in practice, lower SpO_2 targets did not yield higher average HVRs. The benefit of this generally linear relationship is that when conducting step-down HVR tests, one could choose any two SpO_2 levels across this linear line and obtain the same HVR value.

Weil et al. postulated about the cause of this linear relationship between ventilation and arterial PO_2 and SpO_2 in their influential 1970 paper “Hypoxic ventilatory drive in normal man.” In this paper, they observed that the ventilation-PO_2 curve resembled the oxygen-hemoglobin dissociation curve and that both show inflections over the same PO_2 range. This seemed to indicate that only oxygen tensions that are low enough to lower hemoglobin oxygen saturation would stimulate ventilation. Total arterial

oxygen content is primarily composed of oxygen bound to hemoglobin, and dissolved oxygen contributes minimally to the total arterial oxygen content (Siggaard-Andersen *et al.*, 1990). Thus, a hypoxic ventilatory response in which ventilation only increases when PO_2 is low enough to decrease hemoglobin oxygen saturation would represent an elegant evolutionary strategy for minimizing the metabolic demands of increased ventilation when hemoglobin remains highly saturated at PO_2 levels above 70-80 mmHg, and therefore increases in ventilation would lead to minimal improvements in total arterial oxygen content. This remains a useful concept for teaching the physiology of the HVR, although it has been impossible to test experimentally and there is no known physiological mechanism for sensing decreases in O_2-hemoglobin saturation directly.

The relationship between ventilation and PO_2 or SpO_2 becomes more variable at lower SpO_2 levels based on our computational investigation (**Figures 5.2.10 A-B**). However, since few studies use hypoxia targets lower than 75% SpO_2, we are unable to verify that this would occur *in vivo*. Although, among studies utilizing F_IO_2 targets, studies with lower targets did seem to present higher average HVR values (**Figure 5.2.7C**), providing some evidence that the chosen hypoxia targets can potentially impact HVR measurements, even when reporting HVR as a change in ventilation per unit change in SpO_2. The increased variability in the relationship between ventilation and SpO_2 at lower saturation levels may stem from increased individual variation in hemoglobin-oxygen binding affinity. In 2020, Balcerek et al. found that P_{50} ranged over 7 mmHg across 60 healthy individuals, with women presenting lower hemoglobin-oxygen binding affinities due to higher 2,3-BPG and BPGM levels (Balcerek *et al.*, 2020). Thus, at a similar PO_2, and therefore a similar stimulus to peripheral chemoreceptors, different

participants may present significantly different SpO_2 levels, thereby impacting the calculated HVR value.

Nonetheless, the lack of studies using hypoxia targets below 80% SpO_2 is reasonable given that this level of hypoxemia is not necessary to obtain a valid HVR measure experimentally. Furthermore, the accuracy of commercially available pulse oximeters decreases at lower SpO_2 levels (Severinghaus *et al.*, 1989). However, in a clinical setting, it may be expected that exceptionally low PO_2 and SpO_2 levels are experienced by patients routinely, such as in severe obstructive sleep apnea, or in chronic or acute lung disease cases (Zysman *et al.*, 2021). Therefore, it is possible that the high variability in HVR at lower SpO_2 levels may have clinical relevance in these cases and could affect disease progression. For example, high respiratory drive is often observed in patients with severe lung injury. The resulting intense respiratory effort can cause additional lung damage through “patient self-inflicted lung injury” (P-SILI) (Spinelli *et al.*, 2020*b*). On the contrary, a patient with lower respiratory drive may be protected against this but may also experience more severe hypoxemic stress.

Carotid body chemoreceptors play a key role in regulating arterial PCO_2, pH, and PO_2 by modulating ventilation rate, and therefore play a major role in the amplitude and plasticity of the HVR. Additionally, carotid body sensitivity influences many other physiological functions including sympathetic and parasympathetic activity, as well as cardiovascular, renal, endocrine, gastrointestinal, and metabolic effects. As a result, the carotid body dysfunction is implicated in the progression of sympathetic-related diseases such as obstructive sleep apnea, congestive heart failure, resistant hypertension, and metabolic diseases (Iturriaga *et al*., 2021). It is therefore an interesting possibility that the HVR may be used as a marker of carotid body function. HVR measurements have been

used to assess changes in carotid body O_2-sensitivity in whole-animal experiments. Applying this idea to our results might suggest that low O_2-sensitivity in carotid bodies is more common than high O_2-sensitivity in healthy humans. However, the HVR also depends on translating arterial chemoreceptor activity into ventilation, which involves both neural transmission in the respiratory centers and neuromuscular function. It is not easy to separate these different mechanisms in human studies, at least, so we cannot conclude that high O_2-sensitivity in carotid bodies is relatively rare in healthy humans from our analysis.

5.2.6 CONCLUSION

The dataset produced through this meta-analysis provides the most comprehensive overview, to date, of the normal HVR during acute hypoxia exposure, as well as the amplitude of the HVR following acclimatization to hypoxia. It also highlights the high level of variation in this reflex within and across individuals and populations. Our study was not designed to provide “normal values” for clinical settings such as those established for pulmonary function tests and arterial blood gas values. However, all of the data we used is available if it could be useful for such an effort. The data could also be used as a control group for experimental studies if comparable protocols are used. Our main conclusion, which should be applicable to other clinical or experimental studies, is that a low HVR is not necessarily “abnormal”. In earlier experimental studies, we might exclude a subject from further study if they had an extremely low (or negative) HVR measured during screening. Studies have shown that the HVR is reproducible within an individual (MacNutt *et al*., 2015) so a low HVR measured during screening would likely be low in following sessions. However, our analysis suggests this exclusion

of low HVRs is not appropriate and future work may show that a low HVR is even more common than this analysis suggests.

This meta-analysis also has some limitations. First, our analysis of the impact of hypoxia targets on mean HVR values depends on single lower-limit hypoxia targets reported in the methods of each study. However, experimental error will introduce variability in the actual hypoxia targets reached, and most studies do not indicate if HVR values were indeed calculated at these lower-limit points or if other criteria were used to choose data for HVR calculation. Similarly, particularly for rebreathing studies, it was not always stated if the HVR was calculated using a two-point calculation of change in ventilation per change in % SpO_2 (as is used for steady state methods), or if a linear regression across the entire range of SpO_2 levels was used. Nonetheless, with a substantial sample size, our data do not demonstrate significant impacts of SpO_2 targets on the amplitude of the HVR. Second, our data simulations do not account for individual variation and within-subject changes in hemoglobin-oxygen binding affinity, but instead depend on normal range values for estimates. However, these tests occur over short time periods (typically less than 30 minutes) and our simulations adjust for estimated changes in PCO_2 tensions at given PO_2 levels.

In conclusion, we demonstrate that lower HVRs are more common and higher HVRs are rare. This skewness of HVR measurements occurs across study methodologies. While isocapnic methods do produce higher HVR values, there is no impact of steady state or rebreathing methods on overall HVR when comparing across all studies. Finally, the experimental data examined here supports the predicted linear relationship between ventilation and SpO_2, at least at SpO_2 levels above about 75-80%,

and while computational investigations indicate that the level of hypoxia chosen may impact the amplitude of the HVR, this does not occur in vivo.

ACKNOWLEDGEMENTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

ECH, FP, BO, and KP conceived and designed the research. BO, KP, and ECH collected data, interpreted the data analysis, and prepared figures. MO, DJD, and EG provided unpublished HVR data used in the study. BO, KP, and ECH drafted the manuscript. BO, KP, ECH, and FP revised the manuscript. BO, KP, ECH, FP, MO, DJD, and EG approved the final version of the manuscript. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

DATA AVAILABILITY STATEMENT

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

FIGURES

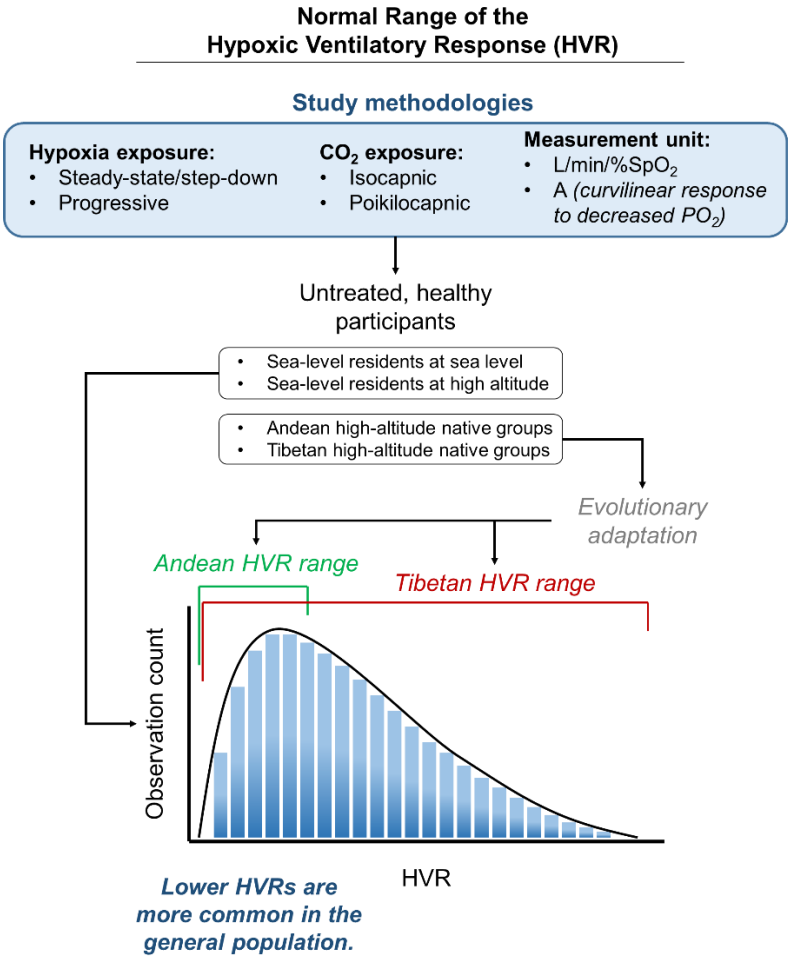

Figure 5.2.1. Abstract Figure In this meta-analysis, we examine hypoxic ventilatory response (HVR) distributions across 78 studies in which healthy, untreated participants from diverse populations were examined under several testing conditions (i.e., step-down or progressive hypoxia, and isocapnic or poikilocapnic CO_2 methods) and units of measurements (i.e., L/min/SpO_2, A unit). We find that lower HVR values are more common amongst the general population and higher HVR values are uncommon. We also impacts of methodology on HVR measurements, including higher HVRs observed in isocapnic protocols, but no significant impact of step-down versus progressive hypoxia methods. These results provide key insight into understanding the evolutionary adaptation of the HVR in high-altitude native populations, as well as comparative interpretations of HVR measurements across studies.

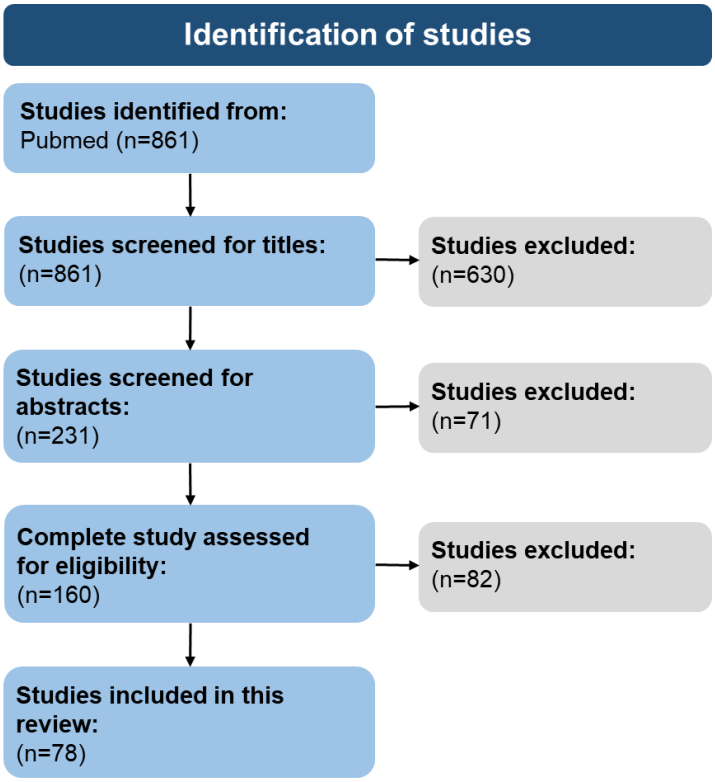

Figure 5.2.2. PRISMA diagram of study filtering. Filtering methods and inclusion criteria used to identify studies for this analysis. A PubMed search conducted on August 12, 2021 using the terms “hypoxic ventilatory response” or “hypoxic chemosensitivity” across any time period yielded 861 reports. All reports were screened by title, and 630 reports were excluded due to the title indicating they were reviews, or providing no indication that any type or respiratory reflex was measured in humans. All remaining abstracts were then screened, and 71 of the remaining reports were excluded because of abstracts which did not indicate that HVR was measured in at least one untreated healthy group. All remaining studies were then read in detail to verify that the study contained compatible study populations, compatible methodological approaches with sufficient detail provided, and compatible units (“L/min/SpO_2” or “A"), and that raw or mean HVR data was available for extraction. 82 reports were excluded during this step (Data not provided/incompatible data presentation (N=7), Insufficient methodological detail (N=5), Lack of healthy adult participants (N=3), Lack of non-intervention group (N=1), Incompatible methodology (N=34), Incompatible HVR unit (N=18), Inability to access report/not available in English language (N=14)). This yielded 78 compatible reports for analysis.

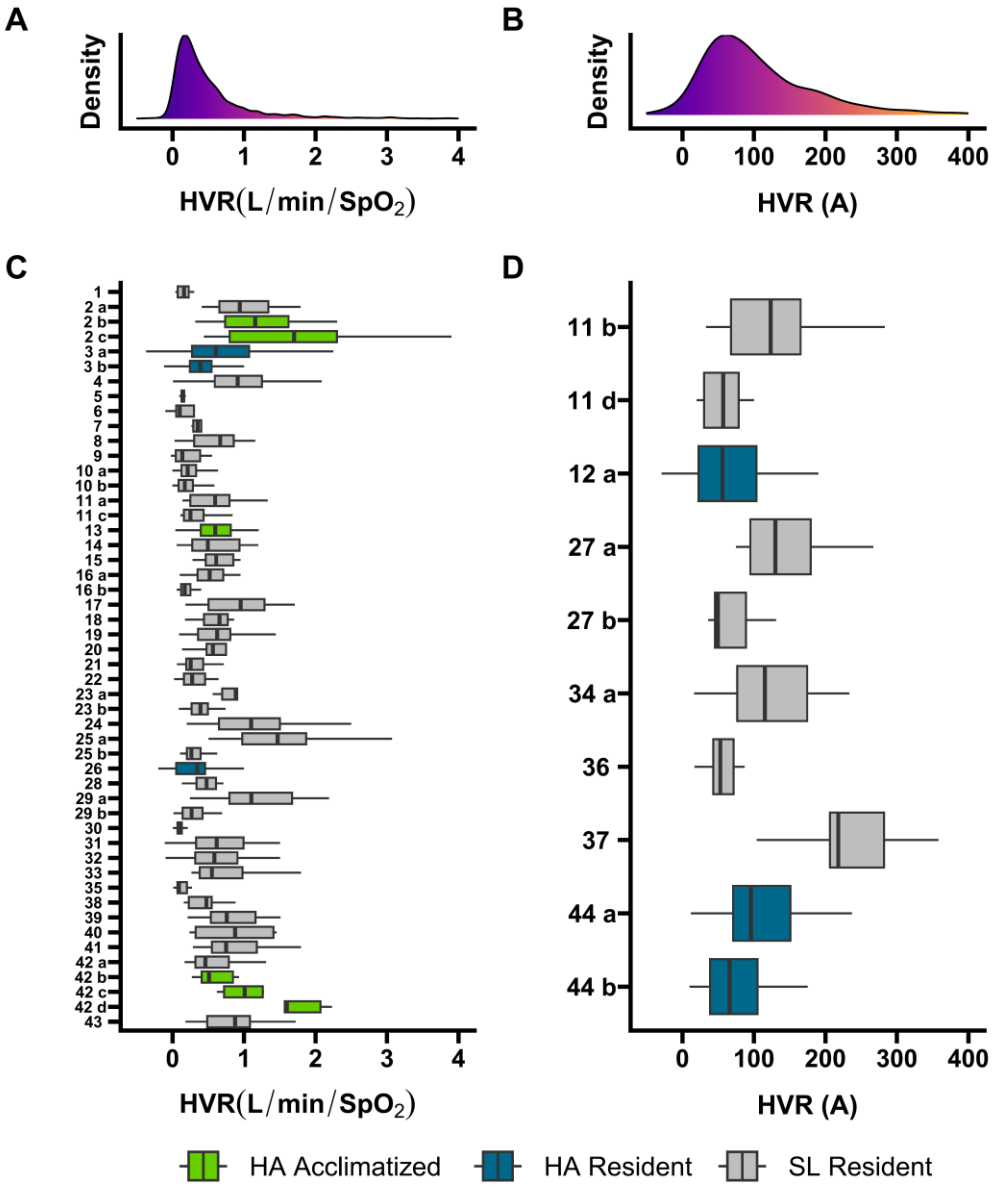

Figure 5.2.3. HVR distributions across studies. (Top) Density plots showing pooled data across all studies, separated by HVR unit, "L/min/%SpO_2" **(A)** or "A" **(B)**. **(Bottom)** Boxplots showing distributions of HVR values within study datasets, separated by HVR unit, "L/min/%SpO_2" **(C)** or "A" **(D)**. For studies examining two distinct populations, data is separated into individual plots for each population within that study (i.e., 2a and 2b). Boxplot colors indicate the population examined in each dataset. Studies 3a, 12a, and 44a represent data collected in Tibetan or Sherpa high-altitude natives, and studies 3b and 26 represent data collected in Andean high-altitude natives. Study 44b represents data collected in high-altitude residents of Han Chinese ancestry.

