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Parikh, Keval A.

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INFLAMMATORY GENE EXPRESSION DURING ACUTE HIGH-ALTITUDE EXPOSURE

By

Keval A. Parikh

A capstone project submitted for Graduation with University Honors

May 6, 2021

University Honors
University of California, Riverside

APPROVED

Dr. Erica Heinrich
Department of Biomedical Sciences

Dr. Richard Cardullo, Howard H Hays Jr. Chair
University Honors

ABSTRACT

Exposure to high-altitude induces oxidative stress due to the low atmospheric oxygen pressure which limits oxygen delivery and can cause tissue hypoxia. Tissues often experience hypoxia and inflammation concurrently at the site of infection or injury due to fluid retention and immune cell recruitment that ultimately reduce the rate of oxygen delivery to tissues. High-altitude hypoxia may lead to systemic inflammation as molecular pathways that mediate the physiological response to hypoxia and inflammation share significant crosstalk. Acute inflammation is also thought to induce stress erythropoiesis, a distinct extramedullary mechanism marked by rapid production of a large burst of new erythrocytes. My project aims to elucidate the role of hypoxia in acute inflammation and stress erythropoiesis by investigating genes of interest related to these mechanisms. I hypothesized that exposure to acute hypoxia should trigger a systemic inflammatory response marked by pro-inflammatory cytokines and related biomarkers, and that molecular signs of stress erythropoiesis would be present in collected samples. We tested this hypothesis by collecting fasting RNA samples from sojourners to high altitude at sea level, and day 1 and 3 at high altitude. Participants were taken to Barcroft station in Bishop, CA, which has an altitude of 3800m. RNA-seq and gene ontology analysis of blood samples from sojourners to high altitude have yielded results that suggest hypoxia plays a role in mediating downstream inflammatory signaling and red blood cell proliferation. We found that no inflammatory cytokines were upregulated, but several mediators of inflammatory cytokines were upregulated, including *TLR4*, *IL6-ST*, and *HMGB1*. Additionally, potential markers of stress erythropoiesis and increased circulating red blood cells were also upregulated, such as *HBG1* (fetal hemoglobin), and *CD47*. While our data does not show evidence for increased inflammatory cytokine expression after 24

hours of exposure to acute hypoxia, it does suggest increased sensitivity to pro-inflammatory cytokines and presents upregulation of potential biomarkers of stress erythropoiesis.

ACKNOWLEDGEMENTS

I would like to thank first and foremost my principal investigator and faculty mentor Dr. Heinrich for her incredible patience, wisdom, and guidance throughout my undertaking of this project. My capstone would not have been possible without her, and I am deeply grateful for the opportunity to work under her. I would also like to thank graduate student Kathy Pham and my fellow Heinrich lab members, who have also provided continual support and help on my project. I would also like to acknowledge the participants that were involved in this study, without whom this research would not have been possible. Lastly, I would like to thank my parents, brother, and friends and family for their everlasting love and support. They have allowed me to grow into the person I am today, and I am forever grateful for their presence in my life.

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Introduction

Molecular signaling pathways that regulate the response to hypoxia and inflammatory stimuli share significant crosstalk. Specifically, the hypoxia inducible factor (HIF) and nuclear factor-kB (NF-κB) act as key regulators of the physiological response to hypoxia and inflammation respectively, and they share significant interdependence (Semenza 2009). This link is significant because while hypoxia-induced inflammation may play an adaptive role in protecting tissue against infection and injury, it may also lead to several maladaptive responses, especially during chronic or systemic hypoxia.

Some studies suggest that acute hypoxia exposure is correlated to increases in candidate inflammatory markers including C-reactive protein (CRP), IL-1β, and IL-6 (Klausen 1997, Boos 2016, Julian 2011). Sojourners to high altitude exposed to acute hypoxia are also at risk of developing hypoxia-induced illness such as Acute Mountain Sickness (AMS), which is characterized by headache, fatigue, nausea, and gastrointestinal issues (Roach et al. 2018). While the exact pathophysiological mechanism behind AMS is unknown, it is thought to result from inflammation-induced disruption of the blood-brain barrier, as well as anatomical factors such as insufficient cerebrospinal fluid production (West 2004; Luks et al. 2017). Individuals who develop AMS at high altitude demonstrate increased inflammatory marker expression (including TNF-α, IL-1β, and IL-6) compared to non-AMS controls (Liu et al. 2017, Wang et. al 2018). Under hypoxic conditions, IL-6 has been shown to increase hematopoietic stem cell proliferation and has a synergistic effect on red blood cell production alongside related pro-inflammatory cytokines including IL-1α and TNF-α (Faquin et al. 1992).

Some high altitude (HA) native populations, such as the Andean residents, experience excessive erythropoiesis (EE), often in conjunction with other maladaptive responses to hypoxia

including blunted respiratory drive (Heinrich et al. 2020). EE is characterized by a large increase in the circulating red blood cell count ([Hb] ≥ 21 g/dL in men and ≥ 19 g/dL in women, compared to 14-18 g/dL in men and 12-16 g/dL in women in control HA populations) (Liu 2018, Villafuerte and Corante 2016, Gonzales and Chaupis 2014, Beall 2007). EE is a hallmark of Chronic Mountain Sickness (CMS), which impacts HA populations and is a maladaptive response to chronic hypoxemia. Over 140 million people live at high altitude, and on average CMS impacts over 10% of these populations (Neigi et al. 2013, Villafuerte 2015, Villafuerte and Corante 2016). Although the specific pathogenesis of CMS is unknown, current hypotheses suggest that various genetic risk factors, some of which are known to contribute to EE in HA populations, as well as prolonged systemic hypoxia play key roles in disease development. Some of these genetic risk factors are known to contribute to EE in HA populations, however, specific mechanisms of EE are unknown.

