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Inflammatory gene expression during acute high-altitude exposure

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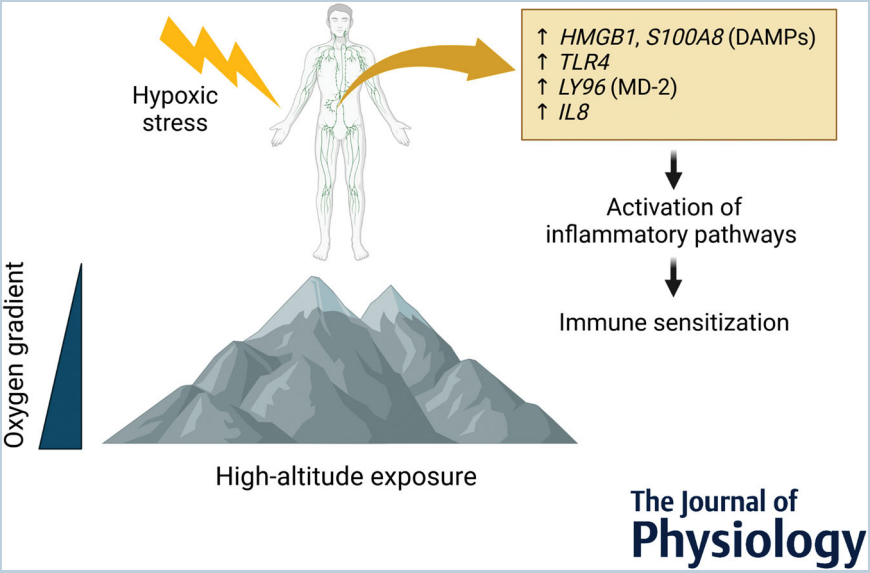

Abstract The molecular signalling pathways that regulate inflammation and the response to hypoxia share significant crosstalk and appear to play major roles in high-altitude acclimatization and adaptation. Several studies demonstrate increases in circulating candidate inflammatory markers during acute high-altitude exposure, but significant gaps remain in our understanding of how inflammation and immune function change at high altitude and whether these responses contribute to high-altitude pathologies, such as acute mountain sickness. To address this, we took 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 3 days of high-altitude acclimatization in healthy sea-level residents (n = 15; five women). Several inflammation-related genes were upregulated on the first day of high-altitude exposure, including

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a large increase in *HMGB1* (high mobility group box 1), a damage-associated molecular pattern (DAMP) molecule that amplifies immune responses during tissue injury. Differentially expressed genes on the first and third days of acclimatization were enriched for several inflammatory pathways, including nuclear factor-κB and Toll-like receptor (TLR) signalling. 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 acute mountain sickness scores and peripheral 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 signalling pathways and might sensitize the TLR4 signalling pathway to subsequent inflammatory stimuli.

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Abstract figure legend 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 damage-associated molecular pattern (DAMP) molecules, Toll-like receptors (TLRs) and chemotactic factors in peripheral blood. These changes are suspected to result in enhanced immune responses to subsequent inflammatory stimuli over this time frame.

Key points

- Inflammation plays a crucial role in the physiological response to hypoxia.
- High-altitude hypoxia exposure causes alterations in the inflammatory profile that might 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 3 days of high-altitude exposure, which might play a role in immune system sensitization.
- These results provide insight into how hypoxia-induced inflammation might contribute to high-altitude pathologies and exacerbate inflammatory responses in critical illnesses associated with hypoxaemia.

Introduction

High altitude is a physiologically stressful environment owing to low oxygen availability, low temperatures and low humidity. Given that 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; Moore, 2017; Scheinfeldt et al., 2012; Simonson, 2015). Although many of the mechanisms that modulate high-altitude acclimatization are well described, it remains unclear how acute high-altitude exposure influences inflammatory signalling and immune function.

In 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). Owing to this vital link between inflammation and hypoxia, the signalling pathways that control these responses have evolved to share significant crosstalk (Bandarra & Rocha, 2013; Corcoran & O'Neill, 2016; D'Ignazio et al., 2016; Görlach & Bonello, 2008; Pham et al., 2021).