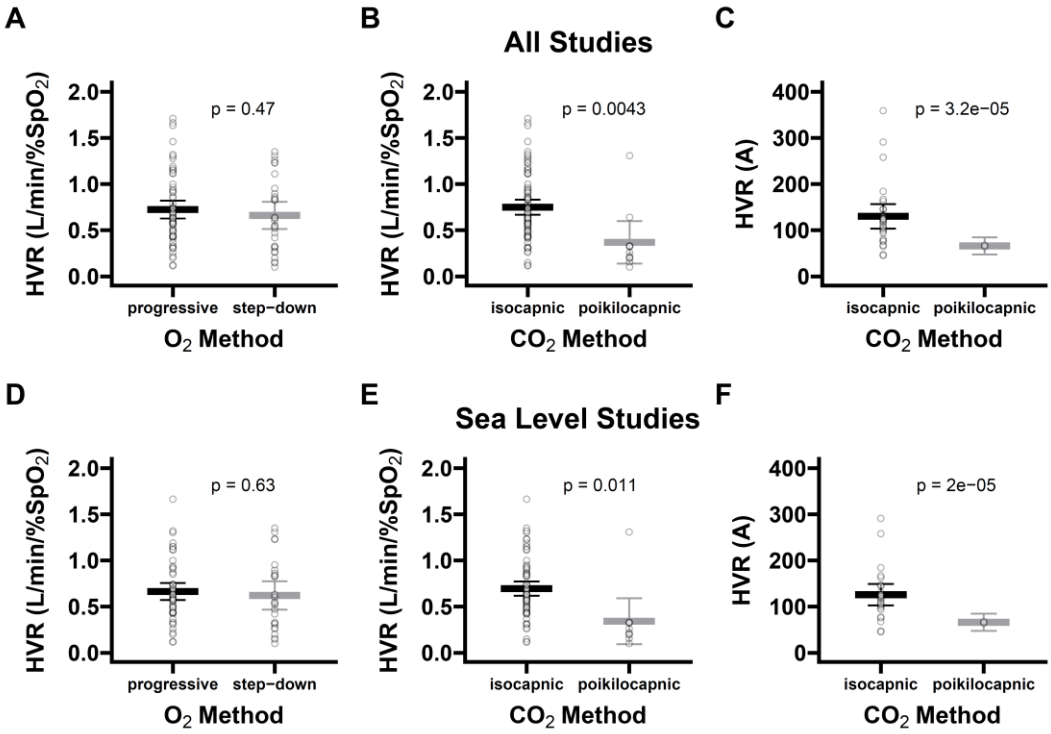

Figure 5.2.4. Impact of methodology on mean HVR. Data points represent mean HVR values within an individual study. Data for all studies, including those in sea-level residents at sea level, high-altitude resident populations tested at high altitude, and sea-level residents acclimatized to high altitude are provided in panels **A-C**. Data for only studies conducted in sea-level residents at sea level are provided in panels **D-F**. Error bars represent 95% confidence intervals.

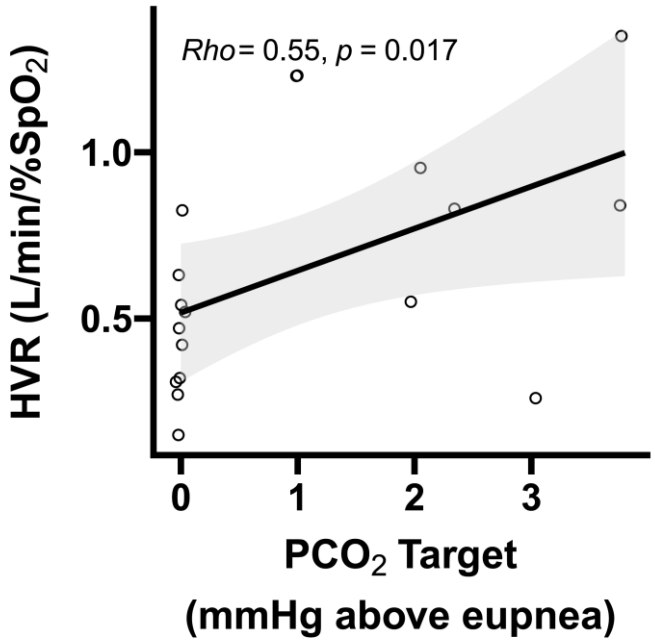

Figure 5.2.5. Spearman correlation plot for relationship between the HVR and end-tidal PCO_2 isocapnic target. Data points represent mean HVR values for individual studies. Points are slightly jittered along the x axis for visibility of overlapping data. R and p values represent values calculated via a Spearman rank correlation analysis.

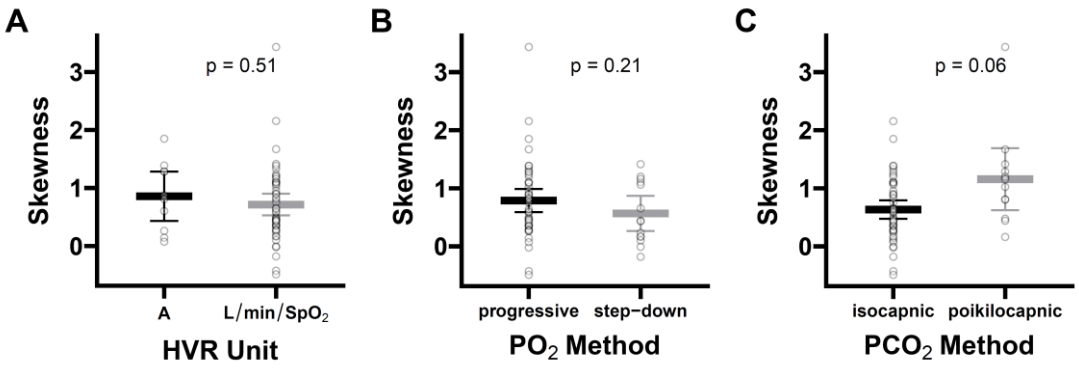

Figure 5.2.6. Impact of methodology on HVR distribution skewness. Data points represent skewness of HVR value distributions within an individual study. Error bars represent 95% confidence intervals.

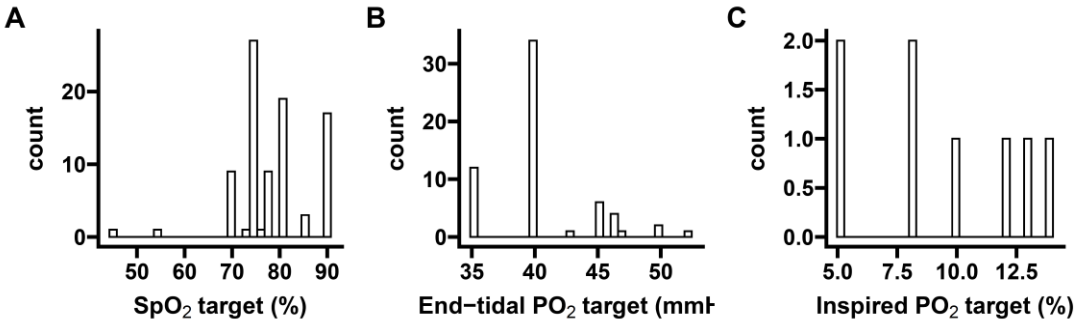

Figure 5.2.7. Histograms of experimental hypoxia targets chosen across all studies. Hypoxia targets were determined for each individual study based on the target SpO_2 **(A)**, or end-tidal PO_2 target in mmHg **(B)** or % **(C)**. Results reported here include both hypoxia targets for step-down tests as well as low-end threshold hypoxia targets for progressive/rebreathing methods.

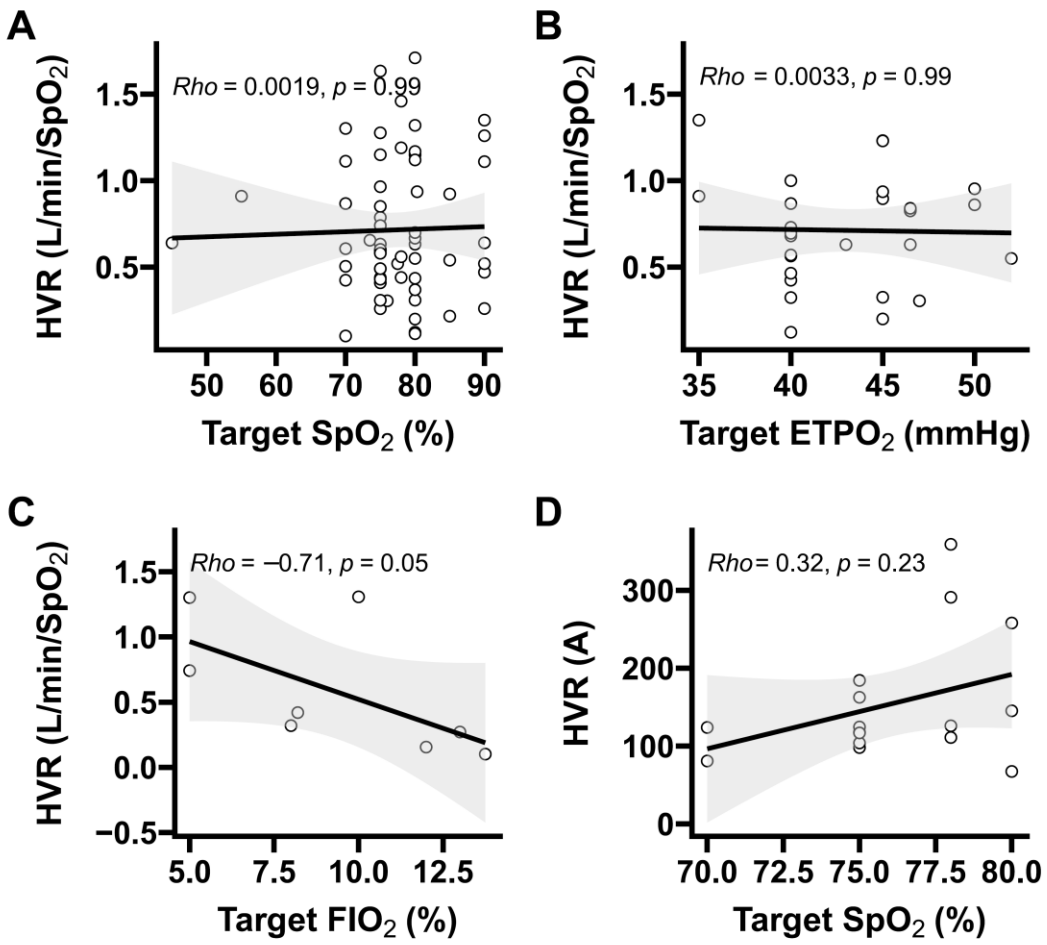

Figure 5.2.8. Mean HVR values as a function of target hypoxia level. Relationships between mean HVR across studies and hypoxia targets for studies indicating SpO_2 **(A)**, end-tidal PO_2 **(B)**, and F_IO_2 targets **(C)** with L/min/SpO_2 units, as well as SpO_2 targets with A units **(D)**.

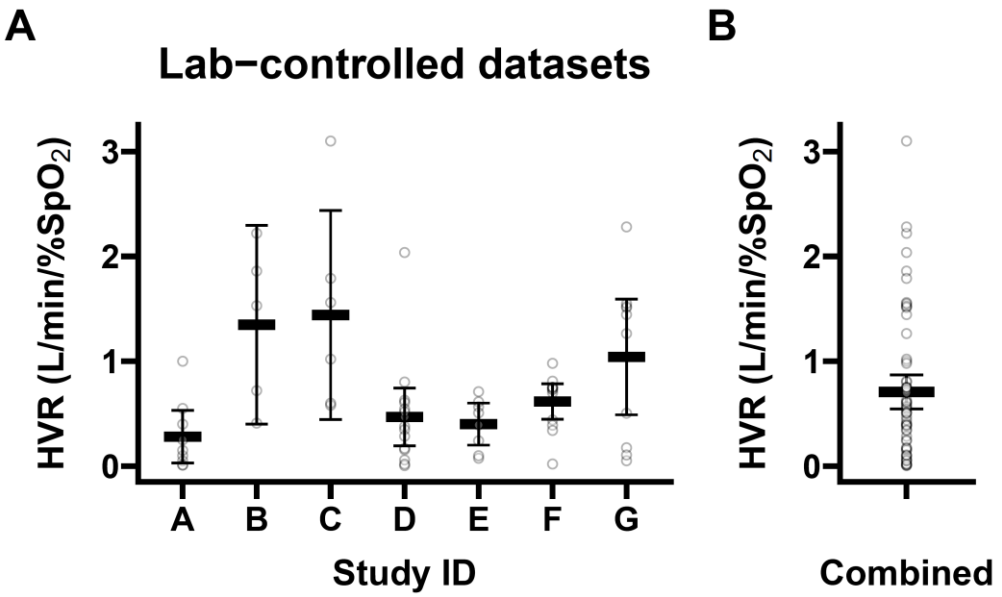

Figure 5.2.9. Distributions of lab-controlled datasets. Datasets collected in the same laboratory all using isocapnic step-down protocols. Study IDs: **A** – Garcia et al. 2000, **B** – Hupperets et al. 2004, **C** – Weigner et al. 1998, **D** – Basaran et al. 1998, **E** – Unpublished 1, **F** – Unpublished 2, **G** – Unpublished 3.

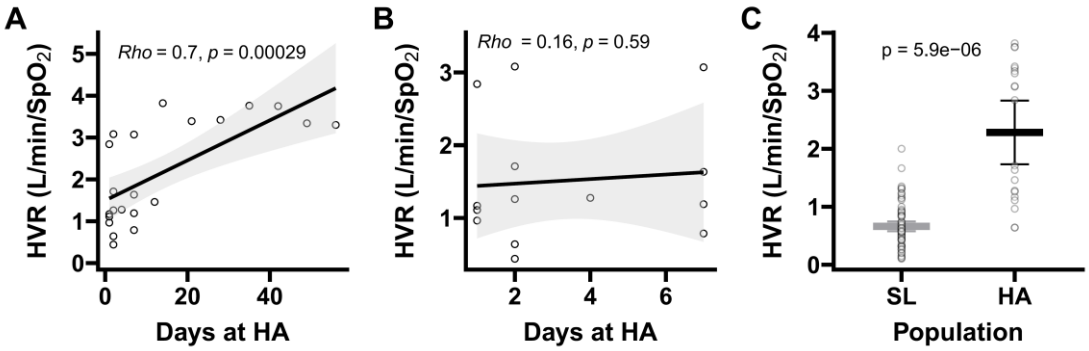

Figure 5.2.10. Impact of high-altitude acclimatization on the HVR. (A) Mean HVR values collected from 19 datasets across 6 studies conducted at 3800 to 4559 m elevation. **(B)** An expanded view of mean HVR values from studies reporting 1-7 days of acclimatization at 3800 to 4559 m elevation. **(C)** Mean HVR values in all studies collecting HVR values in sea-level residents at sea level (SL) compared to sea-level residents acclimatized to 3800-4559 m elevation (HA).

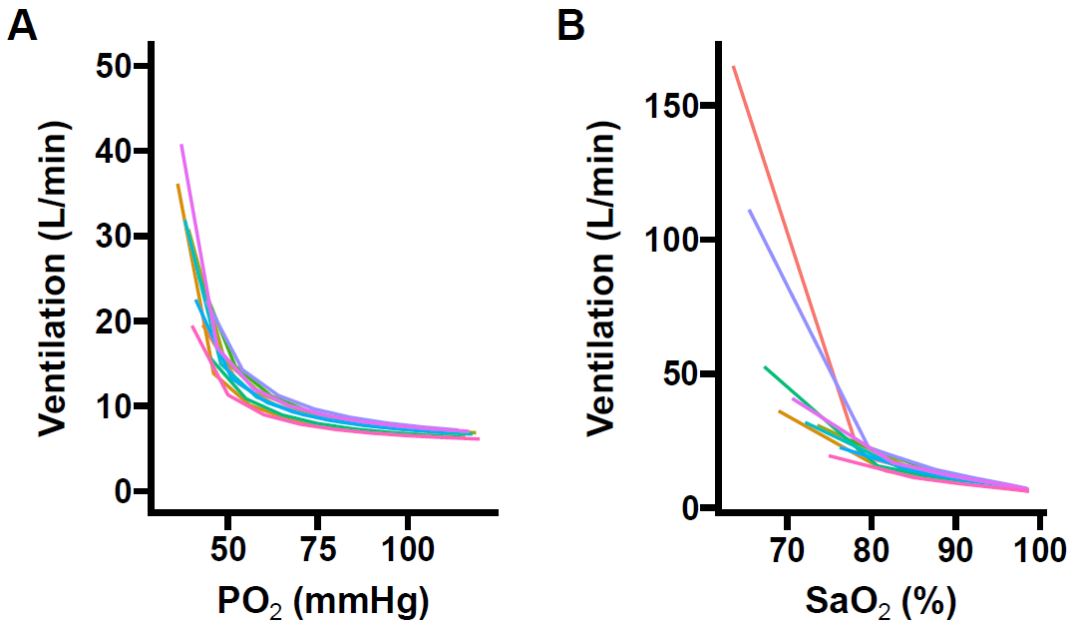

Figure 5.2.11. Simulated HVR curves. A subset of 10 randomly generated HVR curves plotted as a function of PO_2 **(A)** or SaO_2 **(B)**. The same 10 datasets are plotted in A and B, and estimated SpO_2 levels in B were calculated from PO_2 in A as described above.

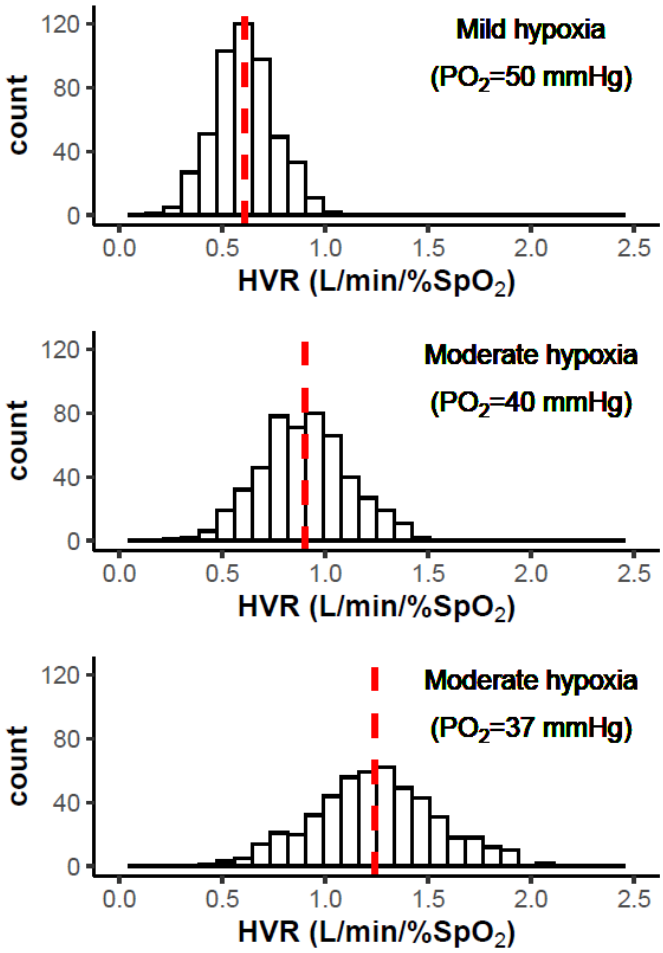

Figure 5.2.12. Distribution of simulated HVR values at different target PO_2 levels. Plots display histogram distributions of 500 simulated HVR measurements with a target PO_2 at three levels across the same curves. The mean HVR in each group is indicated by vertical red dashed lines.