Chronic inflammation is typically associated with anemia because systemic inflammation increases hepcidin levels and can result in impaired iron handling (D'Angelo 2013). However acute inflammatory events can also stimulate stress erythropoiesis, an extramedullary mechanism distinct from steady-state bone marrow erythropoiesis marked by rapid production of a large burst of new erythrocytes. The purpose of stress erythropoiesis is to protect against acute anemia of inflammation. (Bennet et al. 2018, Paulson et al. 2011, Ganz 2019). Studies in mice have explored this potential mechanism, where induced inflammation led to increases in erythroid progenitor cells in the spleen which were distinct from bone marrow erythroid progenitors. (Jackson et al. 2010, Liao et al. 2018). Since acute bouts of hypoxia can trigger stress erythropoiesis, I predict that acute high-altitude exposure, as well as chronic intermittent hypoxia in long-term high-altitude residents also results in activation of extramedullary stress erythropoiesis. While it is known that steady state bone marrow erythropoiesis increases at high altitude in response to increased

erythropoietin expression, the extent to which stress erythropoiesis occurs in humans is unknown. Furthermore, the mechanism behind extramedullary stress erythropoiesis and its role in the physiological response to hypoxia in humans has not been investigated. My study hopes to shed light on the potential role and presence of stress erythropoiesis in response to hypoxia-induces acute inflammation.

The goals of my capstone are (1) to determine if acute sustained hypoxia exposure creates a systemic inflammatory response and (2) identify biomarkers of stress erythropoiesis in sojourners to high altitude. I hypothesize that exposure to acute hypoxia should trigger a systemic inflammatory response marked by pro-inflammatory cytokines and related biomarkers, and that molecular signs of stress erythropoiesis would be present in collected samples. I will investigate this hypothesis by measuring the expression of inflammatory genes in HA sojourners to look for evidence of a systemic inflammatory response and potential stress erythropoiesis as a response to acute hypoxia. In addition to erythropoiesis, hypoxia-induced inflammation influences many other physiological responses that can be seen in the figure below.

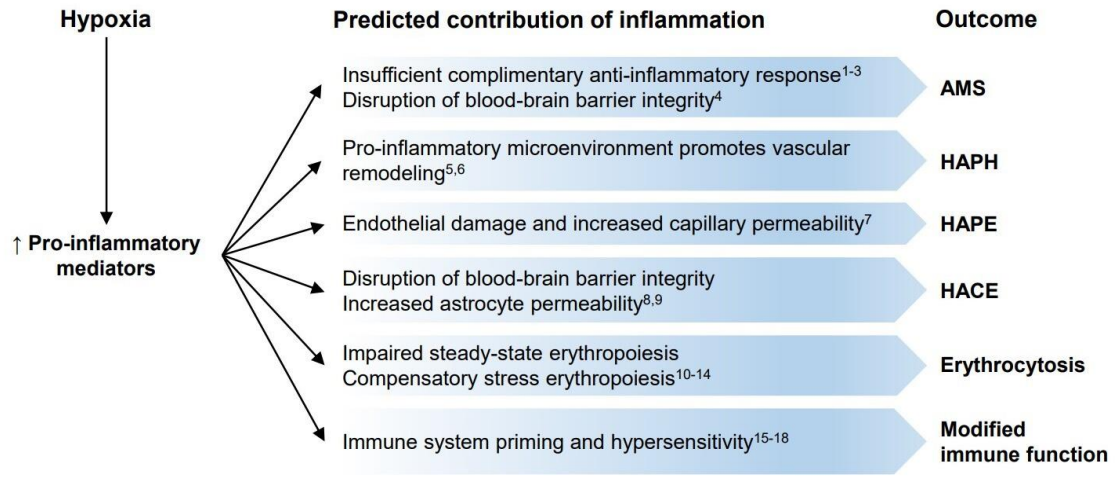


Figure 1. Predicted roles of inflammation in high-altitude illnesses. Hypoxia-induced inflammation can trigger several physiological responses, some of which result in various hypoxia-related diseases. An increase in pro-inflammatory mediators is seen to result in increased AMS incidence and erythrocytosis.

Methods

Ethical considerations: Experiments were approved by the UC Riverside Clinical IRB (HS 19-076). All work was conducted in accordance with the *Declaration of Helsinki*, except for registration in a database. Participants were informed about the risks and benefits of participating in the study and provided informed consent in their native language (English).

Participants: Participants were recruited in the summer of 2019. Exclusion criteria included: age less than 18 or over 65, history of cardiovascular or pulmonary disorders, use of anti-inflammatory medications or other agents that may interfere with ventilatory chemosensitivity, pregnancy in women, travel above 8000 feet within 1 month of the first measurements, current smokers (including cigarettes, e-cigarettes, or marijuana) that regularly smoked once or more per day, and current or recently treated systemic or serious local infection. Recruited participants were screened for medical history to ensure absence of cardiopulmonary disorders and to ensure that they were not taking any interfering medications. Participants were asked to abstain from taking acetazolamide, corticosteroids, aspirin, or other anti-inflammatory medications within 48 hours prior to data collection. Participants were also asked to not ingest alcohol or caffeinated beverages within 8 hours prior to data collection.