The hypoxia-inducible factor (HIF) is a transcription factor that regulates gene expression in response to hypoxic stress (Semenza, 2009). Activity of HIF is regulated by oxygen-sensitive prolyl hydroxylase domain proteins (PHDs) and factor inhibiting HIF (FIH). As a result, in 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 (BelAiba et al., 2007; Bonello et al., 2007; Frede et al., 2006; Taylor, 2008; Zhou et al., 2003). Furthermore, PHD is also involved in regulating NF-kB nuclear translocation through its action on I-kappaB kinase (IKK) activity, providing an additional direct mechanism by which hypoxia regulates inflammatory signalling (Cummins et al., 2006).

Owing to the demonstrated links between HIF and NF-kB signalling, it is reasonable to suspect that hypoxaemia induced by high-altitude exposure might result in a systemic inflammatory response. Indeed, hypoxaemia and systemic inflammation commonly occur simultaneously in critical illnesses, such as sepsis and acute respiratory distress syndrome (ARDS) and, although *in vivo* data are limited, preclinical data suggest 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 might contribute to, or be a downstream consequence of, high-altitude illnesses, such as acute mountain sickness (AMS). Several previous studies have provided evidence that candidate inflammatory mediators are upregulated in peripheral blood during the first few days of acclimatization (Eltzschig & Carmeliet, 2011; Faquin et al., 1992; Hartmann et al., 2000; Heinrich et al., 2018; Lundeberg et al., 2018; Scholz & Taylor, 2013). However, whether these changes are associated with phenotypes including AMS severity remains inconclusive.

In the present study, we expand on this work with a broad, unbiased transcriptomic approach to improve our understanding of how inflammatory signalling is altered in peripheral blood mononuclear cells during acute exposure to high altitude. We use RNA sequencing (RNA-seq) 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 exposure to high-altitude hypoxia and that higher inflammatory cytokine expression levels would be associated with more severe hypoxaemia and AMS severity.

Methods

Ethical approval

This study was approved by the University of California, Riverside (UC Riverside) Clinical Institutional Review Board (HS 19-076). All participants were informed of the purpose and risks of the study. 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 apnoea, and displayed no abnormal findings on ECG or pulmonary function testing. The mean age was 25 ± 4 years for men and 26 ± 5 years for women, and body mass index was 26.7 ± 5.4 kg/m^2 for men and 28.4 ± 6.9 kg/m^2 for women. Exclusion criteria included travel >2500 meters within 1 month of the first measurements, a previous history of high-altitude pulmonary or cerebral oedema, smoking and pregnancy.

Experimental design and physiological measures

In the 2 weeks before ascent to high altitude, participants completed initial screening for eligibility at UC Riverside, at ∼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 the presence of high-altitude ancestry) and medical history, including current medications. Participants then completed pulmonary function testing and ECG to verify the 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 measurements were collected at this time, including blood pressure, peripheral oxygen saturation (S_{pO_2}) by pulse oximetry, 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 after blood sampling, before travel.

The group then travelled by car to Barcroft Station (elevation 3800 m) in the White Mountain Research Centre (Bishop, CA, USA) over a period of ∼6.5 h. At the field station, fasting blood samples and morning measurements were collected each day within 1 h of waking and before 09.00 h to keep timing consistent with SL measurements. Physiological measurements and fasting blood samples were collected every morning for 3 days consecutively (HA1, HA2 and HA3), and end-tidal CO was measured at night. The end-tidal CO concentration was measured with a Micro$^+$ Basic Smokerlyzer (CoVita, Santa Barbara, CA, USA). Participants held their breath for 15 s, then exhaled through the device to residual volume. Measurements were taken in triplicate and averaged. The analyser was calibrated with 50 ppm CO before the measurements each

day. 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 min until values stabilized. Blood pressure measurements were collected in duplicate using a manual sphygmomanometer while participants rested in an upright seated position.

Participants abstained from taking anti-inflammatory medications or other agents that might 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 that participants remained hydrated.

Gene expression

RNA isolation. Peripheral blood was collected into PaxGene Blood RNA tubes (Qiagen, Germantown, MD, USA) using standard venipuncture procedures. Samples collected at SL were incubated at room temperature for 30 min, 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 min, frozen at −20°C at the field station, then transported to UC Riverside in liquid nitrogen and frozen at −80°C until further processing.

Before RNA isolation, PaxGene Blood RNA tubes were allowed to thaw and incubate for 4 h at room temperature according to the manufacturer's instructions. The RNA was isolated using a PaxGene Blood RNA Kit (Qiagen) following the manufacturer's protocols. The quantity and quality of RNA were verified via a Nanodrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) and Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA USA). RNA samples with RNA integrity number (RIN) values >8 were used for downstream sequencing.