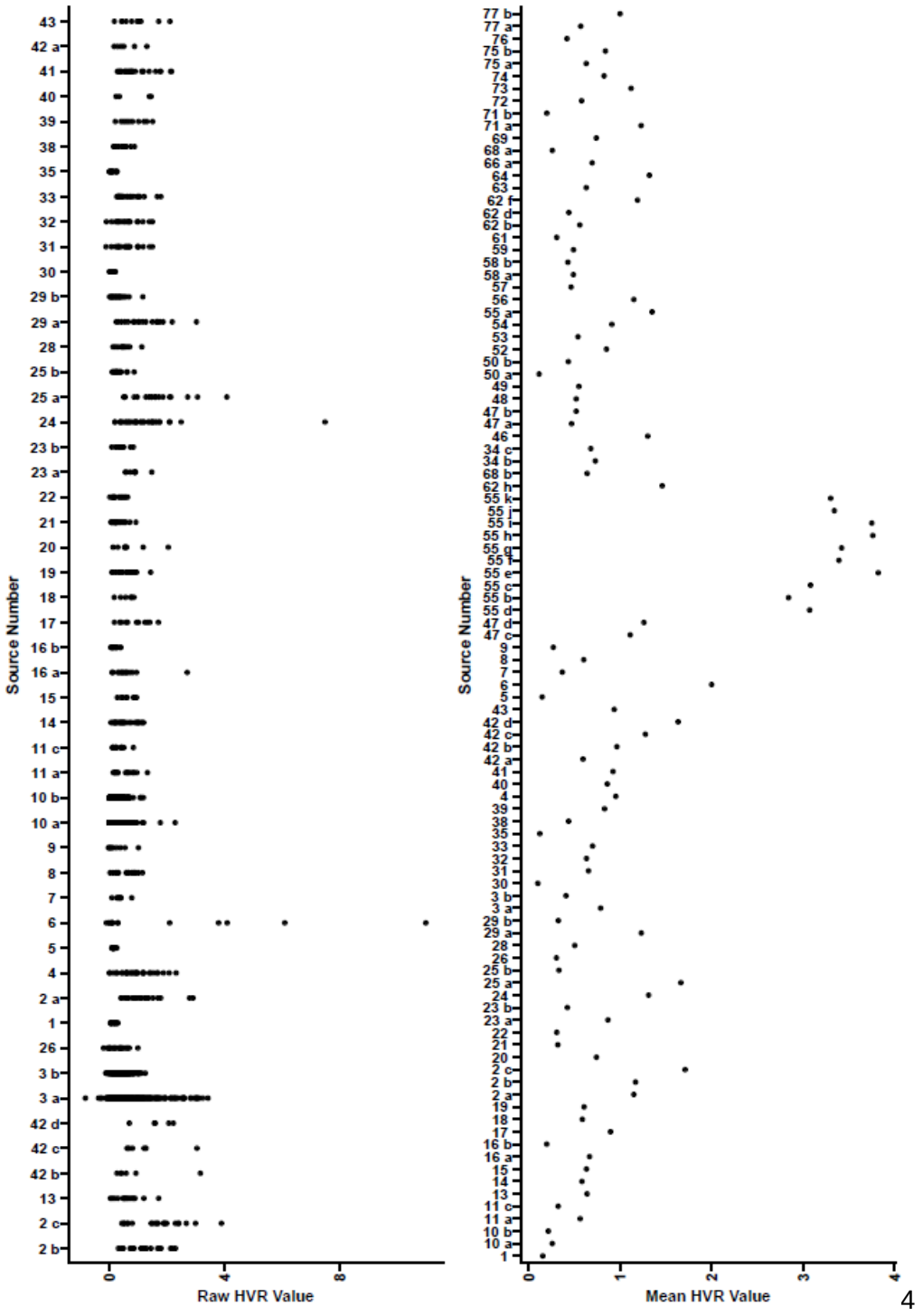

Figure 5.2.S1. Raw and mean HVR values across all studies. Datasets 55b-k are derived from high-altitude acclimatized individuals and therefore clearly demonstrate elevated HVR values compared to other datasets.

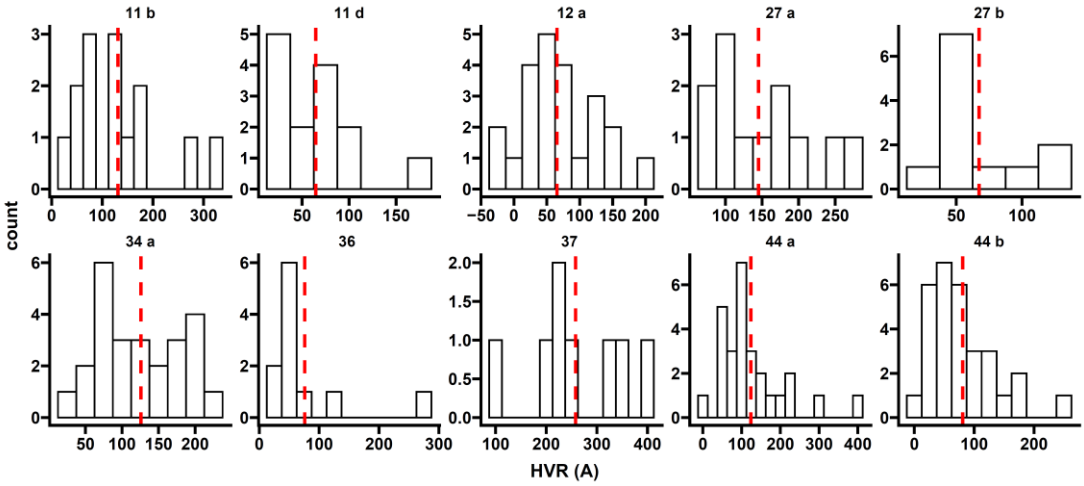

Figure 5.2.S2. Density plots of HVR values across studies/datasets reporting A units. Each study/dataset is plotted as an individual histogram with its identifying number above. The red dotted line represents the mean HVR for each study. Only studies reporting raw HVR data in table or scatterplot form are included.

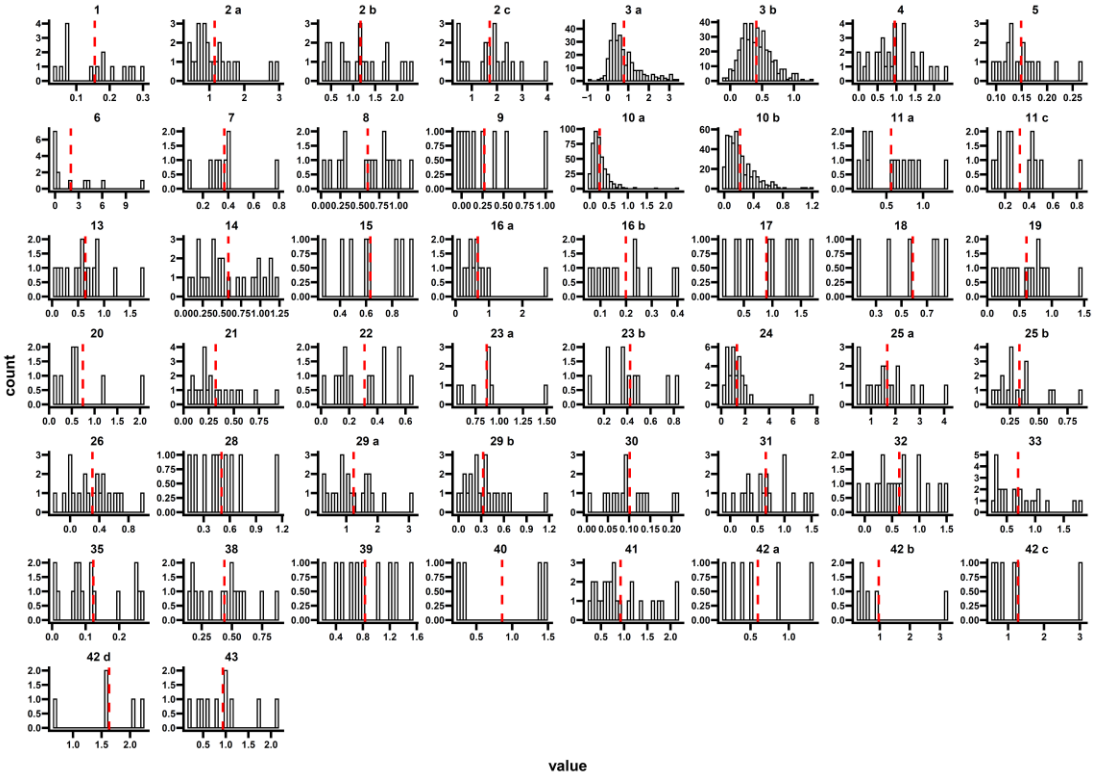

Figure 5.2.S3. Histograms of HVR values across studies/datasets reporting L/min/SpO_2 units. Each study/dataset is plotted as an individual histogram with its identifying number above. The red dotted line represents the mean HVR for each study. Only studies reporting raw HVR data in table or scatterplot form are included.

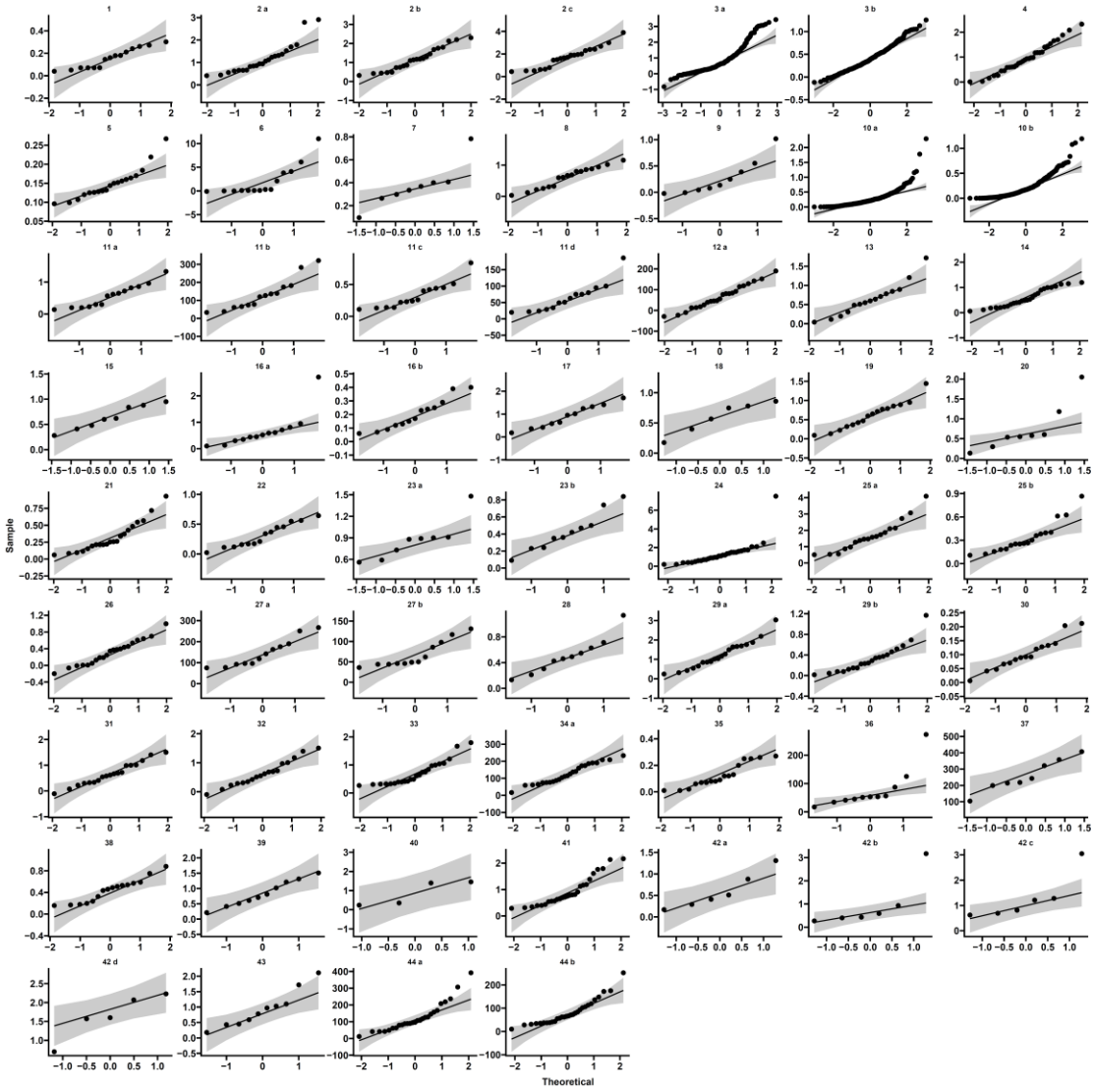

Figure 5.2.S4. Q-Q plots of each study/dataset. Each study/dataset is plotted as an individual panel with its identifying number above. Shaded regions indicate 95% confidence intervals. Each plot checks the normality of the data by drawing the correlation between raw HVR data values within a study dataset and the normal distribution.

Chapter 5.3

Health disparities in COVID-19: Immune and vascular changes are linked to disease severity and persist in a high-risk population in Riverside County, California

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5.3.1 ABSTRACT

Health disparities in underserved communities, such as inadequate healthcare access, impact COVID-19 disease outcomes. These disparities are evident in Hispanic populations nationwide, with disproportionately high infection and mortality rates. Furthermore, infected individuals can develop long COVID with sustained impacts on quality of life. The goal of this study was to identify immune and endothelial factors that are associated with COVID-19 outcomes in Riverside County, a high-risk and predominantly Hispanic community, and investigate the long-term impacts of COVID-19 infection.112 participants in Riverside County, California, were recruited according to the following criteria: healthy control (n=23), outpatients with moderate infection (outpatient, n=33), ICU patients with severe infection (hospitalized, n=33), and individuals recovered from moderate infection (n=23). Differences in outcomes between Hispanic and non-Hispanic individuals and presence/absence of co-morbidities were evaluated. Circulating immune and vascular biomarkers were measured by ELISA, multiplex analyte assays, and flow cytometry. Follow-up assessments for long COVID, lung health, and immune and vascular changes were conducted after recovery (n=23) including paired analyses of the same participants. Compared to uninfected controls, the severe infection group had a higher proportion of Hispanic individuals (n=23, p=0.012) than moderate infection (n=8, p=0.550). Disease severity was associated with changes in innate monocytes and neutrophils, lymphopenia, disrupted cytokine production (increased IL-8 and IP-10/CXCL10 but reduced IFNλ2/3 and IFNγ), and increased endothelial injury (myoglobin, VCAM-1). In the severe infection group, a machine learning model identified LCN2/NGAL, IL-6, and monocyte activation as parameters associated with fatality while anti-coagulant therapy was associated with survival. Recovery from moderate COVID

infection resulted in long-term immune changes including increased monocytes/lymphocytes and decreased neutrophils and endothelial markers. This group had a lower proportion of co-morbidities (n=8, p=1.0) but still reported symptoms associated with long COVID despite recovered lung health. This study indicates increased severity of COVID-19 infection in Hispanic individuals of Riverside County, California. Infection resulted in immunological and vascular changes and long COVID symptoms that were sustained for up to 11 months, however, lung volume and airflow resistance was recovered. Given the immune and behavioral impacts of long COVID, the potential for increased susceptibility to infections and decreased quality of life in high-risk populations warrants further investigation.

5.3.2 INTRODUCTION

The United States alone currently accounts for 103 million of the 760 million confirmed COVID-19 cases world-wide, and 1.1 million confirmed deaths have been reported in the US as of June 2023 (World Health Organization, 2020). Of the 66 million reported cases with ethnicity data available, Hispanic individuals make up 24% of these cases despite only making up 18.45% of the total US population (Center for Disease Control and Prevention, 2022). The Hispanic population also comprises 17% of all reported deaths, one of the largest groups impacted by infection (Center for Disease Control and Prevention, 2022). Despite a decreasing trend observed in COVID-19 hospitalizations and deaths and significant advances in vaccine development and distribution in the last two years, the number of COVID-19 positive cases and deaths in the Hispanic community has continued to rise. Possible reasons include existing health disparities like pre-existing health conditions and higher rates of key co-morbidities (Khatana & Groeneveld, 2020; Zhao *et al.*, 2022; Rao *et al.*, 2023; Gatto *et al.*, 2023). A

higher prevalence of these pre-existing factors is predicted to stem from systemic inequities in healthcare access, quality of care, and inequalities in the built environment. In the county of Riverside, California, Hispanic individuals make up 37 % of the cumulative 741,000 reported COVID cases, almost 2-fold more than reported cases in non-Hispanic white individuals (County of Riverside, 2023). In addition, Hispanic individuals make up 42% of the hospitalizations and deaths in this county. This staggering statistic is concerning given the lack of Hispanic data in COVID-19 research.

COVID-19 infection induces significant immune and vascular responses that change with disease severity. Severe infection often results in overactivation of certain innate immune subsets (Meidaninikjeh *et al*., 2021; Hazeldine & Lord, 2021; Satta *et al*., 2022; Ekstedt *et al.*, 2022; Herrera *et al*., 2023; Rice *et al*., 2023; Root-Bernstein, 2023; Leal *et al*., 2023) while additional studies have reported suppression of adaptive immune subsets that are necessary for antiviral immunity and memory responses (Westmeier *et al.*, 2020; Hanley *et al.*, 2020; Pappas *et al.*, 2022). These highly dysregulated immune responses not only represent their own form of infection-induced pathology but also drive vascular pathology, including coagulopathies, myocarditis, and tissue damage particularly in the lung following infection (Cheon *et al*., 2021; Root-Bernstein, 2023; Wu *et al*., 2023a). Immune-independent mechanisms underlying COVID-19-induced vascular damage, including changes in essential vitamins and platelet aggregation, have also been reported (Getachew *et al.*, 2022). Dysregulated immune responses may therefore play a larger role in COVID-19-induced vascular pathology than originally thought.

Increasing reports of post-acute COVID-19 conditions raise serious concerns for unknown long-term effects of this disease. These conditions are similar to other post-

acute infection syndromes that have long been present but remain widely misunderstood (Choutka *et al.*, 2022). Long COVID is characterized by persistent symptoms, immune dysregulation, and lasting tissue damage that persists after recovery from infection (Kennedy *et al.*, 2021; Li *et al.*, 2022*b*; Wiech *et al.*, 2022; Davis *et al.*, 2023). Recent work has identified a relationship between COVID-19 disease severity and the development of long COVID (Hawar & Dauod, 2022). Prolonged immune changes associated with long COVID are present for months following recovery in the periphery (Phetsouphanh *et al.*, 2022; Ryan *et al.*, 2022) as well as in tissues directly affected by infection (Roukens *et al.*, 2022). Measures have been taken to evaluate and manage long COVID based on current knowledge (Kim *et al.*, 2022), but significant gaps in knowledge remain about the risk factors and mechanisms underlying long COVID (Pinto *et al.*, 2022).