Study design: Recruited subjects arrived at UC Riverside in the morning and provided baseline physiological measurements and fasting blood samples. Participants were then provided with breakfast. The participants and research team then disembarked for Barcroft station (3800 m), a part of the White Mountain Research Center in the UC Natural Reserve System. The station was approximately 6 hours away from the University of California, Riverside. Barcroft Station provides full visitor amenities and research space. Participants acclimatized over a period of 3 days at the HA station.

Sample collection: Each morning at sea level and high altitude, fasting peripheral blood samples of no more than 20mL were collected by a licensed phlebotomist using standard venipuncture procedures. 2.5 ml of blood was also collected in PaxGene blood RNA tube (Qiagen, Germantown, MD, USA). PaxGene tubes were incubated at room temp for 2 hours then frozen at -20 °C. Prior to transport to UCSD, samples were stored in a liquid nitrogen dry shipper for approx. 8 hours then transferred to -80 °C at UCR until further processing. Blood was also collected in EDTA vacutainer tubes, centrifuged at 2500 g for 10 min, and plasma aliquoted into microtubes and frozen at -20 °C. Plasma was then shipped to UCR in a liquid nitrogen dry shipper and frozen at -80 °C until further analysis.

RNA isolation: To investigate genetic markers of systemic inflammatory response, samples were analyzed using RNA sequencing. RNA was initially isolated from whole blood samples collected in PAXgene Blood RNA tubes. RNA tubes were allowed to thaw and incubate at room temperature for 4 hours prior to RNA isolation as per the manufacturer's instructions. RNA was then isolated using PaxGene Blood RNA kit (Qiagen, Germantown, MD, USA), summarized in the following process. Samples were centrifuged to remove the initial supernatant, and then cells were lysed, and proteinases were added to remove protein. Samples were then passed through kit-provided spin columns, treated with DNase to remove DNA contamination, washed, and eluted to attain the final RNA samples. RNA quality and quantity were verified via Bioanalyzer (Agilent, Santa Clara, CA, USA).

RNA sequencing: RNA samples were processed and prepared by the UCR Riverside Genomics Core. The NEBNext Ultra ll Directional RNA Library Prep Kit with poly(A) enrichment (New England BioLabs, Ipswich, MA) was used for library preparation using roughly 500ng input RNA of each sample. The following adjustments were made to the NEBNext Ultra ll Directional

RNA Library Prep Kit: 0.8x beads were used during the first purification step after second strand synthesis, adaptor was diluted 1:15, 0.7x beads were used for purification after adaptor ligand, a total of 13 cycles of enrichment was done, 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 with qPCR. Samples were shipped to UC San Diego for sequencing on the Illumina NovaSeq 6000, which was conducted according to manufacturer's recommendations.

Data analysis: Data analysis was performed in R (R Studio, Boston, MA) using the workflow described by Love et al. (2016). Raw read data was filtered and aligned to Homo sapiens (human) genome assembly GRCh38 (hg38) using the R package *Rsubread* with a gapped index.

Compressed binary sequence alignment/map (BAM) formatted output files were summarized to generate count matrices, as described by Love et al. 2016. A *DESeqDataSet* object class was constructed to store read counts and intermediate estimated quantities during statistical analysis. The control level was set to "Subject", and the variable of interest was set to "Location". Data was pre-filtered to remove rows that have no counts or only a single count across all samples. The pre-filtered count data was then transformed with rlog. Data was normalized using the *DESeq2* package in R.

Data was visualized in several different ways using PCA plots, MDS plots, MA plots, and gene count plots. PCA plots were used to analyze sample-to-sample distances, where the variable was set to "location" and controlled by "participant." Heat maps was also constructed to provide an overview of sample similarities based on Euclidean distances. General linear models were used to compare gene expression across day 1 and sea level values (HA1 vs SL) and day 3 and sea level values (HA3 vs SL), controlling for variation within individuals. P values were adjusted using a Benjamini-Hochberg procedure and a false discovery rate of 5%. Gene expression differentials

with a fold change above 1 or below -1 and an adjusted p-value below 0.05 were considered differentially expressed in response to acute hypoxia. Gene ontology was analyzed via Enrichr to discern the molecular function and related biological processes of associated with differentially expressed genes (GO Molecular function and GO Biological Processes).

Results

Participant data is recorded in **Table 1**. Participants were between the ages of 19-32, with 10 men and 5 women. The mean age for men was 25, and the mean age for women was 26. **Figure 2** presents the relative similarity between samples in the form of an MDS plot. Circles have been placed around samples for each location (SL, HA1, HA3), which can be used to visualize similarity between subjects. The MA plot in **Figure 3** plots log2 fold change versus mean expression counts. All significant (log2fold change>|1|) genes are represented by a blue dot. The genes that are most highly differentiated have a log2 fold change above |1| and are located towards the right side of the plot. Essentially a higher mean of normalized counts increases significance of differential gene expression.

Table 1. Participant demographics.

Variable	Men (N = 10)	Women (N=5)
Age (years)	25 ± 4.00	26 ± 5.13
Height (m)	1.74 ± 8.13	1.63 ± 9.41
Weight (kg)	81.3 ± 37.5	89.8 ± 49.6
Body Mass Index (kg/m^2)	26.7 ± 5.43	28.4 ± 6.88
FVC (L)	4.29 ± 1.41	3.01 ± 1.11
FEV1/FVC (%)	69.2 ± 9.66	81.9 ± 9.08

Values are presented as Mean ± SD. Participant demographic data of 15 participants that were a part of the study. Forced vital capacity (FVC) was used to establish the status of participant lung function and is measured in liters. Forced expiratory volume (FEV) measures how much air a person can exhale during a fully exhaled forced breath.