RNA sequencing. Samples of RNA were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's protocols, with the following adjustments: 0.8× beads were used during the first purification step after second strand synthesis; the adaptor was diluted 1:15; 0.7× beads were used for purification after adaptor ligand; 13 cycles of enrichment were conducted; and a dual bead size selection (0.5× and 0.7×) 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, San Diego, CA, USA), which generates 50 bp paired-end reads.

Raw sequence data were aligned using *Rsubread* in RStudio (RStudio, Boston, MA, USA) (R v.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). Paired contrasts were made for the first (HA1) and third (HA3) mornings at high altitude *vs*. sea level (SL) separately. The *P*-values were adjusted with the Benjamini–Hochberg adjustment method for a false discovery rate of 5%, and genes with adjusted *P*-values <0.01 were considered to be significantly differentially expressed. Differentially expressed genes were examined for gene set enrichment in Enrichr using GO Biological processes (2021) for gene ontology (GO) 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). Given that we aimed to determine whether these expression levels were associated with phenotypes, this analysis was conducted on samples collected at SL and after one night at high altitude (HA1), because AMS scores were highest, S_{pO_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 the manufacturer's 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 (v.4.0). Counts were normalized to the following normalization probes: *PGK1*, *CLTC*, *GADPH*, *GUSB*, *TUBB* and *HPRT1*. These normalization probes are automatically selected using the *geNorm* algorithm, which selects probes that minimize the pairwise variation statistic during housekeeping gene selection. Genes within a sample with mRNA counts ≤2× the maximum background (the average of the housekeeping gene count

Table 1. Physiological measures at baseline and over 3 days at high altitude

Variable	SL	HA day 1	HA day 2	HA day 3	ANOVA
P_{sys} (mmHg)	128 ± 7	125 ± 12	126 ± 45	126 ± 13	0.537
P_{dia} (mmHg)	79 ± 10	83 ± 9	83 ± 7	85 ± 7	0.054
HR (beats/min)	78.0 ± 8.1	88.3 ± 13.2	89.7 ± 12.1*	95.6 ± 12.8***	<0.001
S_{pO_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 (ppm)	3.9 ± 1.4	5.2 ± 1.5	5.0 ± 1.9	—	0.080

Abbreviations: CO, carbon monoxide; HA, high altitude; HR, heart rate; P_{dia}, diastolic blood pressure; P_{sys}, systolic blood pressure; SL, sea level; and S_{pO_2}, peripheral oxygen saturation. Overall *P*-values for repeated-measures ANOVA are provided. Significant differences from SL: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, via *post hoc* pairwise comparisons with Bonferroni-adjusted *P*-values.

of negative controls) in >20% of all samples were excluded from analysis. Differential expression analysis included location (SL *vs*. HA1) as a predictor and subject as a confounder. The *P*-values were adjusted with the Benamini–Yekutieli method. Adjusted *P*-values <0.05 were considered significant. Given that the NanoString gene expression panel included a preselected 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 Probe Descriptive module of the nSolver Advanced Analysis software was used to examine gene-by-gene correlations, with location set as a covariate (SL or HA1) and interval identity, and participant as a series identity. 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 (v.4.1.0; R Foundation for Statistical Computing). To identify changes in physiological variables at high altitude compared with baseline sea-level measures, we used repeated-measures ANOVA and *post hoc* Student's paired *t* tests with Bonferroni corrections. To determine whether fold changes in gene expression were associated with physiological measures at high altitude (S_{pO_2} and AMS score), Pearson correlation coefficients and *P*-values were obtained with the *rcorr* function from the *Hmisc* package in R. Throughout the paper, data are presented as the mean ± SD.

Results

Physiological measures

Table 1 provides an overview of physiological measures at sea level and over 3 days of acclimatization to high altitude. On the first morning at high altitude, eight of the 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 one of the 15 subjects scored positive for AMS. The S_{pO_2} decreased by ∼10 percentage points on the first day at high altitude and remained lower than SL 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 CO levels were elevated on average at high altitude, although this was not significant by repeated-measures ANOVA.