The primary goal of this study was to identify immune and endothelial biomarkers associated with disease severity in Riverside County, CA. Additionally, a machine learning model was used to dissect large data output and predict risk factors for COVID-19 disease severity. A secondary goal was to determine if Hispanic individuals were disproportionately affected by COVID-19 infection. Finally, a third goal of this study was to explore immune and vascular factors associated with long COVID in this cohort and investigate the impact on lung health and immune homeostasis.

5.3.3 METHODS

Study Population

Men and women with active COVID-19 infection, who had recovered from a past mild-to-moderate COVID-19 infection or had no history of COVID-19 (healthy control) were recruited. Individuals who were currently incarcerated or were not residents of Riverside

County, CA, were excluded, as were individuals under the consenting age of 18. Pregnant women were excluded from the study due to exposure to acute hypoxia and hypercapnia in breathing tests not reported here. Severe group participants who received treatment (e.g., dexamethasone) prior to sampling were not excluded. Inclusion criteria for each study group were: 1) healthy controls with no prior history of confirmed COVID-19 infection (via PCR or rapid test); 2) moderate infection participants that were outpatients with active COVID-19 infection (confirmed by PCR test), who were offered monoclonal antibody treatment; 3) severe infection participants with active COVID-19 infection (confirmed by PCR test) requiring hospitalization and supplemental oxygen; 4) recovered participants with no active COVID-19 infection but prior history of confirmed positive (via PCR or rapid test) mild-to-moderate COVID-19 infection.

Study Recruitment and Design

Study recruitment

Moderate infection participants were invited to participate by fliers distributed by their physician. Severe infection participants were recruited from Riverside University Health System (RUHS). For Control and Recovered groups, participants were recruited from University of California Riverside Health Clinic, Riverside Free Clinic, and University of California, Riverside.

Study design

Participants completed a medical history questionnaire which provided information regarding demographic information, current medications, as well as current and past medical conditions. Recovered participants also completed the Yorkshire Rehabilitation Scale questionnaire which provides self-reported health status before and

after recovery from COVID-19 (Sivan *et al.*, 2021; O'Connor *et al.*, 2022). For recovered participants and healthy controls, basic physiological parameters including height, weight, blood pressure, and lung function parameters via spirometry were recorded. Peripheral venous blood samples were collected from all participants using standard phlebotomy procedures. For ICU patients, data was collected from hospital medical records, hospital personnel drew blood, and specimens were transported to the University of California, Riverside for processing. For all groups, samples were processed for downstream analysis within 4 hours of collection. Samples were collected between January 2021-February 2022 from RUHS for the severe (hospitalized) group, January-July 2022 for the moderate (outpatient) group, April-October 2022 for healthy control group, and April-October 2022 for follow-up (recovered) group.

Laboratory Evaluation of Samples

Whole blood and plasma isolation

Peripheral venous blood was collected in 10 ml vacutainer tubes containing EDTA (BD, Franklin Lakes, NJ, USA) and maintained at room temperature until processing within 4 hours of collection. Following blood collection, an aliquot of whole blood was set aside for flow cytometry analysis. Plasma was isolated according to previously published protocols (Bonenfant *et al.*, 2022). Briefly, gradient centrifugation with Histopaque-1077 was performed, and plasma was recovered for quantification of cytokines, Resistin, and endothelial parameters.

Flow cytometry

100µl whole blood was incubated in human Fc Receptor Block (Biolegend) followed by anti-human fluorophore-conjugated antibodies: PerCP/Cyanine5.5 anti-

CD3), APC/Cyanine7 anti-CD11b, Brilliant Violet 650 anti-CD25, PE/Cyanine5 anti-CD62P/P-Selectin, Brilliant Violet 711 anti-CD45, APC anti-CD66b, Brilliant Violet 605 anti-CD56/NCAM, Alexa Fluor488 anti-CD14, Brilliant Violet 785 anti-CD8, PE anti-CD16 (all from Biolegend); Alexa Fluor594 anti-ACE-2 (R&D Systems), PerCP-eFluor710 anti-CD19 (eBioscience), and BV421 anti-HLA-DR, DP, DQ (BD Biosciences). Red blood cells were lysed and cells were fixed using 1-step Fix/Lyse Solution (eBioscience), rinsed with FACS buffer, and resuspended in FACS buffer (0.5% BSA, 0.005% EDTA, 1x PBS). Samples were analyzed using the NovoCyte Quanteon flow cytometer, NovoSampler Q, and NovoExpress Software. An average of 1 x 10^5 events were collected from the "Live Cell" gate for analysis. Analysis was conducted using FlowJo software version 10. The complete gating strategy is provided in **Figure 5.3.S2**.

Participant-specific immune tracking

Within each study group, some participants (Moderate, n=3; Severe, n=9; Control/Recovered, n=5) provided blood specimens at multiple timepoints. These samples were used for within-subject time course analyses to determine changes in parameters throughout the course of infection and following recovery. Flow cytometry for different immune cell populations was analyzed for each of these participants to track changes over time. A representative participant was then selected for each group, and UMAP projections were created to visualize immune cell changes.

Cytokine and endothelial marker analysis

Plasma samples were assayed for cytokine and endothelial marker analysis using LEGENDplex kits and the manufacturer's protocols (Biolegend): "Human Anti-Virus Response Panel" (13-plex) at 1:2 dilution and "Human Vascular Inflammation

Panel" (13-plex)"at 1:100 dilution in 5% TX100 (in PBS) for virus inactivation. Samples were run on a Novocyte Quanteon flow cytometer, and data analysis was conducted using BioLegend's LEGENDplex Data Analysis Software.

ELISA

The ELISA assays for Resistin and spike protein receptor-binding domain (RBD) were performed with the following ELISA kits: Human-Resistin-Mini ABTS ELISA Development Kit (PEPRO TECH, Catalog #900-M235). Human SARS-CoV-2 RBD ELISA Kit (Themo Fisher, Catalog # EH492RB). Prior to measurement, the plasma samples were centrifuged at 1000g for 5 minutes, supernatant was then diluted 1:100 for Resistin and 1:4 for spike protein. The absorbance was acquired by a plate reader (BioTek Synergy HT).

Viral reverse transcription quantitative PCR

RNA was extracted from nasal swab samples using a Quick-RNA Viral Kit (ZYMO Research, Catalog # R1034). QPCR was performed using primers (N2) and probes from the 2019-nCoV RUO Kit (IDT, Catalog # 10006713). SARS-CoV-2 RNA was quantified using GoTaq® Probe 1-Step RT-qPCR System (Promega, A6120) according to the manufacturer's protocol. 1000 copies/µL of 2019-nCoV plasmid (IDT, Catalog # 10006625) was used as positive control.

Lung function spirometry tests

To measure lung function in recovered participants and healthy controls, standard spirometry procedures were conducted in triplicate and mean values were collected for forced vital capacity (FVC), forced expired volume in 1 second (FEV_1), and FEV_1/FVC. Values were adjusted for predicted values based on the National Health and

Nutrition Examination Survey (NHANES) III reference values, or Global Lung Function Initiative (GLI) references values for Asian individuals. Respiratory flow was measured with a spirometer and respiratory flow head (1000 L) (ADInstruments, Colorado Springs, CO, 80907), with data acquisition via a PowerLab 8/35 and analysis conducted in LabChart Pro using the ADInstruments spirometry plugin.

Statistical and Bioinformatic Analysis

Statistical analysis for laboratory results

Statistical significance for all experiments was determined by either 2- tailed unpaired Student's t-test or One-Way ANOVA with multiple comparisons and a p-value < 0.05 was considered statistically significant. For self-reported symptom severity on the YRS Questionnaire, data was tested for normality of distributions with Shapiro Wilk's tests, and scores were compared across pre and post COVID-19 infection using paired Wilcoxon-signed rank tests in R using the *stats* package. P-values for these comparisons were adjusted for family-wise error rates using a Holm-Bonferroni method. The type of statistical test run for each experimental result is indicated in the corresponding figure and table legends.

Correlation analyses

To examine correlations between parameters in the dataset, pairwise Pearson correlations were conducted using the R package *Hmisc*. Using the min-max scaling method, all continuous variables were scaled from 0 to 1 to guarantee that they were all on the same scale. Binary variables were converted to 1 (yes) or 0 (no). Correlation analysis results were visualized using the *corrplot* function in R. Correlation coefficients

with p-values less than 0.05, 0.01, and 0.001 are denoted with “*,” “**,” and “***” respectively.

Machine learning (ML)

We trained the model using three sets of data: one set of parameters acquired from the hospital, one set of parameters lab-based assays, and one set with both types of parameters. The parameters from the hospital included 17 features “Sex”, “Hispanic”, “Diabetes”, “Hypertension”, “SBP”, “DBP”, “RBCs”, “WBC”, “Platelets”, “AST”, “ALT”, “LDH”, “CRP”, “Ferritin”, “D-dimer”, “HgbA1C”, “Anticoagulant”. The parameters from lab-based assays included 19 features including “Platelets Percentage of Whole Blood”, “Neutrophils”, “Monocytes”, “B cells”, “MHCII Monocytes”, “Resistin”, “IL-8”, “IP-10”, “IL-6”, “IFNλ2/3”, “MHCII+Platelets”, “LCN2/NGAL”, “Myoglobin”, “CRP”, “OPN”, “MPO”, “ICAM-1”, “VCAM-1”, “Cystatin C”. Prior to training the ML models, the data was pre-processed by addressing missing values. For the missing values, the average value was used for imputation. In the present study, missing values of HgbA1C were imputed using a HgbA1C threshold approach. Specifically, for non-diabetic participants, missing values were filled with a value of 5.7, which is the lower limit of the normal HgbA1C range. For diabetic participants, missing values were filled with a value of 6.5, which is the diagnostic cut-off for diabetes. For the undetected values from ‘IL-6’, ‘IFN’, and ‘Cystatin C’, half of the lowest measurable value was used to impute. Random forest was applied from the scikit-learn library to train the data. The ‘leave-one-out’ cross-validation was used to evaluate the performance of the model and the feature_importances function provided by the random forest to select the features as the best combination predictors. In random forest, feature importance is computed by averaging the importance of a feature across all trees in the forest. The feature importance score is calculated as the

average decrease in impurity (measured by Gini impurity) caused by splits involving the feature. These selected features were then utilized to train a Simple Decision Tree Classifier. A visualization of the decision tree generated from this classifier was created to provide an intuitive representation of the relationships between the features and the outcome.

5.3.4 RESULTS

Hispanic individuals and co-morbidities are over-represented in severe infection

A total of 112 participants were included in this study and were placed into groups based on their COVID-19 status: 1) healthy control (n=23), 2) outpatients with moderate COVID-19 infection (n=33), 3) ICU patients with severe COVID-19 infection (n=33), and 4) recovered (n=23) (**Figure 5.3.1**). Baseline characteristics are shown in **Table 5.3.1**. P-values indicate results of comparisons between each experimental group and healthy control group for each listed characteristic. Participant ethnicity data is based on self-reporting. Non-Hispanic group included individuals self-reporting as White (not of Hispanic origin), Asian or Pacific Islander, Black (not of Hispanic origin), or multiracial. The number of male and female participants were balanced between each experimental group compared to healthy controls. The mean age of moderate and severe infection groups was balanced (p=0.398), and recovered and healthy control groups had comparable mean ages (p=0.270). Active infection groups had increased age compared to healthy controls (p<0.001). Hispanic individuals were significantly over-represented in the severe infection group (69.7%, p=0.012) compared to other groups and the demographic data for Riverside County (51.6% Hispanic) (US Census Bureau, 2022). There were also significantly more co-morbidities, including diabetes and hypertension, reported in the severe (p<0.001) and moderate (p=0.002) infection groups

compared to healthy controls. Vaccination status also differed significantly in moderate ($p=0.001$) and severe ($p<0.001$) infection groups compared to healthy controls while the recovered group had comparable vaccination status ($p=0.480$). This difference may be partially explained by differences in vaccine availability during sample collection from infected individuals compared to healthy participant recruitment. Overall, these data indicate that Hispanic individuals and reported co-morbidities were over-represented in the severe infection group which correlates with hospitalization and death statistics in Riverside County for the time period of the study (2021-2022) (County of Riverside, 2023).

Confirmation of COVID-19 viral burden in moderate and severe infection

To confirm active COVID-19 infection and determine viral demographics, nasal viral burden and circulating spike protein in plasma were evaluated in moderate and severe infection groups. Participants with moderate and severe infection had detectable levels of nasal viral burden with significantly higher viral load in the severe infection group (**Figure 5.3.S1A**). Circulating spike protein concentrations were also compared across mortality outcomes within the severe infection group, and there was no significant difference between COVID-19 survivors or non-survivors (**Figure 5.3.S1B**).

Immune cell subsets and circulating immune and endothelial factors are dependent on COVID-19 disease severity.

Immune cell subsets and plasma factors were measured to identify infection-induced changes and associations with disease severity. Flow cytometry gating of the peripheral blood was performed to quantify innate and adaptive immune cells and platelets (**Figure 5.3.2A-C, Figure 5.3.S2**). Flow plots of major immune populations from

concatenated data for each study group demonstrated differences in key immune cell subsets (**Figure 5.3.S3**). In severe infection, innate neutrophils were significantly elevated compared to moderate infection (p=0.010) and healthy controls (p=0.031), and monocytes were significantly decreased compared to moderate infection (p<0.0001). B cells (p=0.015) , NK T cells (p=0.002), CD8- T cells (p=0.0002), and CD8+ T cells (p<0.0001) were all significantly decreased in the severe infection group compared to healthy controls and moderate infection (B cells: p=0.002; NK T cells: p=0.020; CD8- T cells: p=0.030; CD8+ T cells: p=0.009) (**Figure 5.3.2A**). These results demonstrate alterations in both innate and adaptive immune subsets that are dependent on disease severity.

Significant changes were observed in circulating chemokines and interferons relating to chemotaxis, inflammatory, and anti-viral responses in severe infection (**Figure 5.3.2B**). The neutrophil chemoattractant IL-8 was significantly elevated compared to both healthy controls (p=0.0004) and moderate infection (p= 0.002). The chemoattractant IP-10 (CXCL10) was also significantly increased compared to healthy controls (p<0.0001) and moderate infection (p= 0.002). Pro-inflammatory IL-6 was significantly increased in severe infection (p= 0.009) while TNFα was decreased (p= 0.016) compared to healthy controls. Anti-viral IFNλ2/3 (p= 0.002) and IFNγ (p= 0.0003) were also significantly decreased in severe infection.

Indicators of endothelial damage were also altered based on disease severity (**Figure 5.3.2C**). Myoglobin (p=0.02), VCAM1 (p<0.0001), and myeloperoxidase (MPO) (p<0.0001) were all significantly upregulated in the plasma of the severe infection group compared to healthy controls. There were also multiple endothelial markers that that were downregulated in severe infection including ICAM-1 (p=0.02) and C-reactive

protein (CRP) (p=0.0003). Lipocalin (LCN)-2 (NGAL) was not significantly altered in severe infection (p=0.77) but was significantly decreased in the moderate infection group (p=0.001). Collectively, this data demonstrates severity-dependent changes in immune populations, cytokine response, and endothelial damage.

Immune and endothelial biomarkers are impacted by the presence of co-morbidities during severe infection and are associated with fatal outcomes

To discern potential correlations between Hispanic ethnicity, infection severity, and immune/endothelial changes, correlation matrices were plotted for immune and endothelial parameters (**Figure 5.3.3**). Hispanic individuals showed strong positive correlations with severe infection (**Figure 5.3.3A-B**). Hispanic ethnicity was also positively correlated with elevated neutrophils and IL-8 but negatively correlated with monocytes, MHC II expression by monocytes, B cells, and CD8+ T cells (**Figure 5.3.3A**). Analysis of endothelial parameters revealed that Hispanic ethnicity was positively correlated with VCAM-1 and MPO (**Figure 5.3.3B**) which corresponds to elevated levels of these markers seen in severe infection (**Figure 5.3.2**).

The clinical co-morbidities diabetes and hypertension that were over-reported in the severe infection group showed significant impacts on immune and endothelial parameters that were associated with severe disease (**Figure 5.3.3C-D**). Participants with diabetes had increased circulating neutrophils (p=0.04), IL-6 (p=0.004) and myoglobin (p=0.02) but no differences in circulating LCN2/NGAL (p=0.19) (**Figure 5.3.3C**). Participants with hypertensions had increased circulating myoglobin (p=0.02) and LCN2/NGAL (p=0.02), but no changes in VCAM-1 (p=0.15) or platelets (p=0.30) (**Figure 5.3.3D**). These results demonstrate that both immune and endothelial

parameters are correlated with disease severity and are altered based on the presence of co-morbidities that may reflect health disparities in the severe infection group.

To investigate relationships between demographic, immune, and endothelial parameters based on the outcome of severe infection, correlation matrices were performed for survival vs. fatality outcomes (**Figure 5.3.S4**). Severely infected participants with a fatal outcome had more significant correlations between these parameters (**Figure 5.3.S4A**) compared to those that survived (**Figure 5.3.4B**). In fatal infection outcomes, there were significantly negative correlations between Hispanic ethnicity and the cytokine Resistin, but there were no strong positive correlations between Hispanic ethnicity and any other parameter (**Figure 5.3.S4A**). In the survival group, Hispanic ethnicity was not significantly correlated to Resistin or IL-6 but exhibited negative correlations with the endothelial parameter Cystatin C (**Figure 5.3.4B**).

The factors that were differentially correlated in fatal vs. survival outcomes were investigated further to determine if these differences contributed to a fatality (**Figure 5.3.4**). Neutrophils were significantly increased in the fatal ($p=0.03$) compared to survival group (**Figure 5.3.4A**). Expression of MHCII by monocytes was significantly downregulated in the fatal group ($p=0.04$) despite there being no significant changes in monocyte percentages ($p=0.07$) (**Figure 5.3.4A**). The fatal group also had significantly increased plasma Resistin ($p=0.008$) and IL-6 ($p=0.003$) compared to the survival group (**Figure 5.3.4B**). Finally, the endothelial damage indicators myoglobin ($p=0.01$) and LCN2/NGAL ($p=0.007$) were also significantly upregulated in fatal outcome (**Figure 5.3.4C**).

To determine the relationships between the significantly altered factors in fatal vs. survival infection outcomes, correlation analyses were performed (**Figure 5.3.4D-E**).