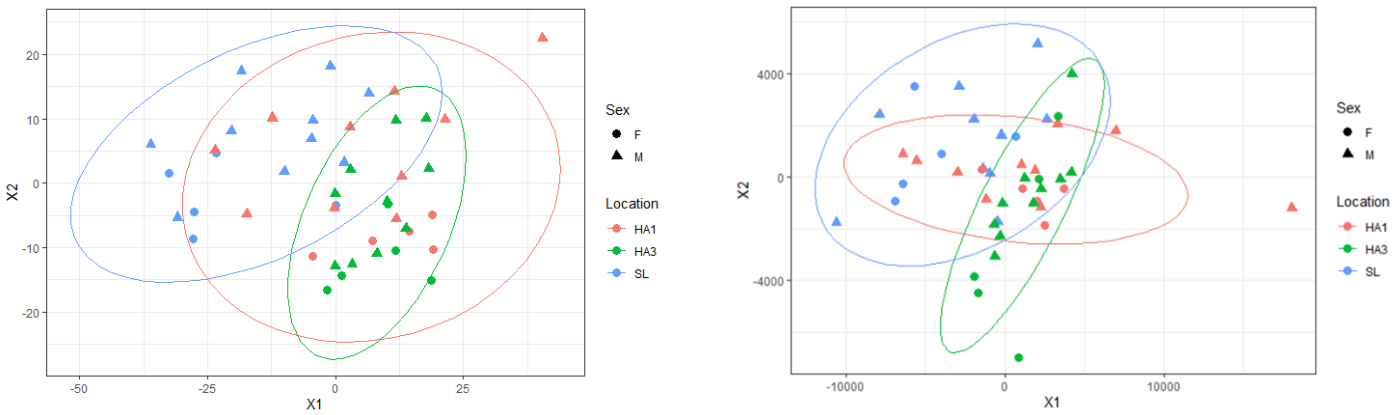


Figure 2. Sample relationships. MDS plots based on the rlog-transformed values (left) and the *Poisson Distance* (right). Samples cluster by location/day as well as by sex. Points correspond to individual samples. Subject sex identified by circles (women) and triangles (men). Point color represents the location at which samples were collected.

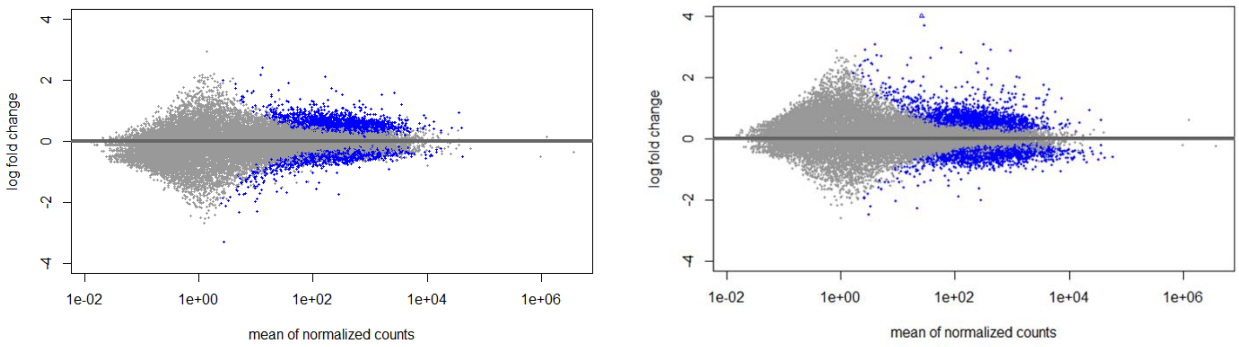


Figure 3. Significantly differentially expressed genes as a function of mean normalized counts. MA plots for HA1 v SL (left) and HA3 v SL (right). The log2 fold change for a particular comparison is plotted on the y-axis and the average of the counts normalized by size factor is shown on the x-axis. Each gene is represented with a dot. Genes with an adjusted *p* value ≤0.01 are colored in blue.

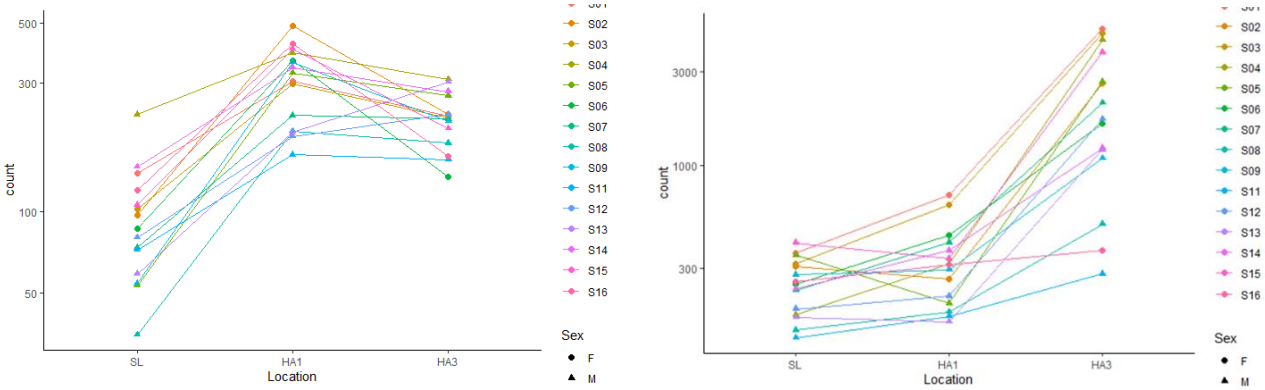


Figure 4. Top differentially expressed genes. These are the top differentially expressed genes for HA1 v SL (left) which was BCL2A1, and HA3 v SL (right) which was BPGM. Plot titles represent NCBI Gene IDs, and gene counts are shown on the left axis.