Gene expression at high altitude

RNA sequencing. Three thousand nine hundred and fifty-eight genes were differentially expressed (adjusted $P < 0.01$) on HA1 (2177 upregulated and 1781 downregulated), and 4219 genes were differentially expressed on HA3 (2190 upregulated and 2029 downregulated) compared with SL. When applying a fold change threshold of one, 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. Figure 1 demonstrates that the genes most differentially expressed on HA1 remained differentially expressed throughout HA3. However, the genes most differentially expressed on HA3 were slower responding and were typically not differentially expressed on HA1. Several genes associated with inflammation and the immune response were in the top 20 upregulated genes on HA1, including *BCL2A1*, *S100A8*, *HMGB1* and *B2M*. Additional genes likely to be 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)

Table 2. Top 20 differentially expressed genes on days 1 and 3 at high altitude

Gene	FC	Adjusted *P*-value
SL *vs*. HA day 1		
BCL2A1	1.73	1.46×10^{-24}
EVI2A	1.52	7.87×10^{-20}
ERGIC2	0.98	5.60×10^{-18}
PPIG	0.66	3.25×10^{-15}
PDCD10	1.22	7.17×10^{-15}
TXNDC9	1.20	1.28×10^{-14}
RGS18	1.04	3.09×10^{-14}
SUB1	1.09	3.09×10^{-14}
TAF7	0.66	9.58×10^{-14}
S100A8	1.58	1.88×10^{-13}
NFXL1	0.97	2.53×10^{-13}
CCDC82	1.02	2.79×10^{-13}
RSL24D1	1.63	4.17×10^{-13}
HMGB1	0.80	4.71×10^{-13}
MAN1A1	0.89	7.26×10^{-13}
NDUFA5	1.35	7.26×10^{-13}
ANKRD12	0.76	1.20×10^{-12}
B2M	0.94	1.59×10^{-12}
NORAD	0.55	1.59×10^{-12}
BLOC1S2	0.88	1.70×10^{-12}
SL *vs*. HA day 3		
BPGM	2.86	3.65×10^{-38}
HEMGN	2.18	3.33×10^{-24}
GYPA	4.64	9.63×10^{-24}
IFIT1B	2.91	1.35×10^{-22}
CA1	3.09	1.35×10^{-22}
XK	2.45	1.65×10^{-22}
SACS	1.51	2.85×10^{-21}
TENT5C	1.69	1.61×10^{-19}
FECH	1.95	1.61×10^{-19}
EVI2A	1.44	5.45×10^{-18}
MBNL3	1.47	8.16×10^{-18}
PDCD10	1.30	2.45×10^{-17}
ZNF292	1.04	4.31×10^{-17}
RIOK3	1.34	4.92×10^{-17}
CAPZB	−0.56	9.42×10^{-17}
YOD1	1.34	1.97×10^{-16}
PI3	−1.61	6.91×10^{-16}
CREG1	0.99	8.01×10^{-16}
NORAD	0.62	1.02×10^{-15}
LOC644285	−1.35	1.12×10^{-15}

and *PDCD10*, in addition to several other genes associated with inflammation and immune function, including *GYPA*, *IFIT1B* and *RIOK3*.

A gene ontology analysis revealed that on 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-kB signalling (Fig. 2*A*). There were also eight significantly enriched pathways, including apoptosis signalling, CCKR signalling, ubiquitin proteasome pathway, PDGF signalling, T-cell activation, Toll-like receptor (TLR) signalling, RAS and FAS signalling (Table 3). On HA3, the I-kappaB kinase/NF-kB signalling GO biological process remained enriched, in addition to other inflammation and immune function processes, including neutrophil activation involved in immune response (Fig. 2*B*). Nineteen pathways were significantly enriched on HA3, including several involved in inflammation and immune function and angiogenesis (VEGF signalling pathway) (Table 3).

After the GO analysis, we examined key genes of interest that 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 interleukin-8 (*IL8*) (Fig. 3). We found that there was a significant upregulation of *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 because 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 examine specific changes in inflammation-related gene expression further, we conducted a NanoString analysis on samples collected on HA1. Of the 250 genes on the human inflammation panel, 18 genes showed significant upregulation (Figs 4 and 5). Of the 18 genes identified as differentially expressed by NanoString, 13 were also detected with RNA sequencing. However, five differentially expressed genes were identified by NanoString alone, including *LY96*. This might be because the NanoString nCounter targets all isoforms of each gene and does not need to convert RNA to complementary DNA for amplification. Seven of the 18 significantly differentially expressed genes are involved in the significantly enriched GO pathway 'Regulation of binding (GO: 00 51098)' (adjusted $P < 0.0001$; *TGFBR1*, *IFIT2*, *IFIT1*, *MAPK8*, *DDIT3*, *HMGB2* and *HMGB1*). Other processes in which significantly upregulated genes are involved include positive regulation of apoptotic processes (*MAPK8*, *DDIT3*, *HMGB1*, *TGFBR1* and *IFIT2*), regulation of DNA binding (*MAPK8*, *HMGB2* and *HMGB1*), DNA conformational change (*HMGB2* and *HMGB1*) and positive regulation of endothelial cell proliferation (*HMGB2*, *HMGB1* and *TGFBR1*).