In the fatal group **(Figure 5.3.4D)**, neutrophils were negatively correlated with B cells, MHCII expression by monocytes, and platelets. Resistin, IL-6, and myoglobin were all positively correlated with each other in fatal infection. In the survival group (**Figure 5.3.4E)**, monocytes were negatively correlated with the endothelial marker VCAM-1 and platelets. Monocyte MHCII was also significantly negatively correlated with neutrophils. Taken together, these results demonstrate key immune and endothelial markers that differ depending on the outcome of severe infection. Neutrophilia, low MHCII expression by monocytes, and elevated plasma Resistin, IL-6, myoglobin, and VCAM-1 correlated with fatal infections.

Immune and endothelial changes are sustained following recovery from moderate COVID-19 infection and correlate with long COVID

Given the serious and poorly understood complication of long COVID development after infection, we examined prolonged immune and vascular changes associated with long COVID in recovered individuals in Riverside County and investigate the impact on lung health and immune homeostasis. We were unable to re-recruit individuals from the severe, ICU patient group due to high mortality and declination to participate in the follow-up study. Flow cytometric analysis revealed long-lasting alterations in both innate and adaptive immune cells despite resolution of infection (**Figure 5.3.S5A** and **Figure 5.3.5A**). Notable differences were observed in the frequencies of neutrophils, monocytes, B cells, NK T cells, CD8- T cells, and CD8+ T cells after recovery from moderate infection (**Figure 5.3.S5A**). The frequency of neutrophils ($p<0.0001$) decreased significantly in recovered participants compared to healthy controls (**Figure 5.3.5A**). Monocytes ($p=0.002$), B cells ($p=0.02$), NK T cells ($p<0.0001$), and CD8- T cells ($p<0.0001$) remained upregulated following recovery while

CD8+ T cells were not changed (p=0.74) (**Figure 5.3.5A**). These alterations in both innate and adaptive immune populations were sustained as long as 11 months post-positive COVID-19 test and indicate prolonged effects of COVID-19 infection on immune homeostasis.

Circulating cytokines returned to baseline levels in recovered participants (data not shown), but there were endothelial damage indicators that were altered following recovery from infection. Myoglobin (p= 0.02), VCAM1 (p=0.002), MPO (p=0.03), and LCN2 (NGAL) (p=0.008) were all significantly downregulated in the recovered group compared to healthy controls (**Figure 5.3.5B)**. These results demonstrate long-lasting effects of COVID-19 infection that could impact endothelial health.

Prolonged changes in the expression of immune and endothelial biomarkers following recovery suggested long COVID presence. To test this, all recovered participants completed the COVID-19 Yorkshire Rehabilitation Scale (YRS) questionnaire for self-reporting of the presence and severity of symptoms defining long COVID. The average reported “Functional Disability” score was significantly increased following COVID-19 infection in the recovered group (**Figure 5.3.5C**). This score encompasses symptoms related to communication problems, mobility, personal care, daily activity, and social role. The most highly impacted function was communication (adj. p = 0.04). There was also a significant increase in “Overall Symptom Severity” after COVID-19 infection. This score encompassed several different physical symptoms such as breathlessness, cough, swallowing, fatigue, continence, pain, cognition, and other psychological symptoms. The most highly impacted symptom was fatigue (adj. P = 0.012), but several other symptoms were also significant prior to correction for multiple family-wise comparisons (**Table 5.3.2**).

The symptoms “Fatigue” and “Communication Problems” were significantly altered after infection following multiple comparison corrections (**Figure 5.3.5C**). Other symptoms that were significantly altered following recovery from infection were “Breathlessness,” “Cough/Throat Sensitivity,” “Pain,” “Daily Activity Problems,” “Cognition Problems,” “Anxiety,” and “Depression” (**Figure 5.3.S5B**). When overall lung health was examined using spirometry testing, lung health was not significantly altered in the recovered group compared to healthy controls indicating a return to baseline lung health (**Figure 5.3.5D**). In summary, these results demonstrate sustained immunological and endothelial changes long after recovery from infection that suggest prolonged effects of COVID-19 on overall immune health and correlate with the presence of long COVID.

Participant-specific tracking of immune changes over time during and following COVID-19 infection

Tracking of immune composition within individual participants at different time points (TP1 and TP2) was performed (**Figure 5.3.6**). UMAP projections of flow cytometry data enabled the visualization and quantification of immune subsets in severely infected participants at enrollment in the ICU (TP1) and three days later (TP2) (**Figure 5.3.6A-B**). Participants with moderate infection were also tracked before (TP1), and after treatment with monoclonal antibody (TP2) (**Figure 5.3.6C-D**). Last, healthy control subjects who subsequently were infected with COVID then recovered, were analyzed for changes in the peripheral blood immune subsets pre- and post-infection (**Figure 5.3.6E-F**). While the majority of immune cells present did not change from TP1 to TP2, innate monocytes decreased over time in severe infection ($p=0.05$). In moderate infection, there were decreasing trends in neutrophils, monocytes, and NK T cells and an increasing trend in B cells (**Figure 5.3.6D**). Paired tracking from uninfected timepoints and six weeks after

recovery demonstrated visible changes in all evaluated immune cell-types following recovery from infection (**Figure 5.3.6E**). Quantification of these immune cells showed increases in B cells (p=0.02), NK T cells (p=0.02), a trend towards monocyte increase (p=0.06) and a decreasing trend in neutrophils (p=0.07) (**Figure 5.3.6F**). Taken together, these changes observed between individually paired participants show dynamic changes in immune subsets in infection, and also indicate that COVID infection leads to persistent changes in the peripheral immune subsets.

Machine learning model identifies predictive markers of infection outcome in severe disease

We employed a random forest algorithm as the machine learning model to determine the optimal combination of parameters for predicting survival in severe COVID-19 patients. The metrics mainly investigated were precision and recall. Precision and recall are two of the most relevant metrics to consider when predicting patient survival using machine learning models because they directly measure the ability of the model to correctly identify true positive cases, which are the patients who survive. The results indicated that when utilizing solely parameters obtained from the clinic, the strongest predictor of fatality was [‘Diabetes’, ‘Hispanic’] with a precision of 76% and recall at 100%. When utilizing only parameters from lab-based assays for immune cells and circulating cytokines and endothelial factors, the optimal combination of predictors was ‘‘LCN2(NGAL)’’,‘‘MHCII Monocyte’’,‘‘IL-6’’], which achieved a precision of 86% and recall at 98%. Notably, when combining all parameters in a single random forest model, the strongest predictors for survival were ‘‘LCN2’’,‘‘MHCII Monocyte’’,‘‘IL-6’’,‘‘Anticoagulant’’], resulting in a precision of 86% and recall at 100% (**Figure 5.3.7A**).

This data suggests the strength of combining data collected from the clinic with lab-based assays for improved prediction of survival outcomes in severe COVID-19 patients.

The decision tree analysis using the selected features, namely LCN2, MHCII Monocytes, IL-6, and Anticoagulant, revealed distinct patterns associated with patient survival and fatality outcomes (**Figure 5.3.7B-C**). In patients receiving anticoagulant therapy (Anticoagulant = 1), there was a notable tendency towards survival, with 72% (18 out of 25) of the patients surviving compared to 28% (7 out of 25) with fatal outcomes (**Figure 5.3.7B**). In contrast, in the absence of anticoagulant treatment (Anticoagulant = 0), a higher fatality rate of 75% (3 out of 4) was observed, as opposed to a 25% (1 out of 4) survival rate. Furthermore, patients with LCN2 levels below 159ng/mL exhibited a higher likelihood of survival, as demonstrated by the n=12 in this category (**Figure 5.3.7C**). Among these, patients with monocytes expressing MHCII at a mean fluorescence intensity lower than 15,550 showed a higher fatality rate, as evidenced by n=7 in this group. In contrast, patients with LCN2 levels above 197ng/mL had a higher risk of fatality, as indicated by n=2 in this category. Among patients with LCN2 levels below 197ng/mL, a lower amount of circulating IL-6 (< 435pg/mL) was indicative of a higher survival rate, with all 7 individuals surviving (**Figure 5.3.7C**). This suggests that lower LCN2 and IL-6 levels might be associated with improved prognosis in this patient population. In summary, the decision tree analysis highlights the potential predictive value of LCN2, MHCII expression by monocytes, IL-6, and Anticoagulant treatment in determining survival and fatality outcomes in patients within our study cohort and warrants further investigation in larger cohorts to validate these findings.

5.3.5 DISCUSSION

Study Overview and Novelty

There are currently alarming health disparities such as inadequate access to healthcare among Hispanic communities, especially among those with pre-existing health conditions. These inequities place individuals at a higher risk of severe or fatal disease as shown by previous work (Bender Ignacio *et al.*, 2022; Rao *et al.*, 2023). Determining the impacts of COVID-19 infection within at-heightened-risk populations is of vital importance. As a result, the goal of this study was to determine links between immunological and endothelial changes and COVID-19 disease severity in Riverside County, CA, a predominantly high-risk and Hispanic community. Results from this study suggest a higher prevalence of severe disease in individuals of Hispanic ethnicity, which may be driven by increased rates of key clinical co-morbidities, and other sociodemographic factors that impact disease risk and outcomes.

This study is unique as it describes immune and vascular biomarkers associated with disease severity in a high-risk population. It also evaluates the relationship between immune and vascular alterations, long COVID, and lung health which have not previously been demonstrated. The biological relevance of our findings is demonstrated through paired tracking of individual participants' immune responses at multiple timepoints during infection and after recovery. A machine learning model was used to learn the features that predict patient survival from severe COVID-19 infection and has the potential to uncover hidden insights that might otherwise be overlooked (Davenport & Kalakota, 2019).

Health Disparities and Severity of COVID-19 Infection

This study revealed that a significant proportion of individuals suffering from severe COVID-19 infection in Riverside County, CA, are Hispanic, and these participants report higher instances of co-morbidities, particularly diabetes, hypertension, and obesity. These results highlight likely healthcare disparities in preventative healthcare access in the severe group. According to the RUHS, 84% of their payors use Medi-Cal insurance (Riverside University Health System, 2020). Statewide Medi-Cal enrollment data shows that 59% of those patients enrolled are Hispanic/Latino youth, which is more than double the enrollment of Asian/Pacific Islander and White ethnicities (Patel, 2017). Furthermore, the Riverside area is home to a high number of uninsured individuals (Meconis, 2017). It has been reported that Medi-Cal patients with co-morbidities requiring specialist visits have difficulties in accessing specialist care (i.e., endocrinologists for those with diabetes or cardiologists for those with hypertension). According to the U.S. Bureau of Labor Statistics 2021 data, the average Hispanic/Latino median household income was $55,321 compared to $74,912 for non-Hispanic white households (Occupational Outlook Handbook, 2022). In 2020, the U.S. Census Bureau reported that 17% of Hispanic individuals were living at poverty level (U.S. Census Bureau, 2023). As a result, few insurance options are available in these cases, and a large percentage of underserved populations utilize Medi-Cal, where they experience difficulties accessing routine preventative care and specialist visits, potentially leading to the worse outcomes seen in the severe infection cohort. Furthermore, limited healthcare access, fear of financial burden, or avoidance of the healthcare system due to documented status can result in patients waiting longer to seek care and therefore

receiving treatment only after the disease has advanced to a more severe stage, also leading to increased mortality risk (Lopez *et al.*, 2021).

Previous work has investigated the impact of health disparities on socioeconomic status and overall psychological health as a result of the pandemic (Pinto *et al.*, 2022; Tao *et al.*, 2022; Zhao *et al.*, 2022; Rao *et al.*, 2023). However, it is unknown how health disparities that are prevalent in underserved Hispanic communities relate to infection-dependent biological alterations and infection outcome. The severe infection group in this study was predominantly composed of Hispanic individuals, and this group demonstrated significant changes in immune cell populations and vascular factors which corresponded to a positive correlation with disease severity. As diabetes has been directly linked to increased COVID-19 disease severity (Shkurnikov *et al.*, 2022), the increased frequency of diabetes, which often leads to complications with viral infections, reported by Hispanic individuals in the severe group offers a prime example of health disparities negatively impacting COVID-19 disease outcomes. Based on these cumulative data, further studies are needed to determine the impact of health disparities on the risk of Hispanic communities developing long COVID.

Severity-Dependent Changes in Immune and Endothelial Factors and Their Predictive Capability

Several immune factors including both innate and adaptive cell populations are necessary to successfully combat COVID-19 infection (Westmeier *et al.*, 2020; Hartley *et al.*, 2020; Meidaninikjeh *et al.*, 2021; Röltgen & Boyd, 2021; Hazeldine & Lord, 2021; Li *et al.*, 2022*a*; Roukens *et al.*, 2022). The severe infection group showed significant alterations to all immune subsets compared to healthy controls and moderate infection, indicating relationships between immune parameters and disease severity. Neutrophils

and the neutrophil chemoattractant IL-8, which are known to cause increased damage during COVID-19 infection, were significantly increased in this group compared to both healthy controls and the moderate infection group (Hazeldine & Lord, 2021; Herrera *et al.*, 2023; Rice *et al.*, 2023; Leal *et al.*, 2023). In our global correlation analyses, neutrophils were also positively correlated with the inflammatory cytokine IL-6, and all of these immune parameters were positively correlated with severe infection. These same factors and Resistin, which has been identified as an indicator of disease severity and early predictor of mortality in sepsis (Bonenfant *et al.*, 2022), were also increased in individuals who had a fatal infection outcome. This data demonstrates a direct link between neutrophils, neutrophil-chemotactic biomarkers, and disease severity in our high-risk study cohort. Contrary to neutrophil patterns observed, all other immune subsets and Type I interferons were significantly decreased in the severe group. These immune parameters were also negatively correlated with severe infection and positively correlated with healthy participants in our correlation analyses. These opposing immune populations in healthy versus severe infection supports the importance of these cells for combatting infection as previously reported (Westmeier *et al.*, 2020; Hartley *et al.*, 2020; Roukens *et al.*, 2022; Satta *et al.*, 2022). Lack of these cells in the severe group could indicate an impaired antiviral response.

Measurement of endothelial biomarkers showed severity-dependent changes in circulating vascular damage indicators. The severe infection group demonstrated significantly elevated circulating levels of myoglobin, VCAM-1, and MPO which were significantly correlated with severe infection. MPO and VCAM-1 were also positively correlated with Hispanic ethnicity in this group, and myoglobin was significantly elevated in fatal infection. Myoglobin has been directly linked to oxidative vascular damage

(Ohashi *et al.*, 1998; Leoncini *et al.*, 2008; Bains *et al.*, 2010), which could explain increased lung pathology in fatal COVID-19 infection. The elevated levels of MPO seen directly correspond to the observed increases in neutrophils in severe infection, and MPO production by neutrophils has been associated with COVID-19 disease pathology (Hazeldine & Lord, 2021). In addition, MPO showed significant positive correlations with Resistin and IL-6 in fatal infection. Currently, techniques are being developed to create potential drug targets for MPO (Chaikijurajai & Tang, 2020) and myoglobin (Pimentel *et al.*, 2021) in COVID-19 disease that could be used to mitigate COVID-19 disease severity caused by vascular damage.

Our machine learning results demonstrate that the inclusion of lab-based assays in addition to routine assays performed in the clinic can significantly improve the accuracy of the predictive models, indicating the complementary nature of the two types of parameters investigated in this study. We found that 'LCN2(NGAL),' 'MHCII expression by monocytes,' 'IL-6,' and 'Anticoagulant treatment' revealed distinct patterns associated with severe infection outcomes. In severe COVID-19 patients receiving anticoagulant therapy, a tendency towards survival was observed, matching previous studies that have reported the potential benefits of anticoagulant treatment in this patient population (Bikdeli *et al.*, 2020; Paranjpe *et al.*, 2020). Emerging evidence from our study also suggests that lower LCN2 levels might be associated with improved prognosis in severe COVID-19 patients. LCN2, or Lipocalin-2 (also known as neutrophil gelatinase-associated lipocalin or NGAL), is an acute-phase protein involved in immune response and inflammation (Flo *et al.*, 2004). Recent work has indicated that elevated LCN2 levels are correlated with the severity of COVID-19 infection and may serve as a potential biomarker for disease progression (Huang *et al.*, 2022). Our finding that higher

MHCII expression by monocytes is associated with improved prognosis confirms results found in recent studies (Schulte-Schrepping *et al.*, 2020; Zhang *et al.*, 2021). The increased expression of MHCII by monocytes in severe COVID-19 patients that survive infection suggests that a more robust immune response contributes to better clinical outcomes and resistance to re-infection (Giamarellos-Bourboulis *et al.*, 2020). Recent studies have indicated that severe COVID-19 patients with lower inflammatory interleukin-6 (IL-6) levels may exhibit improved prognosis (Aziz *et al.*, 2020; Ruan *et al.*, 2020). This association between the combination of anticoagulant therapy, lower LCN2 levels, increased expression of MHCII by monocytes, lower IL-6 levels, and improved prognosis warrants further investigation as it could aid in developing targeted therapies and personalized treatment strategies for COVID-19 patients. In the context of healthcare, even modest improvements in predictive accuracy can have significant impacts on patients' lives, informing treatment decisions and resource allocation (Rajkomar *et al.*, 2019). By leveraging machine learning to analyze these limited datasets, researchers can gain valuable insights into disease mechanisms, prognosis, and therapeutic approaches, ultimately improving patient care and outcomes.

Recovery from infection and biomarkers for long COVID

Previous studies have identified sustained immune alterations following recovery from mild and severe COVID-19 infection (Hartley *et al.*, 2020; Kennedy *et al.*, 2021; Li *et al.*, 2022*a*). In addition, the presence of long COVID and subsequent immune changes have been investigated previously (Phetsouphanh *et al.*, 2022; Wiech *et al.*, 2022; Ryan *et al.*, 2022). Recent studies reported that women, black individuals, and Hispanic individuals are more likely to experience long COVID (Sivan *et al.*, 2022). Consistent with these studies and the primarily Hispanic makeup of Riverside County,

our recovered group was composed of 43% Hispanic individuals which accounted for more than any other single ethnicity.

Our work shows significant increases in monocytes, B cells, NK T cells, and CD8- T cells following recovery from mild-to-moderate infection as well as significant decreases in neutrophils and circulating vascular biomarkers compared to healthy controls. This indicates a prolonged systemic inflammatory response despite successful clearance of active infection in these recovered participants, which has been hypothesized with previous work demonstrating increased immune activation. These results were also seen across our paired timepoint analyses of healthy controls who later returned as recovered participants. As a result, our data suggests potential risks of autoimmunity and/or overactive immune responses following COVID-19 infection.