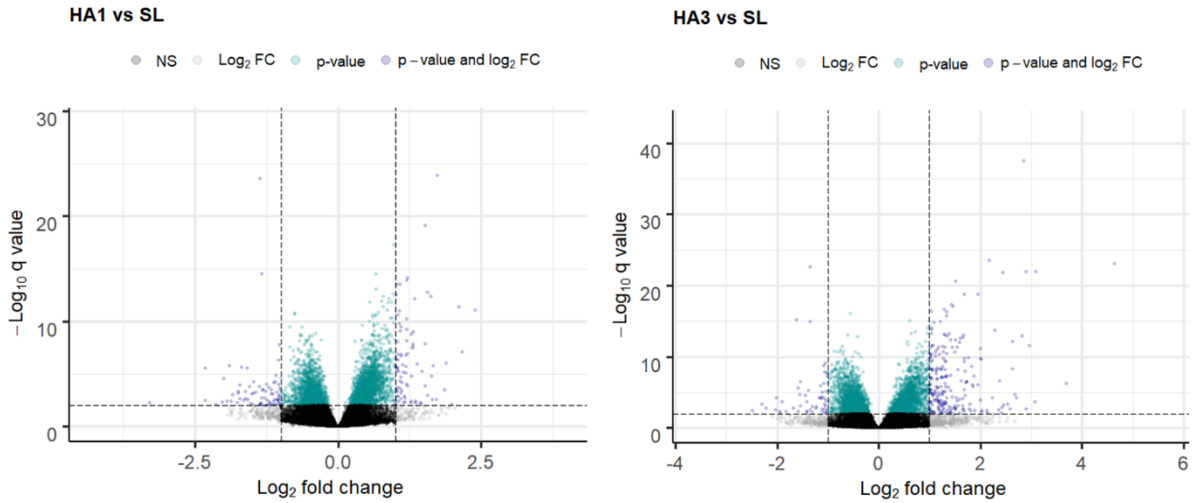


Figure 5. Volcano plot of RNA-seq data. Dotted lines represent p-adj threshold <0.01 and log2fold change threshold >1 for HA1 vs SL (right) and HA3 vs SL(left). Dark blue points represent significantly differentially expressed genes with adjusted p-values less than 0.01 and log2fold change greater than |1|. Teal points represent genes with adjusted p-values less than 0.01 and log2fold change less than |1|. Gray points represent genes with adjusted p-values greater than 0.01 and log2foldchange greater than |1|. Black points represent genes with adjusted p values greater than 0.01 and log2fold change less than |1|.