Given that 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 signalling and inflammatory gene expression *in vivo*. Expression of *HIF1A* was significantly associated with expression of *PTK2*, *MAPK1*, *HMGB1*, *TLR8* and *NFE2L2* (Fig. 6).

Phenotype associations

Given that the highest AMS scores and lowest S_{pO_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

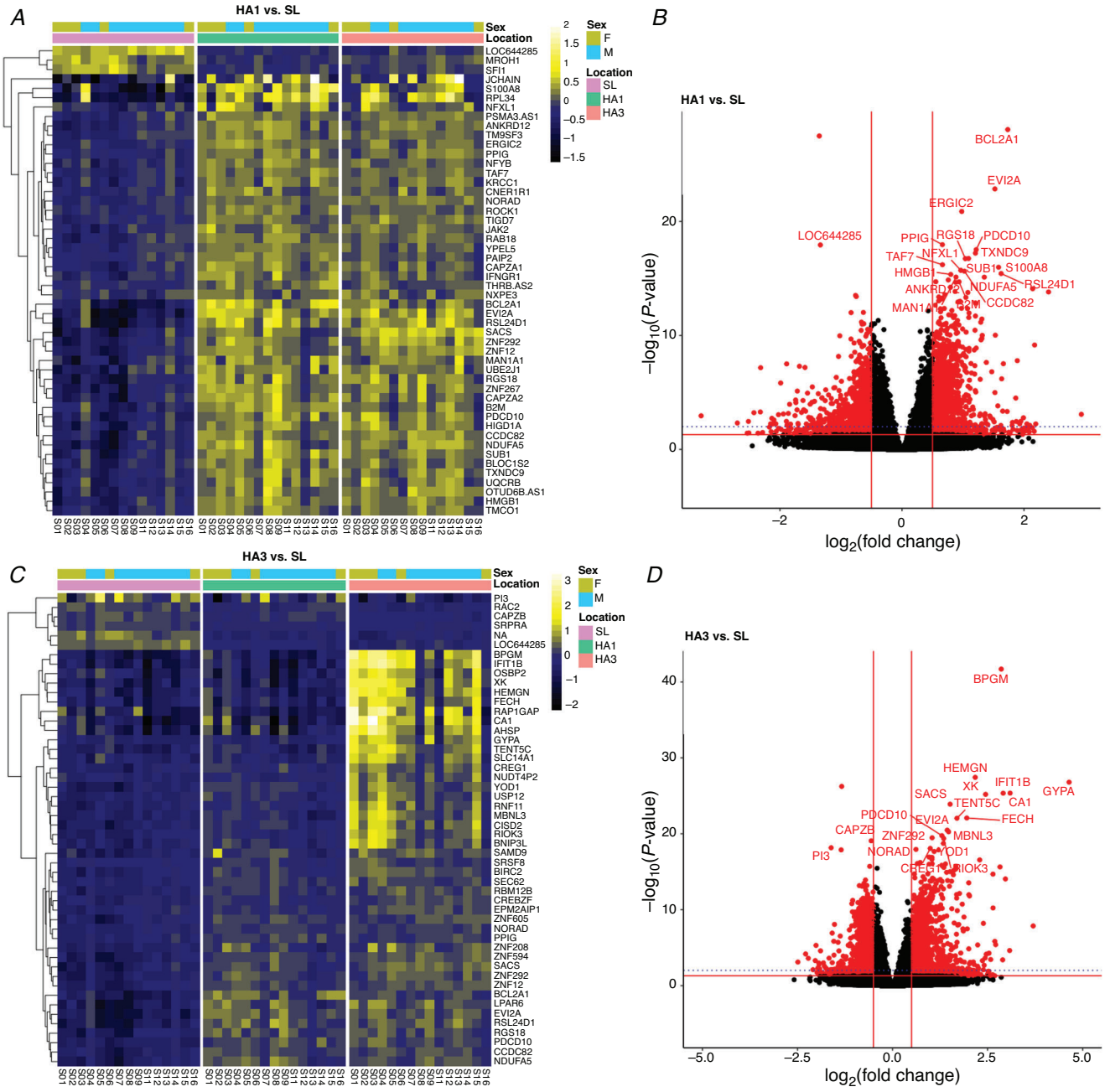

Figure 1. Top differentially expressed genes at high altitude
Heat maps with hierarchical clustering of the top 50 differentially expressed genes and volcano plots from high altitude day 1 (HA1; *A* and *B*) and high altitude day 3 (HA3; *C* and *D*) *vs*. sea-level (SL) baseline. In *A* and *C*, columns represent data for individuals. Sample locations are identified by pink (SL), green (HA1) and red (HA3) markers at the top of each row. Sex differences are identified by green (female) or blue (male). Colours represent relative $\log_2$ fold changes from sea level. In *B* and *D*, red points represent genes with adjusted *P*-values <0.01 and absolute $\log_2$ fold change >0.5. [Colour figure can be viewed at wileyonlinelibrary.com]

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

Term	Adjusted *P*-value	Overlap
High altitude day 1 *vs.* sea level		
Apoptosis signalling pathway Homo sapiens P00006	0.0003	40/102
CCKR signalling map ST Homo sapiens P06959	0.0003	57/165
Ubiquitin proteasome pathway Homo sapiens P00060	0.0024	20/43
PDGF signalling pathway Homo sapiens P00047	0.0033	39/112