5.3.6 CONCLUSION

Our study adds to current knowledge of long-term COVID-19 impacts by showing previously undemonstrated prolonged vascular alterations. We also offer insights into the impact of prior COVID-19 infection on lung health via the use of spirometry measurements. Recent work has identified specific immune signatures that underly certain post-acute COVID-19 sequalae in the lung linking prolonged immune dysregulation with lasting tissue pathology caused by infection (Cheon *et al.*, 2021). This indicates the potential for sustained immune alterations to have deleterious effects on overall lung health following recovery from infection and during long COVID. Our results indicate that despite sustained immune changes and self-reported symptoms suggesting the presence of long COVID, overall lung function is recovered as no significant change was observed in spirometry tests between our recovered and healthy control participants.

Limitations and Caveats

While our study offers novel insights into the long-term health impacts of COVID-19 infection, there remain limitations. First, there was missing information for various participants in each group, such as missing blood collection for some participants due to logistic issues. Second, different treatment regimens offered and given to severe and moderate infection groups (such as Remesdivir/dexamethasone for severe group, monoclonal antibody treatment for moderate group, and Paxlovid for certain recovered participants) could have affected the immune and/or vascular endothelial responses observed. Third, our modest sample size, including our paired participant time point data, limits our ability to generalize these results, and increased participant numbers are needed to perform more rigorous statistical analysis in the future. The addition of increased data representing the Hispanic population in the future could help with comparisons of Hispanic versus non-Hispanic groups and discern population-specific immune alterations. There are also age differences between the Moderate/Severe infection groups and the Healthy Control/Recovered groups which could contribute in part to differences observed in certain biomarkers. The sample size for the Yorkshire Rehabilitation Scale Questionnaire is also limited. Despite this smaller sample size, our study agrees with several other studies worldwide that utilize this questionnaire (Halpin *et al.*, 2021; Ayuso García *et al.*, 2022; Sivan *et al.*, 2022; Straudi *et al.*, 2022). Lastly, it should be considered that the immune response is extremely complex and can vary greatly between individuals which could contribute to some of the variation seen in the results of our study. In addition, our machine learning model made use of a small sample size that warrants further testing to confirm validity of our results for this portion of our study. Further prospective clinical studies that address these caveats are needed to

better understand the relationships between COVID-19 disease severity and immune changes as well as the relationship between long COVID and sustained immune alterations.

DECLARATIONS

Ethics Approval and Consent to Participate

This study was approved by the institutional review boards (IRB) of Riverside University Health System Medical Center (RUHS) and the University of California Riverside (UCR) (HS-20-121 and HS-20-128). Study participants were informed of the risks of participation and provided written informed consent in their native language, English or Spanish. For some ICU patients who were unable to provide consent, written informed consent was waived by the IRB committee at RUHS (HS-20-121), and instead, a designated surrogate decision-maker provided written consent. This study complies with the *Declaration of Helsinki*, except for registration in a database.

Consent for Publication

Not applicable.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing Interests

The authors declare that they have no competing interests.

Funding Statement

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Author Contributions

KVB, KP, JL, YH, IHC, SF, and MK performed laboratory evaluation of samples. KVB, KP, JL, YH, SA, XQ, KI, and AG performed statistical and bioinformatics analysis. MTU and PM supplied severe and moderate infection samples and clinical data. KVB, KP, XQ, KI, MGN, and ECH wrote manuscript.

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FIGURES

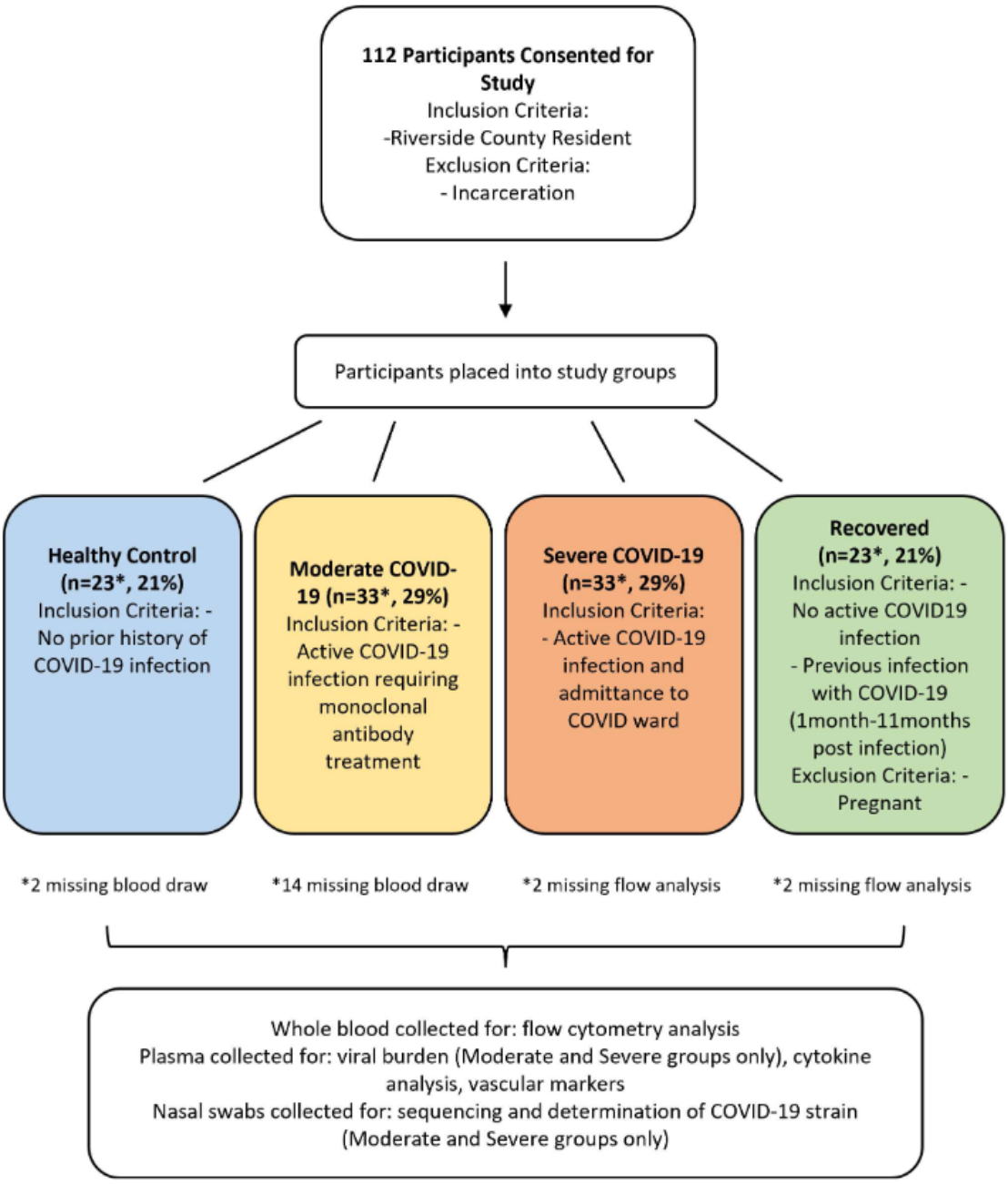

Figure 5.3.1. Flow diagram of study design, subject enrollment, grouping criteria, and experimental methods.

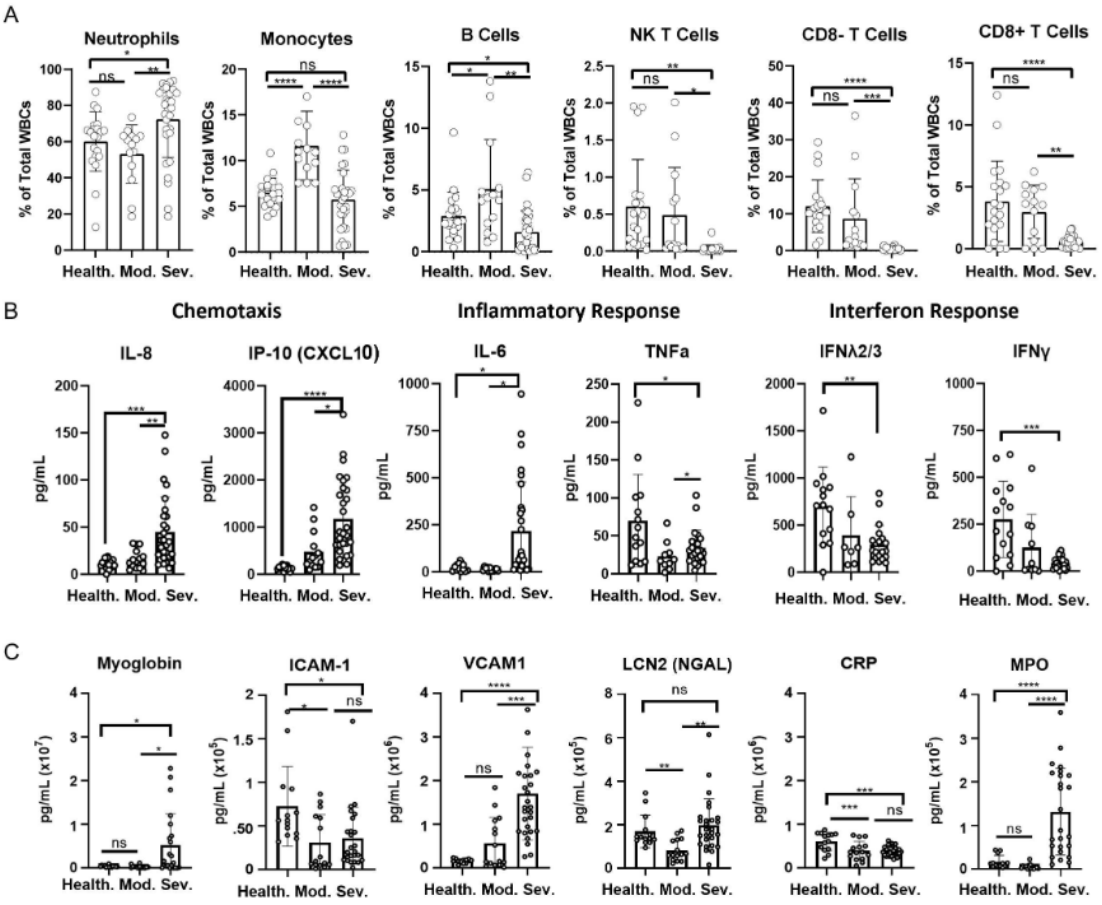

Figure 5.3.2. Immune and endothelial damage analysis during active COVID-19 infection. A) Quantification of significantly altered immune populations from analyzed flow cytometry data. B) Circulating cytokine levels from plasma of control and infected groups. C) Circulating endothelial damage markers from plasma of control and infected groups. For all experiments, “Healthy” n=21, “Outpatient/Moderate” n=19, “ICU/Severe” n=31, “n” altered for some results depending on availability of samples. Statistical significance determined via One-Way ANOVA using multiple comparisons.

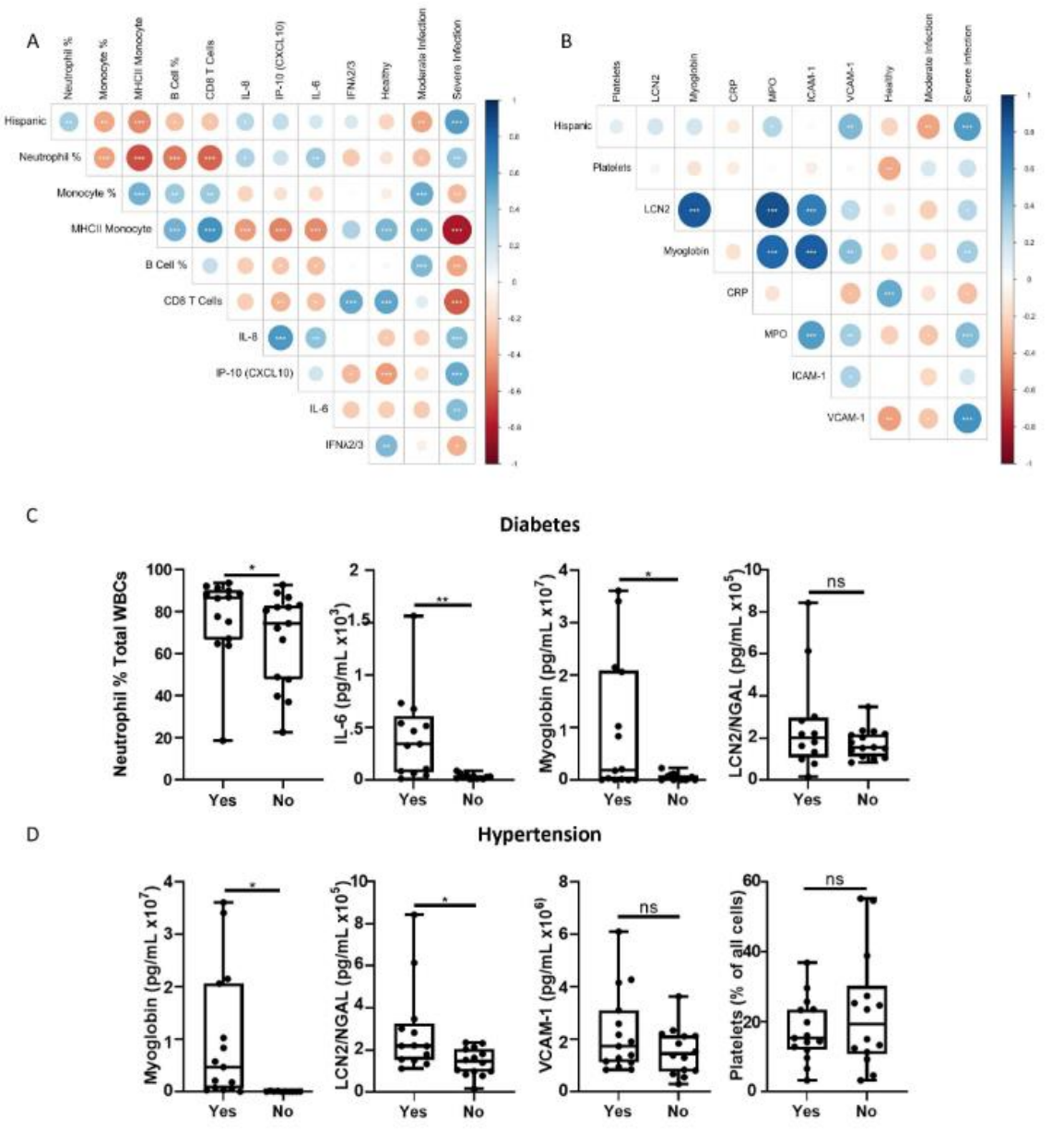

Figure 5.3.3. Correlation analyses of immune, endothelial, and clinical parameters. A-B) Correlation matrix of immune (control n=21, outpatient/moderate infection n=19, ICU/severe infection n=31) **(A)** and endothelial (control n=15, moderate infection n=19, severe infection n=31) **(B)** parameters across control and infected groups. **(C-D)** Circulating levels of immune and endothelial parameters that have significant correlations with the clinical parameters of diabetes **(C)** and hypertension **(D)** in the severe COVID-19 group (n=29). Statistical significance determined via unpaired student's t-test.

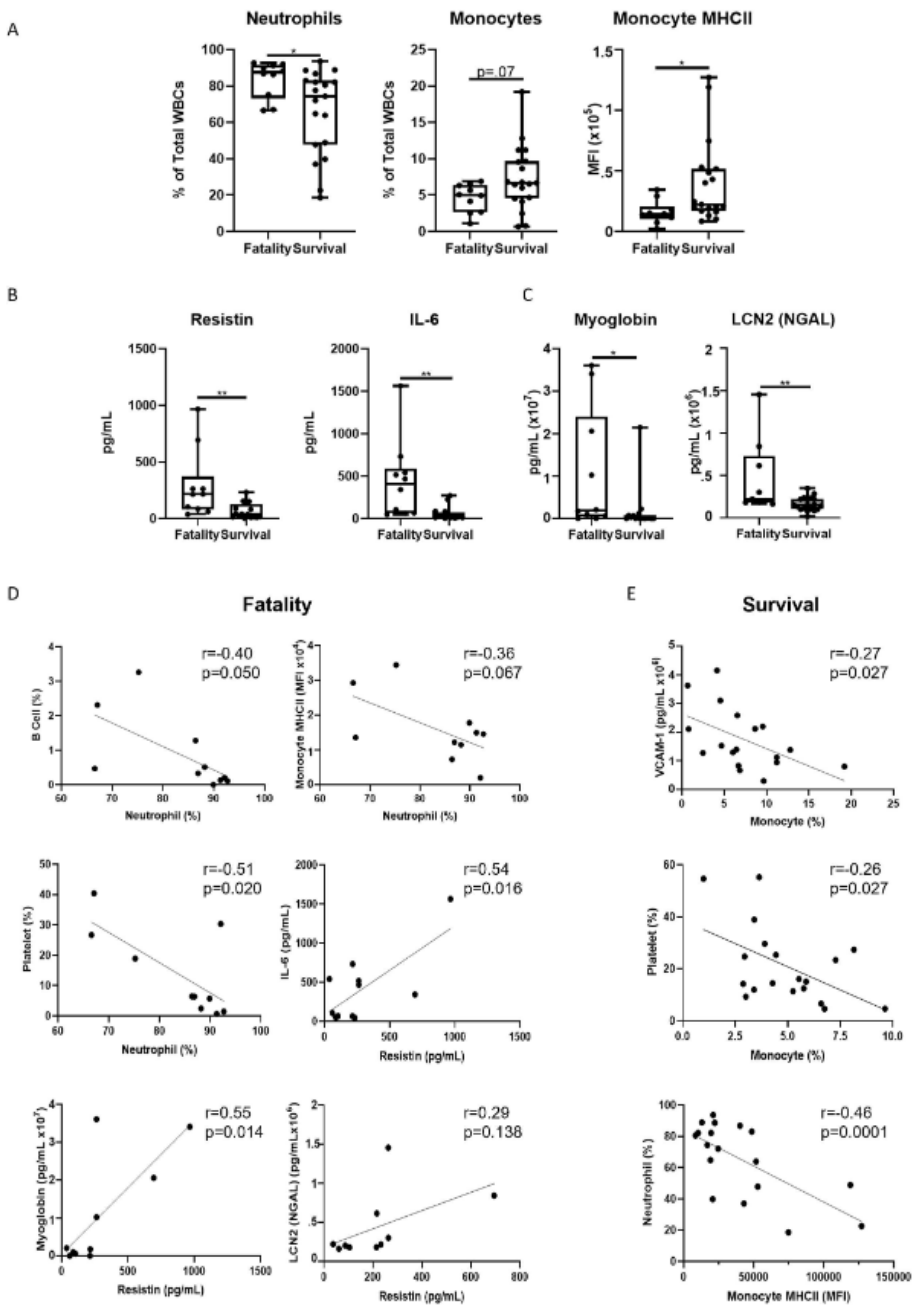

Figure 5.3.4. Analysis of immune and endothelial factors during severe COVID-19 infection based on infection outcome. (A) Significantly altered immune populations from flow cytometry data distinguished by fatality or survival. **(B)** Significantly altered circulating cytokine levels from fatal vs. survival groups. C) Significantly altered circulating endothelial damage markers from plasma of fatal vs. survival groups. **(D-E)** Correlations between parameters within fatal **(D)** and survival **(E)** groups. Statistical significance determined via unpaired student's t-test.