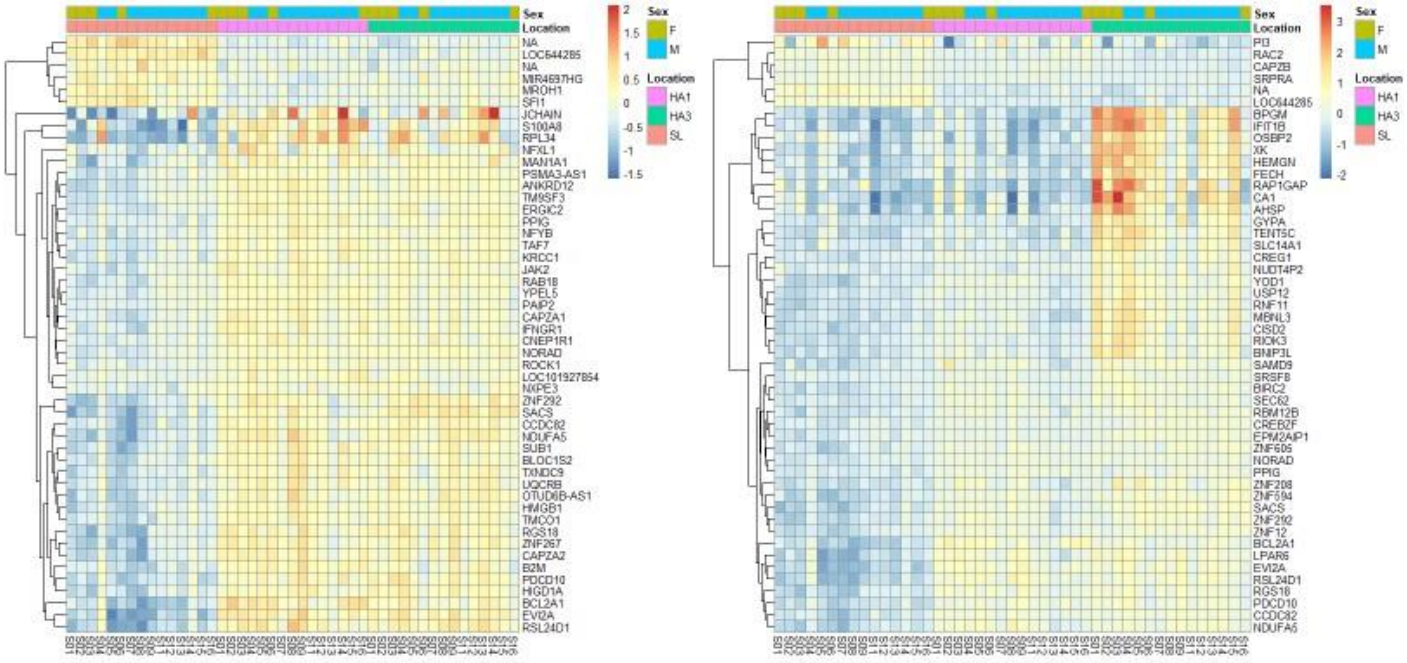
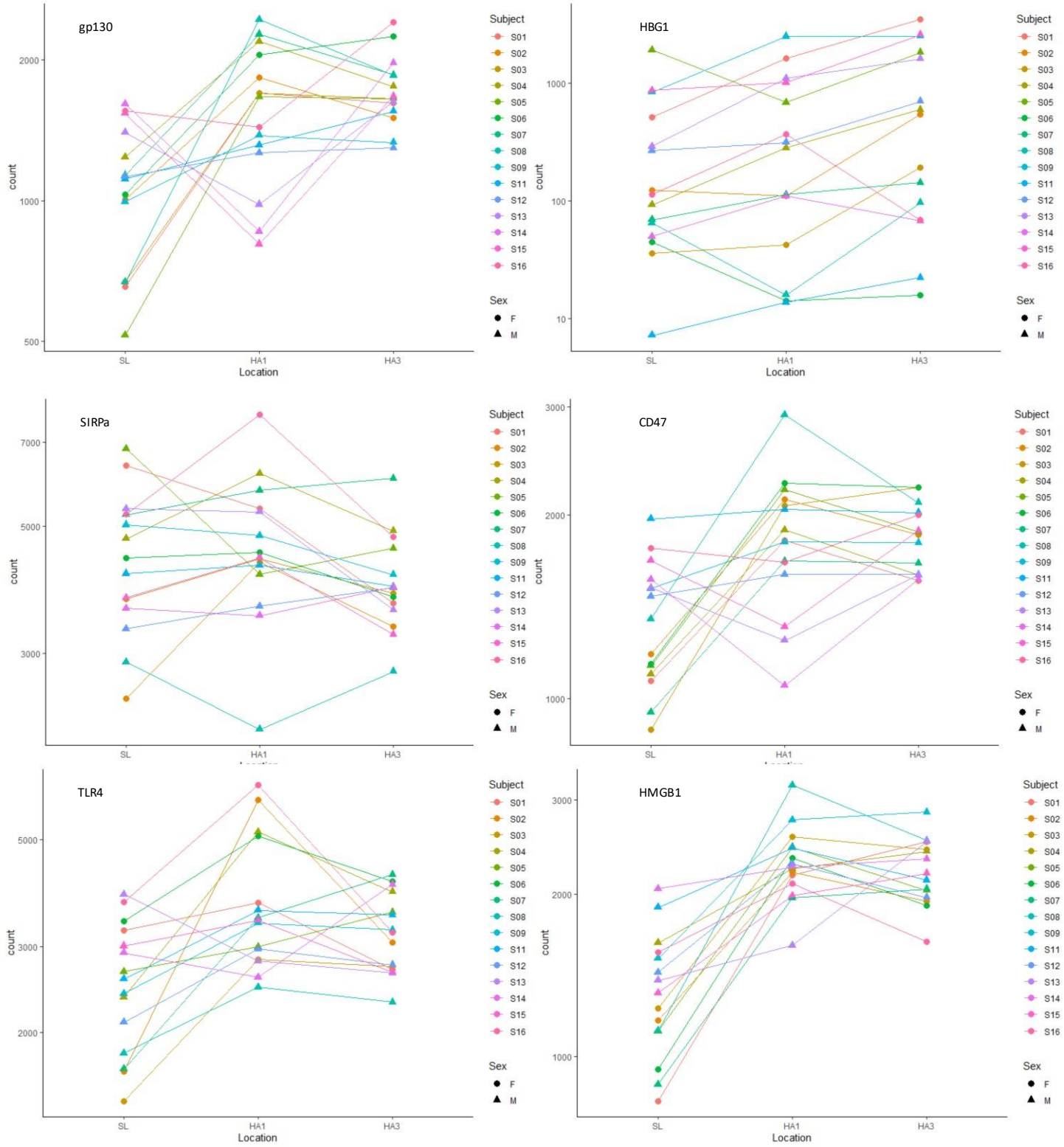


Figure 6. Heat maps using r-log transformed values for top differentially expressed genes from (A) HA1 v SL and (B) HA3 v SL s. Participant ID for each sample is provided at the bottom for each column. Individual gene expression levels for each sample are provided in each box. Normalized gene expression levels are indicated by the colored legend (red = increased expression, blue = decreased expression). Sample locations are identified in the top row by red (sea level; SL), green (high altitude day 1; ALT 1), pink (high altitude day 3; ALT 3) markers along the top of each row. Sex differences are identified by yellow (female) or blue (male).