T cell activation Homo sapiens P00053	0.0033	28/73
Toll receptor signalling pathway Homo sapiens P00054	0.0033	21/49
Ras Pathway Homo sapiens P04393	0.0064	26/69
FAS signalling pathway Homo sapiens P00020	0.0156	14/31
High altitude day 3 *vs.* sea level		
Apoptosis signalling pathway Homo sapiens P00006	0.0000	49/102
CCKR signalling map ST Homo sapiens P06959	0.0000	62/165
T cell activation Homo sapiens P00053	0.0011	31/73
Toll receptor signalling pathway Homo sapiens P00054	0.0011	23/49
PDGF signalling pathway Homo sapiens P00047	0.0011	42/112
VEGF signalling pathway Homo sapiens P00056	0.0053	23/54
Interleukin signalling 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 signalling 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 signalling through G proteins and beta-arrestin Homo sapiens P05911	0.0176	15/34
Inflammation mediated by chemokine and cytokine signalling 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 signalling pathway Homo sapiens P00018	0.0464	34/109

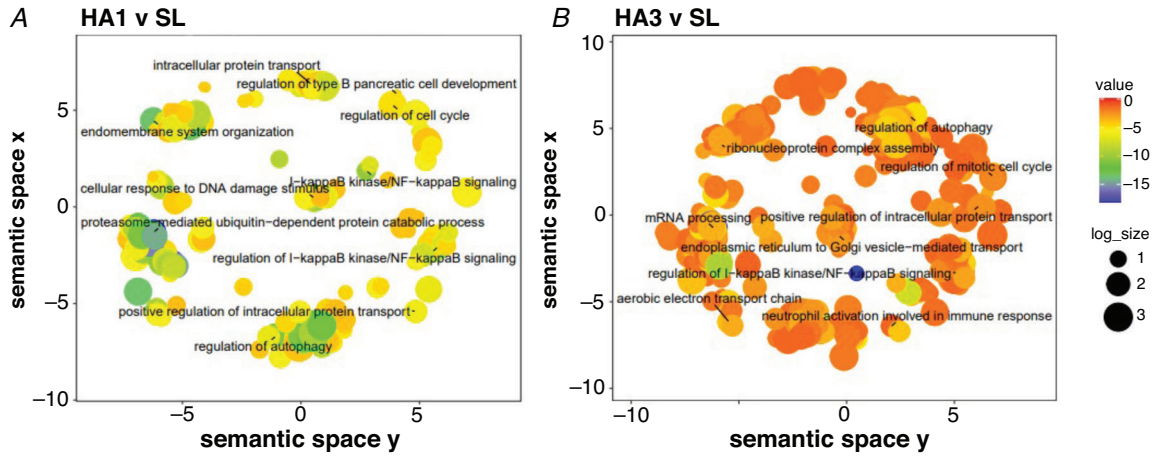

Figure 2. Enriched gene ontology terms with highly variable genes
Clustering of significantly enriched gene ontology (GO) terms into representative subsets using semantic similarities. Significantly enriched GO terms are provided for genes differentially expressed on day 1 (HA1; *A*) and day 3 (HA3; *B*) at high altitude compared with a sea-level (SL) baseline. Bubble colour indicates the $\log_{10}$ *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). [Colour figure can be viewed at wileyonlinelibrary.com]