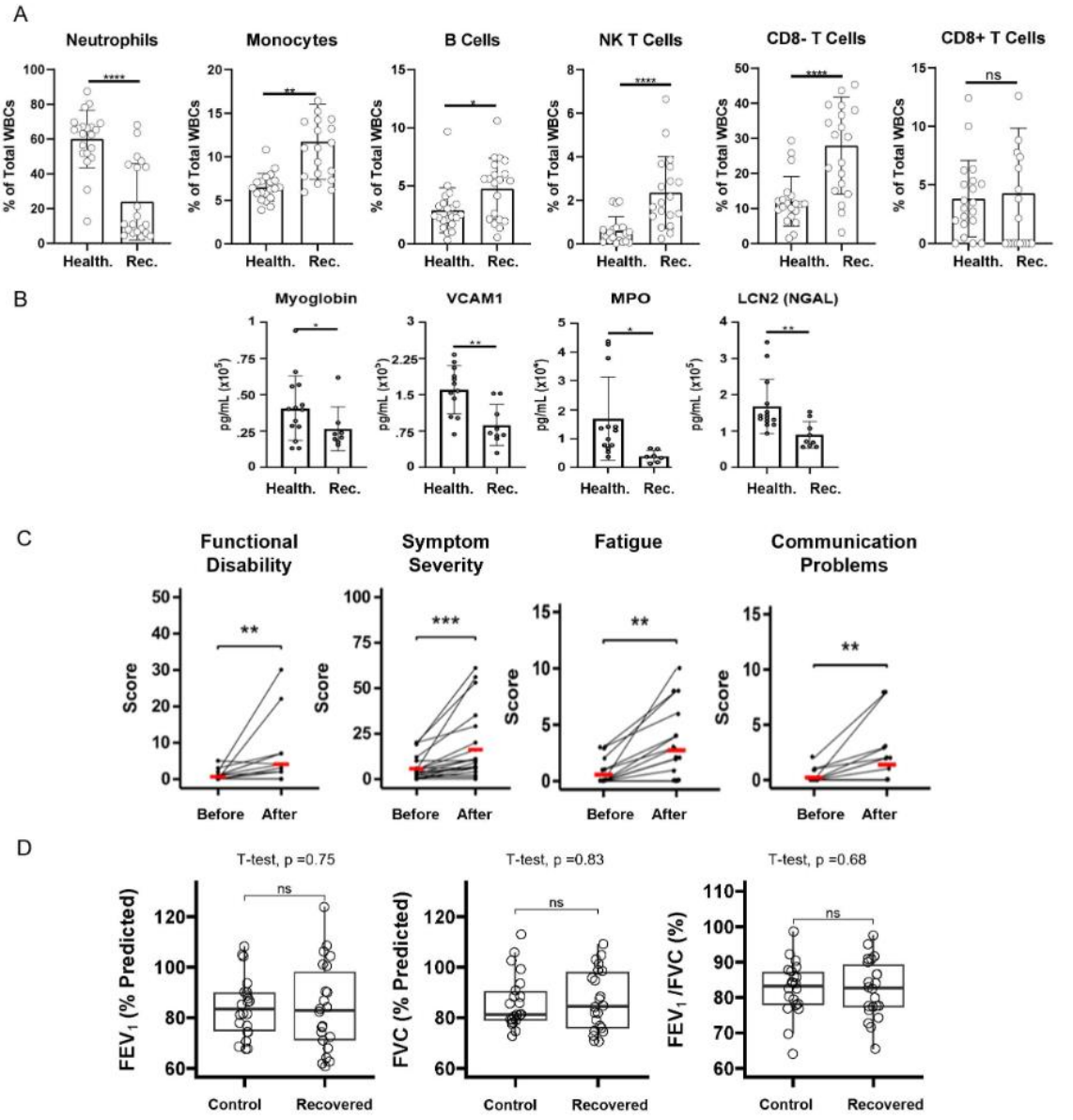

Figure 5.3.5. Immune analysis following recovery from infection including long COVID parameters and lung function tests. (A) Quantification of immune populations from healthy control and moderate recovered flow cytometry data (n=18/group, significant outliers removed from analysis). **(B)** Circulating endothelial damage markers from plasma of control and recovered groups ("Healthy" n=14, "Recovered" n=9). **(C)** Correlation of self-reported COVID-19 symptoms associated with long COVID as assessed by YRS questionnaire (n=23); points are slightly jittered for visibility. **(D)** Results of lung spirometry tests measuring overall lung function. Statistical significance determined via unpaired student's t-test.

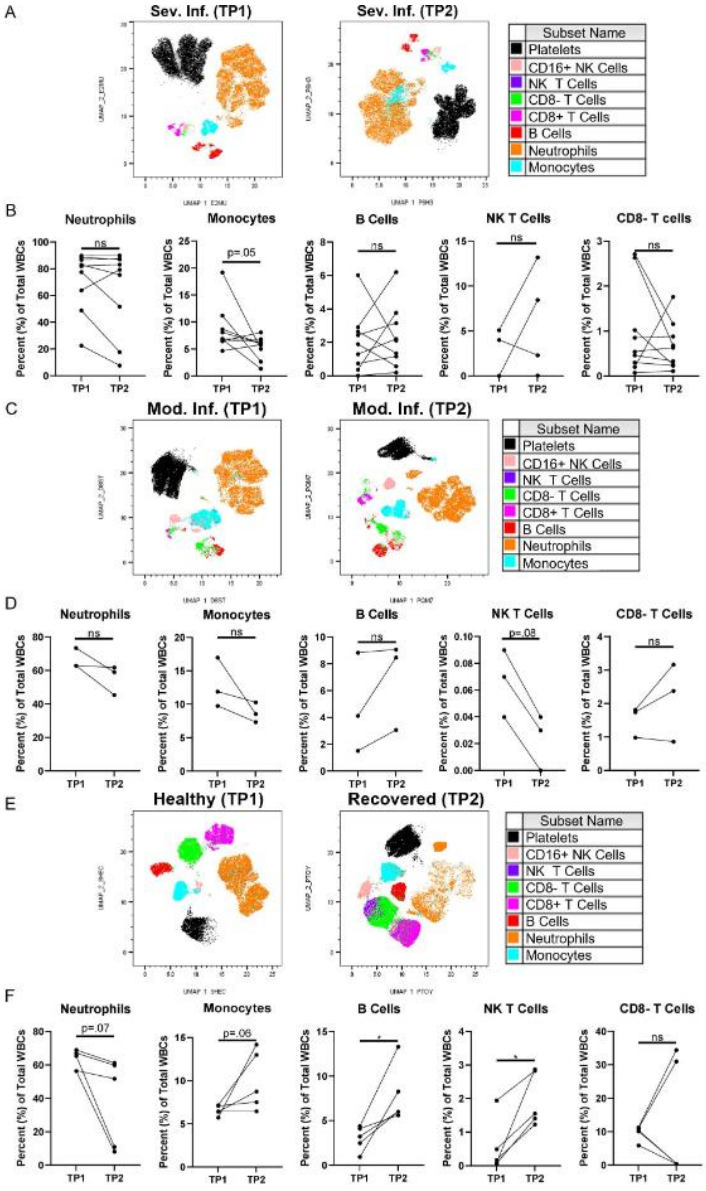

Figure 5.3.6. Paired time point analyses of flow cytometry data during severe infection, moderate infection, and following recovery. UMAP analyses **(A, C, and E)** and graphical quantification **(B,D, and F)** of flow data from single participants with paired time points for different stages of infection: **A-B)** severe infection at 1 day (TP1) and 3 days (TP2) post admission; **C-D)** moderate infection at monoclonal antibody treatment start date (TP1) and 7 days aftertreatment start (TP2); E-F) prior to COVID-19 infection (TP1) and following recovery (varying number of days post COVID+ test result) (TP2). Statistical significance determined via unpaired student's t-test; severe (n=9), moderate (n=3), and control/recovered (n=5) participants at time point one (TP1) and time point two (TP2).

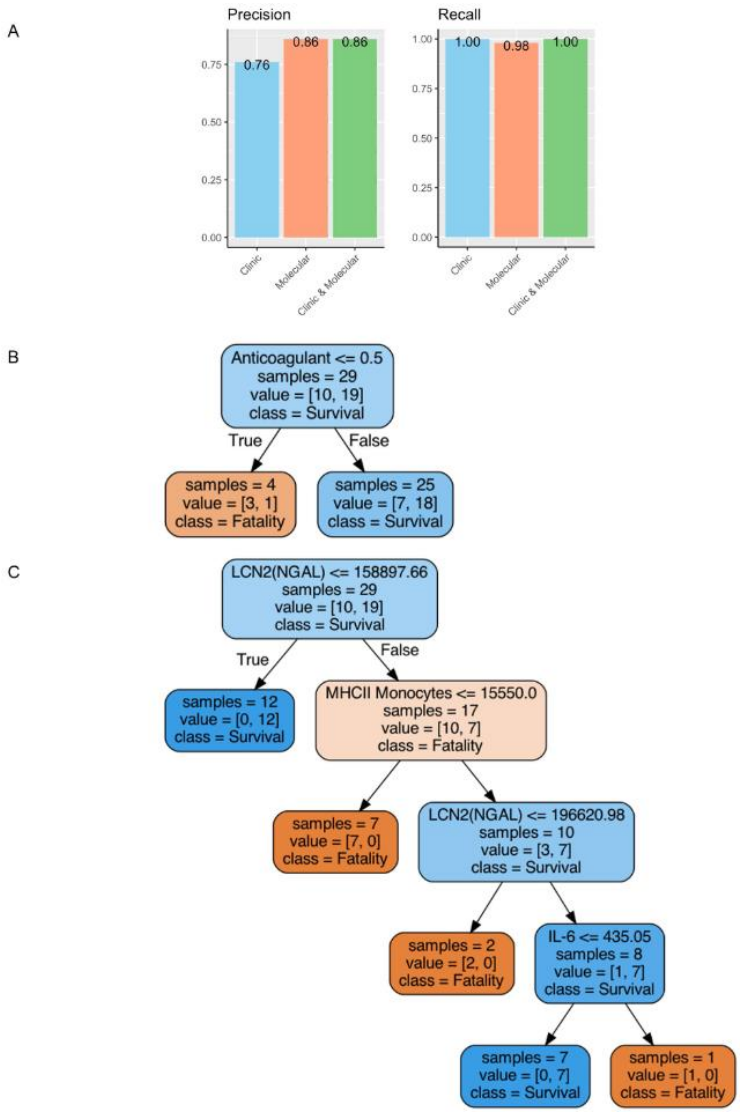

Figure 5.3.7. Machine learning analysis. (A) Feature analysis and comparison of our machine learning models across precision and recall metrics predicting survival. Performance of our parameters from the clinic (blue), parameters from molecular (orange) methods vs. parameters from both the clinic and molecular (green) across four metrics. **(B-C)** Decision Tree for Predicting Survival from Severe COVID-19. This figure illustrates the decision tree used to identify significant parameters for predicting survival from severe COVID-19. **(B)** The tree is first divided based on whether a patient had Anticoagulation treatment. **(C)** The tree is further divided based on patients' "LCN2(NGAL)," "MHCII Monocytes," and "IL-6" levels. With different thresholds from Anticoagulation, LCN2, MHCII Monocytes, and IL-6, the branches lead to different outcomes. The figure also includes the threshold for each parameter, represented by the numbers at the end of each branch, and the sample size from under the threshold from the parameter. Additionally, the figure includes the value which represents the number of possible outcomes of a decision and the decision made.

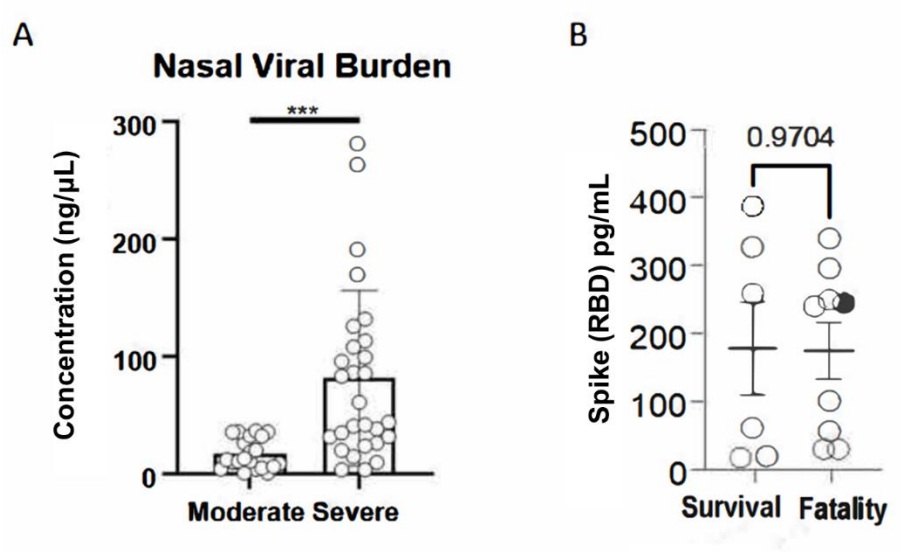

Figure 5.3.S1. Confirmation of active COVID-19 infection for severe and moderate infection groups. (A) Quantification of viral burden from nasal swab of severe and moderate infection groups. **(B)** Quantification of blood spike protein in severely infected participants based on survival vs fatality from infection.

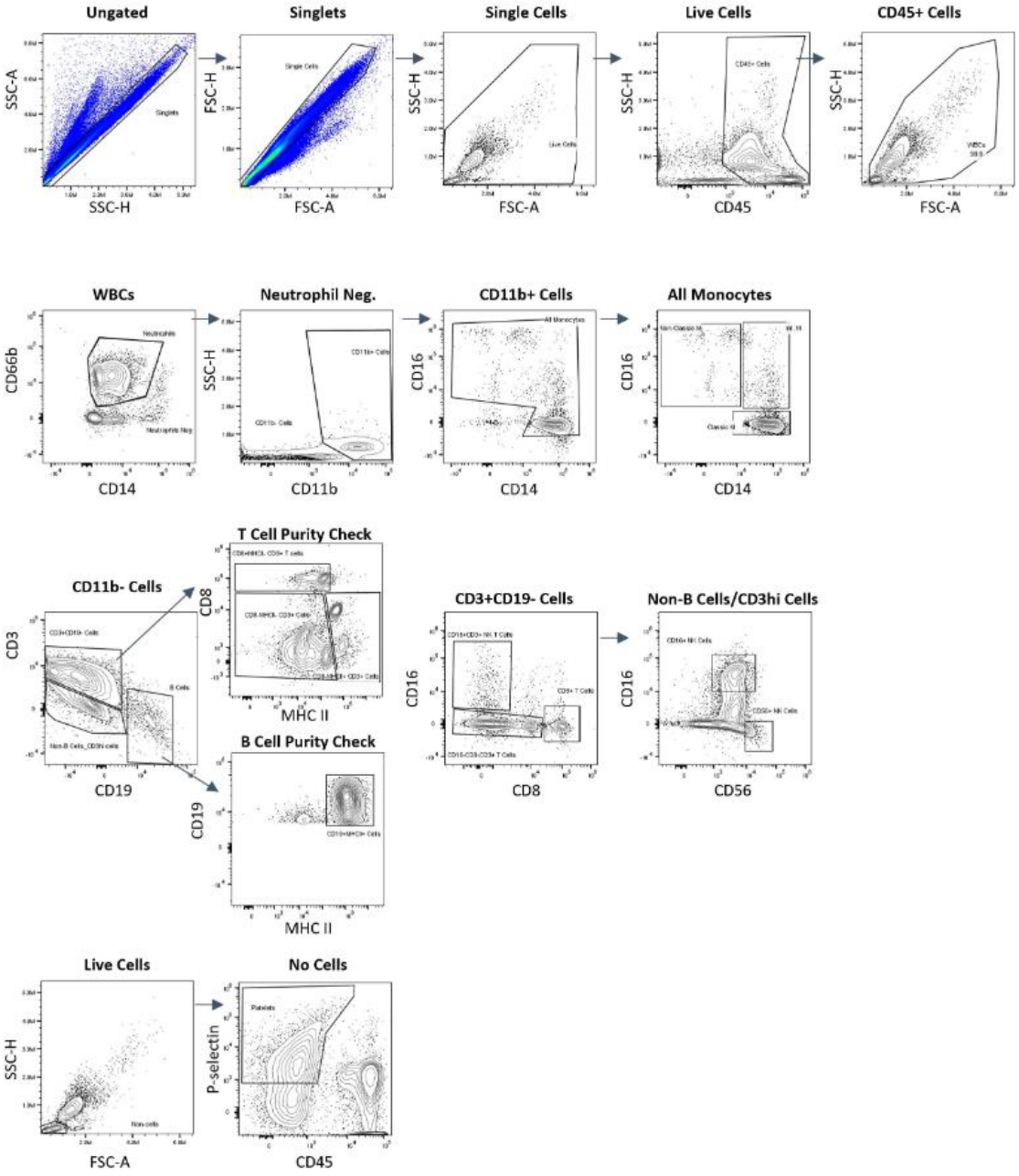

Figure 5.3.S2. Full gating strategy for whole blood flow cytometry. Concatenated healthy control sample used as in Figure 5.3.2.