To determine if acute high-altitude exposure induced a systemic inflammatory response, we analyzed RNA expression profiles in peripheral blood collected at sea level and over three days of acclimatization. Genes were chosen primarily based on related inflammatory function and mediation. The gene expression data for IL-6 and IL-1B is reported in **Figure 7**, however

these genes alongside other pro-inflammatory cytokines were not found to be differentially upregulated. However Gene Ontology analysis displayed in **Table 2** shows that regulation of the IκB and NF-κB was associated with the most highly differentiated genes. Both of these act as mediators for downstream inflammatory response. Our data also shows that *TLR4*, *HMGB1*, *gp130* were all upregulated in the sample data, visualized in **Figure 7**. TLR4 and gp130 serve as direct receptors to various pro-inflammatory cytokines. HMGB1 is a co-receptor of pro-inflammatory cytokine in conjunction with TLR4.

To determine if markers of stress erythropoiesis were observed during acture high-altitude expsoure, we examined expression of fetal hemoglobin in peripheralblood at sea level over three days of acclimitization. Our data also showed marked increases (($p<0.05$) in fetal hemoglobin (*HBG1*), which is a potential biomarker of stress erythropoeisis, and *CD47*, a positive regulator of circulating red blood cell concentration, in expression over the 3 measurement days. Additionally, *SIRPa*, another positive upregulator of circulating red blood cell concentration was shown to be significantly upregulated on day 1 at HA.


gp130
HBG1
SIRPa
CD47
TLR4
HMGB1
count
Location
SL
HA1
HA3
Subject
S01
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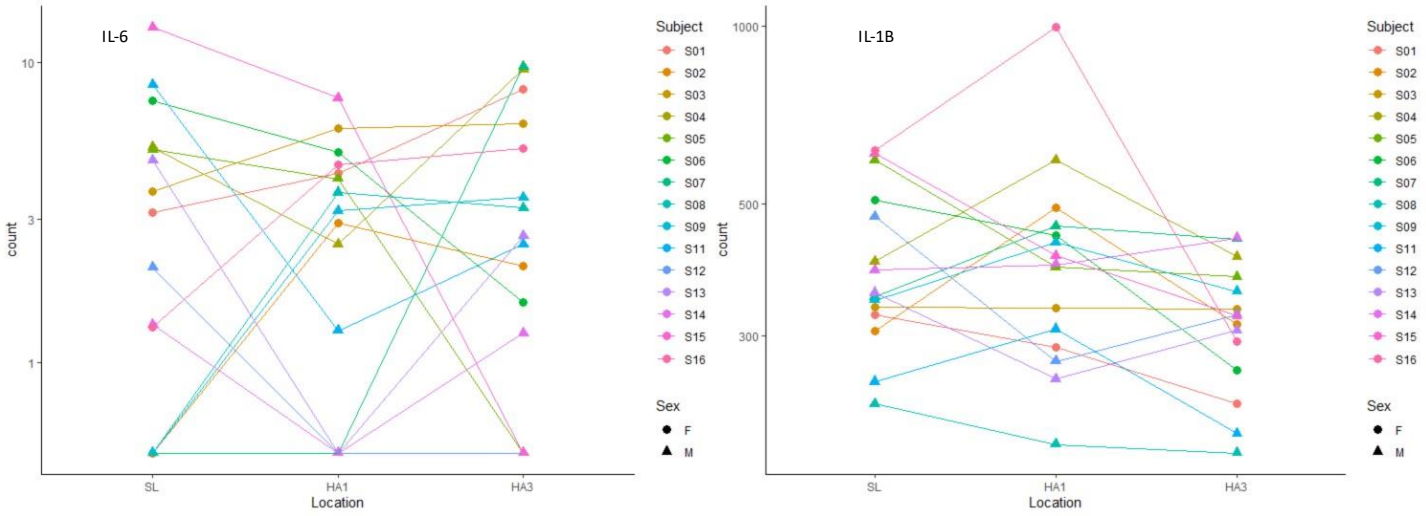

Figure 7 Normalized counts over treatment using *ggplot2* styles. The plots visualize genes of interest over the three measurement days. Each sample is marked by a different color, and circles were used to represent females, while traingles were used to represent male participants.

Term	P	Adj. P	Odds Ratio
mRNA processing	7.364e-21	3.590e-17	3.10
RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	6.409e-19	1.562e-15	3.23
Neutrophile activation involved in immune response	4.322e-18	6.555e-15	2.25
Neutrophil degranulation	6.268e-18	6.555e-15	2.25
Neutrophil mediated immunity	6.723e-18	6.555e-15	2.23
mRNA splicing, via spliceosome	3.661e-17	2.974e-14	2.88
Protein deubiquitination	5.202e-17	3.623e-14	2.89
Ubiquitin-dependent protein catabolic process	4.458e-16	2.717e-13	2.45
Protein modification by small protein removal	8.356e-16	4.26e-13	2.75
Proteasome-mediated ubiquitin-dependent protein catabolic process	2.791e-15	1.361e-12	2.56
Regulation of cellular macromolecule biosynthetic process	6.341e-14	2.710e-11	1.86
Protein ubiquitination	2.563e-13	1.041e-10	1.96
Proteasomal protein catabolic process	3.905e-13	1.464e-10	2.60
Regulation of transcription, DNA-templated	6.224e-12	2.167e-9	1.45
Regulation of nucleic acid-templated transcription	1.861e-11	5.824e-9	1.76
Regulation of autophagy	5.892e-11	1.795e-8	2.54
Regulation of I-kappaB kinase/NF-kappaB signaling	5.331e-10	1.529e-7	2.41

Regulation of gene expression	9.406e-10	2.547e-7	1.50
Cellular response to DNA damage stimulus	1.134e-9	2.910e-7	1.99
Phosphorylation	1.211e-9	2.951e-7	1.89

Table 2. Top 20 GO Biological Processes during HA3. GO analysis was conducted using Enrichr and inputted data consists of all genes differentially upregulated (adj. $p<0.05$) on day 3 at HA. Regulation of the IkB kinase and NF-kB signaling was a highly associated biological process that is linked to mediating the inflammatory response.