measured by NanoString. Resting oxygen saturation (S_{pO_2}, as a percentage) was significantly associated with *FASLG*, *SMAD7*, *PTGER4* and *TRAF2*, with a trend towards a correlation with *IL8* ($P = 0.05$; Table 4). The AMS score was significantly associated with *TNFSF14*, *FASLG*, *IL18*, *CD40LG*, *PTGER4*, *MAPKAPK2*, *HLADRB1*, *SMAD7*, *AGER*, *MAFK* and *IRF5* (Table 4).

Discussion

This study examined how high-altitude acclimatization influences inflammatory signalling by use of 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 signalling (Fig. 2; Table 3), and the expressions of several inflammation-related genes, including *FASLG* and *SMAD7*, were associated with AMS scores and S_{pO_2} levels (Table 4). The release of cellular stress markers during tissue hypoxia is known to trigger downstream mechanisms that promote host defence, such as the TLR signalling pathway (Schaefer, 2014). Therefore, it is not surprising that we found TLR signalling 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* and *S100A8*), interferon-stimulated genes (*IFIT1*, *IFIT1B*, *IFIT2* and *IFI44*) and markers of DNA damage (*DDIT3*; Table 2; Fig. 5; Diamond, 2014; Schaefer, 2014; Yang et al., 2017). Here, we will discuss the impact of DAMPs, particularly *HMGB1* and its synergistic role with *LY96* in TLR4 activation.

Upregulation of the TLR4 pathway at high altitude

LY96 is a potential indicator of immune system priming (Kim et al., 2010; Park & Lee, 2013). *LY96* encodes for MD-2, a coreceptor with 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). Activation of TLR4 by LPS, a pathogen-associated molecular pattern (PAMP), triggers the expression of inflammatory cytokines and chemokines (Park & Lee, 2013). Given that hypoxia has been shown to increase *TLR4* expression, this might lead to exacerbated inflammation in response to subsequent inflammatory stimuli (Kim et al., 2010). In the present *in vivo* study, both *TLR4* and *LY96* gene expression were significantly upregulated after 1 day of high-altitude exposure (Fig. 5). Although *TLR4* upregulation was found to be significant only in the RNA-seq data, *TLR4* expression approached significance in the NanoString data (adjusted $P = 0.057$).