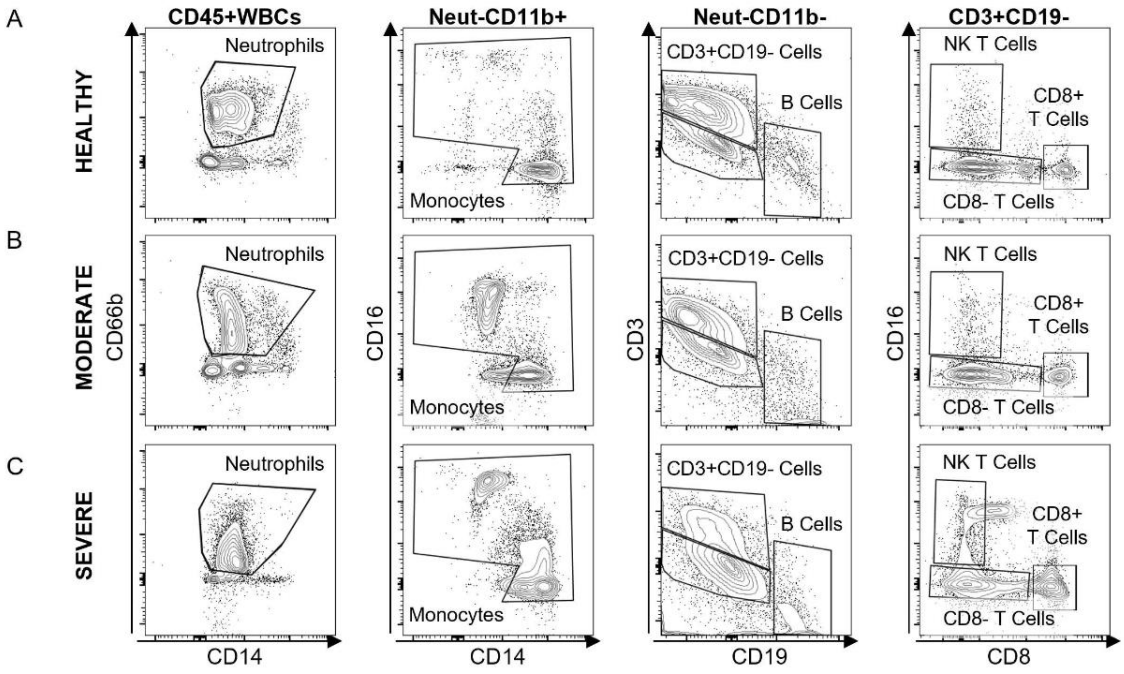

Figure 5.3.S3. Flow plots of significantly altered immune cell types by study group. (A-C) Flow plots of major immune cell populations analyzed for control **(A)**, moderate infection **(B)**, and severe infection **(C)** groups (all individual samples concatenated by group for shown plots).

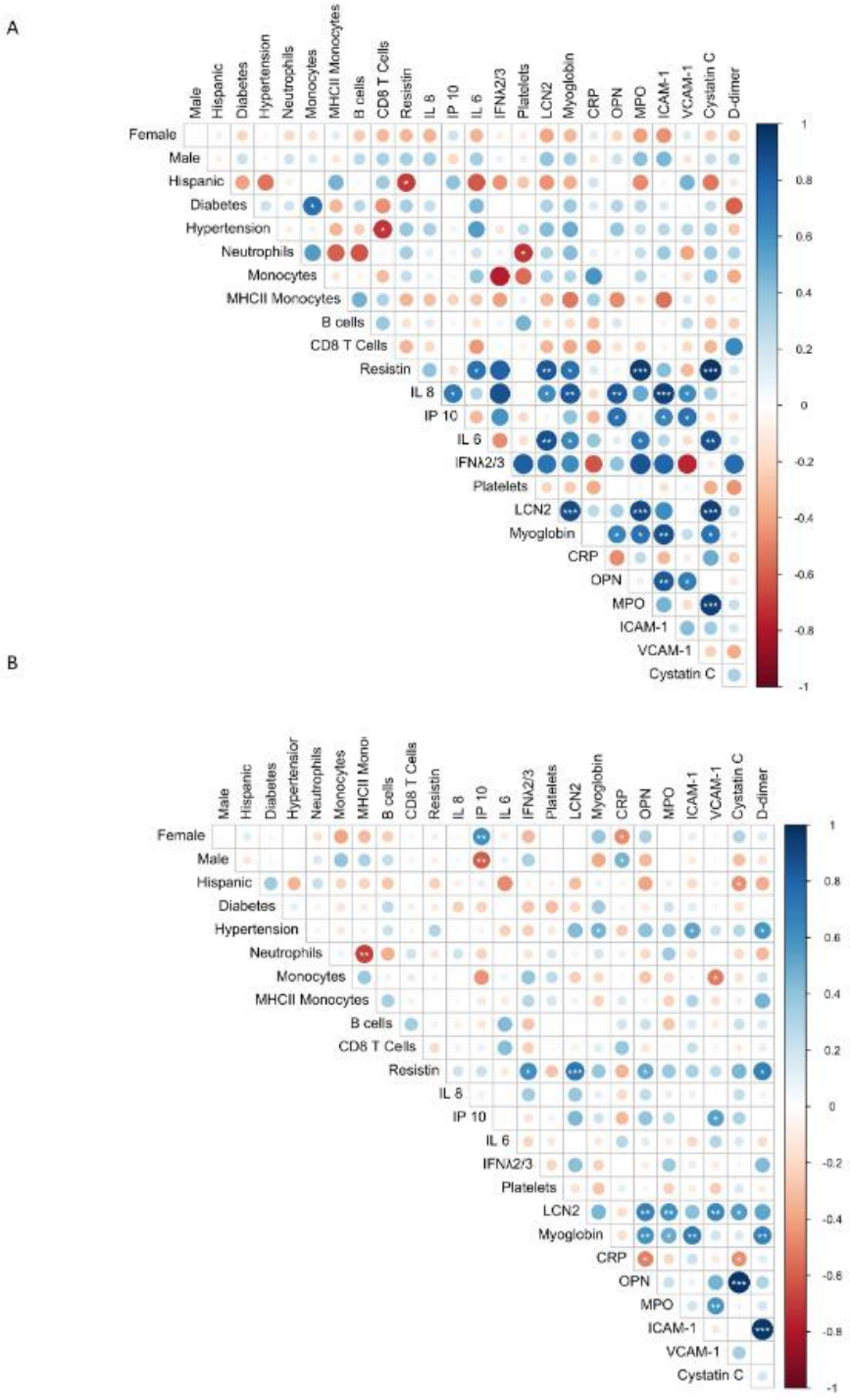

Figure 5.3.S4. Correlation matrices of demographic, immune, and endothelial parameters in severe COVID-19 infection with fatal and non-fatal outcomes. A) Fatal outcome correlation matrix. B) Survival outcome correlation matrix.

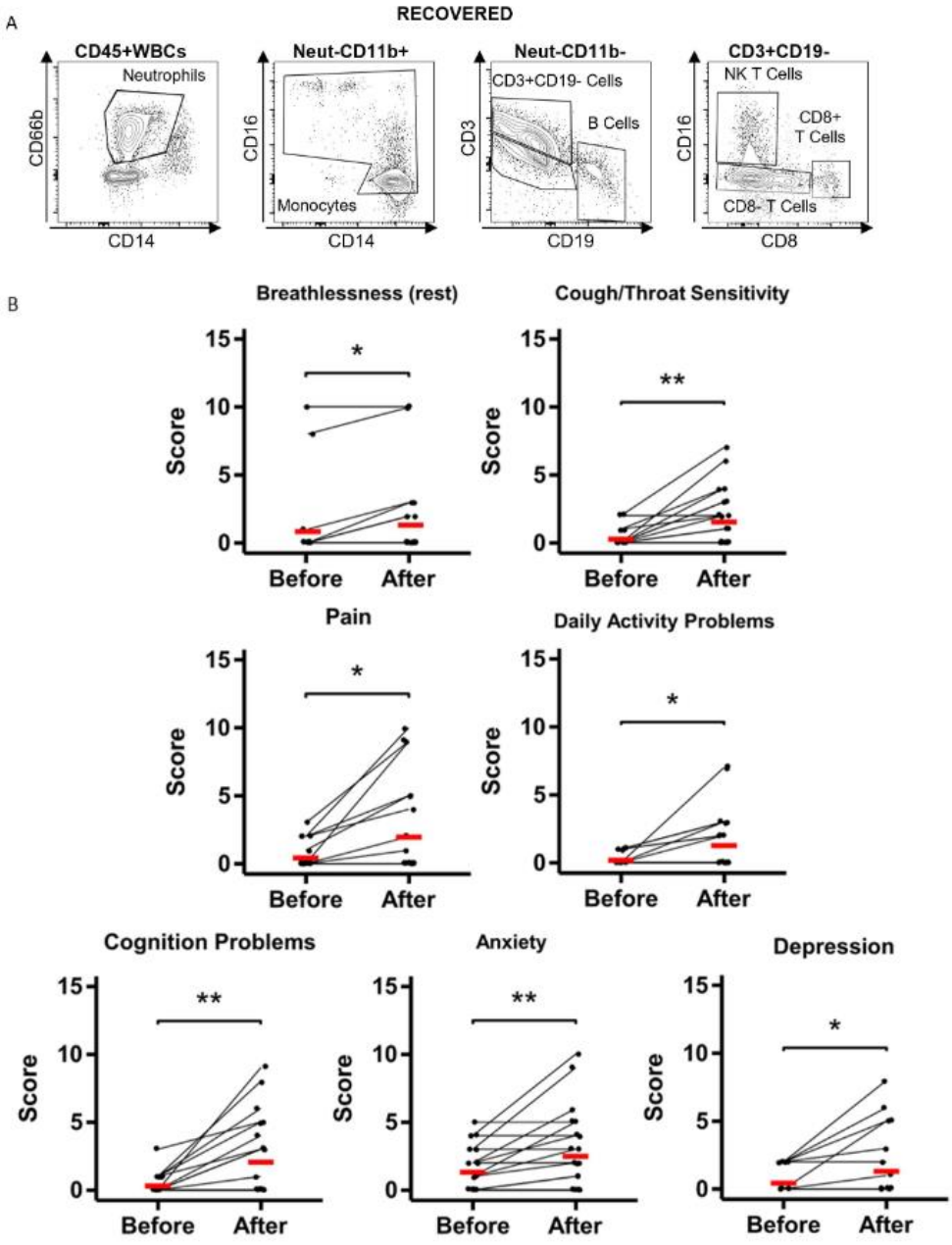

Figure 5.3.S5. Significantly altered immune cell types and long COVID symptoms in Recovered group. (A) Flow plots of major immune cell populations analyzed for moderate recovered group (all individual samples concatenated by group for shown plots). **(B)** Correlation of self-reported COVID-19 symptoms associated with long COVID identified as significantly altered prior to multiple comparison corrections as assessed by YRS questionnaire (n=23); points are slightly jittered for visibility.

Table 5.3.1. Study demographics by group

Characteristic	Healthy Control n=23(%)	Outpatient Moderate Infection n=33(%)	P-value	ICU Severe Infection n=33(%)	P-value	Recovered n=23(%)	P-value
Sex							
Male	**12 (57)**	**13 (39)**	**0.27**	**23 (70)**	**0.39**	**10(48)**	**0.76**
Female	**9 (43)**	**20 (61)**		**10 (30)**		**11(52)**	
Age, years							
Mean (SD)	**27.60 (7.27)**	**52.88 (16.68)**	**0.0003**	**56.09 (13.86)**	**<0.001**	**31.05 (12.17)**	**0.27**
Median (IQR)	**26.00 (19.00-52.00)**	**48.00 (22.00-84.00)**		**52.00 (23.00-80.00)**		**27.00 (19.00-67.00)**	
Ethnicity, No. (%)							
Hispanic	**7 (33)**	**8 (25)**	**0.55**	**23 (70)**	**0.012**	**9 (43)**	**0.75**
Non-Hispanic	**14 (67)**	**24 (75)**		**10 (30)**		**12(57)**	
Co-morbidity, No. (%)							
Yes	**8 (38)**	**27 (82)**	**0.002**	**30 (91)**	**<<0.001**	**8 (38)**	**1**
No	**13 (62)**	**6 (18)**		**3 (9)**		**13 (62)**	
Vaccinated, No. (%)							
Yes	**18 (95)**	**10 (45)**	**0.001**	**9 (28)**	**<<0.001**	**21 (100)**	**0.48**
No	**1 (5)**	**12 (55)**		**23 (72)**		**0 (0)**	

Table 5.3.2. Self-reported symptoms as obtained by YRS Questionnaire results.

	Scores			
Symptom	**Pre-COVID-19**	**Post-COVID-19**	**P**	**P Adj**
Breathlessness (at rest)	0.83 ± 2.61	1.30 ± 2.91	0.05	0.29
Breathlessness (while dressing)	1.00 ± 2.86	1.70 ± 3.10	0.06	0.29
Breathlessness (stairs)	2.04 ± 2.70	2.65 ± 2.64	0.12	0.49
Cough or throat symptoms	0.26 ± 0.62	1.52 ± 2.10	0.01	0.07
Swallowing problems	0.04 ± 0.21	0.30 ± 1.11	0.37	0.74
Fatigue	0.57 ± 1.08	2.74 ± 3.18	0.00	0.01
Continence	0.09 ± 0.42	0.74 ± 2.00	0.17	0.52
Pain and discomfort	0.43 ± 0.90	1.96 ± 3.34	0.01	0.11
Cognition	0.30 ± 0.70	2.04 ± 2.85	0.01	0.07
Anxiety	1.30 ± 1.58	2.48 ± 2.94	0.01	0.08
Depression	0.43 ± 0.84	1.30 ± 2.38	0.04	0.25
PTSD	0.00 ± 0.00	0.13 ± 0.63	1.00	1.00
Communication problems	0.22 ± 0.52	1.39 ± 2.37	0.01	0.04
Mobility problems	0.04 ± 0.21	0.70 ± 2.03	0.17	0.52
Personal care	0.09 ± 0.43	0.23 ± 0.75	0.59	0.59
Daily activity problems	0.17 ± 0.39	1.26 ± 2.14	0.01	0.06
Social role	0.09 ± 0.42	0.48 ± 1.20	0.17	0.52

Chapter 6

Conclusion

Summary

While there are numerous mechanisms that contribute to high-altitude acclimatization, this work both confirms previous findings and provides new evidence regarding the role of inflammation and immune function. It is clear that high altitude is a stressful environment that triggers the release of pro-inflammatory mediators and affects the immunological cell balance. This dissertation examines the changes in the transcriptomic profile, the cytokine profile, and the immune profile, and how these alterations may contribute to development of high-altitude illnesses. Here, we introduce a potential mechanism of hypoxia-induced immune sensitization following acute high-altitude exposure that may exacerbate inflammatory and immune responses. Furthermore, we explored differences in the time domains of high-altitude acclimatization by investigating distinctions in the inflammatory profiles between lowlanders who traveled and acclimatized to high altitude and with native Andean highlanders who have survived in high-altitude hypoxic environments for thousands of generations.

This work identified novel transcriptional changes seen during acute high-altitude exposure in non-native lowlanders. Specifically, we have identified several inflammatory-related genes involved in innate immunity, such as the Toll-Like Receptor 4 signaling pathway, and therefore may indicate a potential mechanism for immune system sensitization. Furthermore, this work has characterized plasma cytokines that are essential to the immune response as well as changes in immune cell populations and function after acute high-altitude exposure. These changes provide insight into the mechanism behind hypoxia-induced inflammation and how high altitude impacts immune

cell function. Additionally, these changes on a transcriptional, plasma cytokine, and immune population levels are found to be correlated with high-altitude illness severity, providing insight into how the inflammatory profile may affect acclimatization. Immune profile characterization in lowlanders traveling to high altitude has revealed shifts from a pro- to anti-inflammatory profile as individuals acclimatize. Our work has found further evidence that immune system sensitization is focused on innate immunity, and we also report potential adaptive immune suppression at high altitude. This study also investigated the impact of hypoxia inducible factor (HIF) stability on CD14 expression, a co-receptor of the TLR4 pathway, and found that HIF stability promotes CD14 expression. The relationship between physiological responses and inflammatory profile changes to high altitude can be further explored to examine how hypoxemia modulates the inflammatory response in critical illnesses.

We demonstrate significant differences between acute and chronic hypoxia exposure time domains. Our work has determined relationships between high-altitude illness severity and immune population shifts with inflammatory cytokines on an acute timescale. In this study, we characterized the chronic hypoxia exposure time domain specifically in Andean highlanders. Here, we have determined that chronic hypoxia exposure in the Andean population have elevated pro-inflammatory cytokine levels that are correlated with Chronic Mountain Sickness severity. Interestingly, this relationship was not seen with excessive erythrocytosis, which is thought to be the main mechanism responsible for Chronic Mountain Sickness development. For both acute and chronic time domains, we identified potential biomarkers of high-altitude illness severity. We have identified IP-10 to not only be significantly elevated in native Andeans compared to acutely exposed sojourners to high altitude, Andeans with excessive erythrocytosis have

significantly higher concentration than healthy Andeans. This distinction, however, is not seen in Andeans with Chronic Mountain Sickness versus healthy Andeans. This indicates that additional underlying mechanisms are in play in Chronic Mountain Sickness development, in which we suspect a pro-inflammatory profile may play a role.

In pulmonary diseases, hypoxemia may exacerbate the inflammatory response to viral or bacterial infections. Of most notable interest at this time, the coronavirus disease of 2019 (COVID-19) pandemic sparked incredible scientific effort to understand how to manage and treat patients with severe hypoxemia, as COVID-induced hypoxemia was associated with mortality rates. Even after recovery from COVID-19, there are long-lasting detrimental health effects that indicate potential risks of autoimmunity as well as potential overactive immune responses. Our meta-analysis study on the normal distribution of the hypoxic ventilatory response (HVR) demonstrates that there is significant prevalence of low HVR values among the general healthy population. Coupled with pulmonary diseases potentially causing profound hypoxemia, individuals who have a low hypoxic ventilatory response may be more susceptible to these diseases and develop exacerbated and severe symptoms.

Potential Future Directions

This dissertation provides novel insights into high altitude hypoxia-induced immune sensitization as well as adds to research regarding the impact of high altitude on the inflammatory and immune profile. However, there still remains several gaps in knowledge that need to be addressed. We have demonstrated the potential mechanism of high-altitude hypoxia-induced immune sensitization, but further characterization of other components of the innate immunity, as well as the impact of this immune sensitization, would be beneficial to elucidate the function of this mechanism.

Additionally, our study focuses on the native Andean highlanders when discussing the impact of chronic hypoxia exposure. However, this is only one of several native high-altitude populations and is considered to be one of the most maladaptive native groups. Characterizing the inflammatory and immune profile in other high-altitude groups, such as the Tibetans, Sherpa, and Ethiopians, would provide insight into how the inflammatory profiles and immune function alterations may contribute to or protect against high-altitude illness pathogenesis. While we have identified potential biomarkers of high-altitude illness in both acute and chronic time domains of high-altitude acclimatization, further work to investigate these differences in time domain responses will be beneficial to those who travel to or live at high altitude. Furthermore, understanding the long-term effects of high-altitude hypoxia exposure would not only provide insight into potential evolutionary adaptations that promote survival in hypoxic environments, but may give rise to new and novel therapeutic targets to treat hypoxemia-induced inflammatory and immune responses in critical and chronic illnesses.

Taken together, this dissertation provides new and supportive evidence of the role of the inflammatory and immune profile in high-altitude illness pathogenesis. Furthermore, through the lens of high-altitude physiology, this work provides a novel perspective into the responses to hypoxemia in critical pulmonary diseases. This research highlights the significant and synergistic crosstalk between the hypoxia and inflammatory transcriptional response. The essential role of this crosstalk highlights the physiological consequences of hypoxia-induced inflammation, and the importance to identify novel therapeutic targets in order to mitigate excessive inflammation with concomitant hypoxemia and systemic inflammation.

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