Data Analysis and Discussion

In this study, we conducted a transcriptome analysis on RNA collected from whole blood of healthy sojourners exposed to hypoxia at high altitude. This data shows upregulation of genes involved in various inflammatory pathways and potential evidence of stress erythropoiesis upon exposure to hypoxia, which are further discussed below. Most genes involved in the physiological inflammatory response indicate an increased sensitization to inflammatory cytokines, such as the toll-like receptor 4 (*TLR4*) signaling pathway. Furthermore, the data shows an upregulation of genes involved in maintenance of increased hematocrit such as *CD47*. This data provides potential insight into the mechanisms by which hypoxia mediates an acute inflammatory response and regulates erythropoiesis.

Previous studies have suggested a link between AMS incidence and the inflammatory pathway (Klausen 1997, Boos 2016, Julian 2011). However, another study notes no significant change in proinflammatory cytokine (including IL-6, IL-1β, and TNF-α) concentration in hypoxic conditions, despite a small observed increase in proinflammatory cytokine mRNA (Swenson 1997). Despite this, the study did demonstrate upregulation of several other components of the inflammatory pathway. Similarly, the data in our study shows no significant increase in proinflammatory cytokine expression, including IL-6, IL-1β, and TNF-α, despite a GO analysis association of the regulation of IκB and NF-κB. However, our data illustrates significant upregulation ($p<0.05$) of *gp130* (*IL6-ST*), which is a primary receptor for IL-6, amongst several other pro-inflammatory cytokines (Silver and Hunter 2010). This data points to an increased sensitivity to IL-6 and other pro-inflammatory cytokines.

Significant increases were demonstrated in TLR4 and HMGB1 concentrations, which play a role in mediating the inflammatory response to bacterial infection. TLR4 is best known for its

role in innate immunity as a primary receptor for lipopolysaccharide (LPS), a pathogen-associated molecule (Park and Lee2013). TLR4 is found to be significantly upregulated (adj. $p<0.05$) in the RNA-seq analysis in both day 1 and day 3 at HA and was found to be positively regulated in the GO analysis. Though TLR4 itself is not a biomarker for inflammation, its upregulation does suggest an increased sensitivity to a physiological inflammatory response. This notion is further supported by the upregulation of HMGB1 in day 1 and 3 at HA (adj. $p<0.05$). Extracellular HMGB1 proteins have been shown to act as pro-inflammatory mediators by binding to proinflammatory cytokines including LPS and IL-1β to activate TLR4 receptors and initiate downstream inflammatory signaling (Yang et al. 2020). Additionally, *TLR4-HIF* and *HMGB1-HIF* are noted to have a positive correlation in gene expression (adj. $p<0.05$, $R = 0.353$). The correlation of *TLR4* and *HMGB1* to *HIF1α* suggests that hypoxia plays a role in physiological inflammatory sensitivity, and potentially regulates inflammation itself. Further studies must be conducted to elucidate the interplay between hypoxia and related inflammatory mediators.

Exposure to hypoxia often results in an increase in RBC production alongside inflammation. Acute inflammation can also stimulate stress erythropoiesis, which protects against acute anemia of inflammation. Stress erythropoiesis an extramedullary mechanism distinct from steady-state bone marrow erythropoiesis marked by rapid production of a large burst of new erythrocytes (Paulson et al. 2011, Ganz 2019). Stress erythropoiesis is also seen in patients affected by sickle cell or thalassemia syndromes during recovery after bone marrow transplantation. Specifically, this stress erythropoiesis is marked by circulating red blood cells containing increased levels of fetal hemoglobin (*HBG1*) (Risso et. al 2012). Our data exhibits an increase in fetal hemoglobin on day 1 and day 3 (adj $p<0.05$) at HA compared to sea level values. This could suggest a potential link between hypoxia and stress erythropoiesis, though no conclusion can be

made to correlate inflammation as a causal factor, as inflammatory cytokines were not upregulated in our data.

Our data also presents other markers of increased circulating red blood cells in sojourner samples. Several studies have suggested that CD47 and SIRPa interaction results in an inhibitory signal for erythrocyte phagocytosis. Specifically, CD47 may act as a marker used by host macrophages to determine if a cell is foreign or innate (Burgur et al. 2012, Oldenborg et al. 2000). *CD47* was shown to be significantly upregulated in sample data (adj $p<0.05$) on days 1 and 3 at HA, while *SIRPa* was shown to be significantly increased on day 1 at HA. This reinforces the notion that an increase in circulating red blood cell concentration is seen upon exposure to acute hypoxia, which could potentially be a result of stress erythropoiesis.

Further studies are necessary to determine the mechanism by which hypoxia mediates inflammatory sensitivity as well as the impact of hypoxia-induced inflammation on stress erythropoiesis. Although RNA-seq is effective in providing an unbiased overview of the entire transcriptome, it requires experienced cDNA library preparation and raw data analysis. As such, many of the genes that were anticipated to be differently expressed in fact did not have significant changes. To better account for our study limitations, future studies could utilize nanoString which uses direct digital detection of individual mRNA and can therefore provide measures of very low abundance transcripts. Proteomic analysis could also be used to investigate presence of circulating protein corresponding to genes of interest.

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