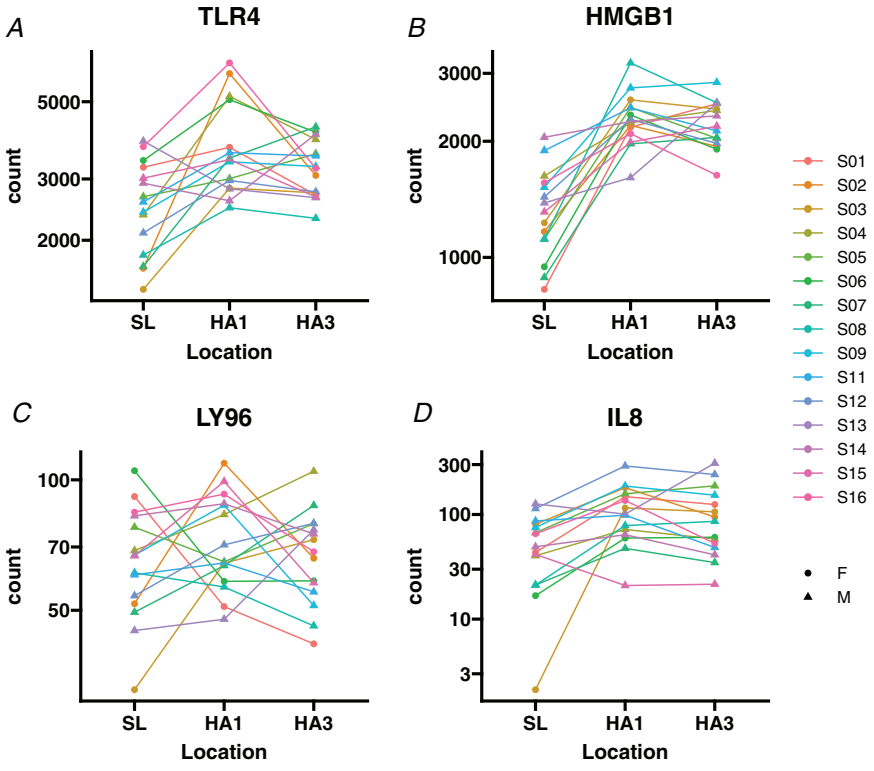

Figure 3. Normalized individual gene counts of RNA-seq data
Normalized individual RNA counts at sea level (SL), first day at high altitude (HA1) and third day at high altitude (HA3) for *TLR4* (*A*), *HMGB1* (*B*), *LY96* (*C*) and *IL8* (*D*). The *y*-axis is a $\log_{10}$ scale. Subject identity is coded by colour and sex by shape. [Colour figure can be viewed at wileyonlinelibrary.com]

These data suggest that over 1−3 days of exposure to hypoxia, hypoxic stress primes the innate immune response and might increase the inflammatory response to infection. This is supported with *in vitro* work by Kim et al. (2010), in which hypoxia exacerbated the TLR4-mediated inflammation in response to LPS in murine RAW 264.7 macrophages. However, the duration of exposure to hypoxia, in addition to the model used, have varying effects on *TLR4* expression. With short-term exposure (2–4 h) in macrophages, *TLR4* was noted to have increased expression (Kim et al., 2010). With longer hypoxic exposure (>24 h) in murine dendritic cells, there was no significant change in *TLR4*, but there was a significant increase in *TLR2* and *TLR6* expression (Kuhlicke et al., 2007). Therefore, although it is clear that hypoxic stress is likely to impact TLR4 signalling in peripheral blood cells, the details and time domains of this effect require further study.

To complement *LY96* and *TLR4* upregulation, *HMGB1* also showed one of the strongest signals of increased expression at high altitude (Table 2). *HMGB1* encodes the high mobility group Box 1 protein, which interacts directly with TLR4 and functions as a DAMP mediator of inflammation. Activity of HMGB1 is dependent on its location and cell type. Intracellular cytosolic HMGB1 has been shown to inhibit apoptosis and to activate the autophagic response, particularly in response to oxidative stress (Tang et al., 2011; Zhu et al., 2015). Upon release

Table 4. Relationships between phenotypes and $\log_2$ fold changes in gene expression

Gene	*R*	*P*-value
S_{pO_2}		
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		
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

The *P*-values for Pearson correlations are provided. Negative values of *R* indicate that increased expression levels at high altitude were associated with lower acute mountain sickness (AMS) scores or peripheral oxygen saturation (S_{pO_2}) levels.

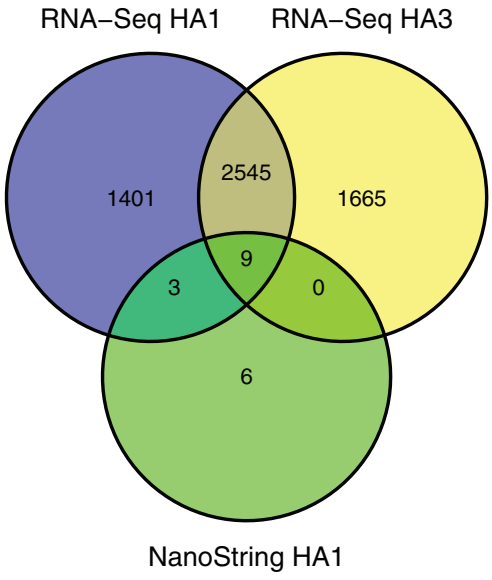

Figure 4. Venn diagram of differentially expressed genes identified by RNA-seq and NanoString
Differentially expressed genes identified via RNA-seq on high altitude day 1 (HA1; purple) and high altitude day 3 (HA3; yellow) are compared with differentially expressed genes identified by NanoString on HA1 (green). NanoString identified six additional differentially expressed genes not identified by RNA-seq. [Colour figure can be viewed at wileyonlinelibrary.com]

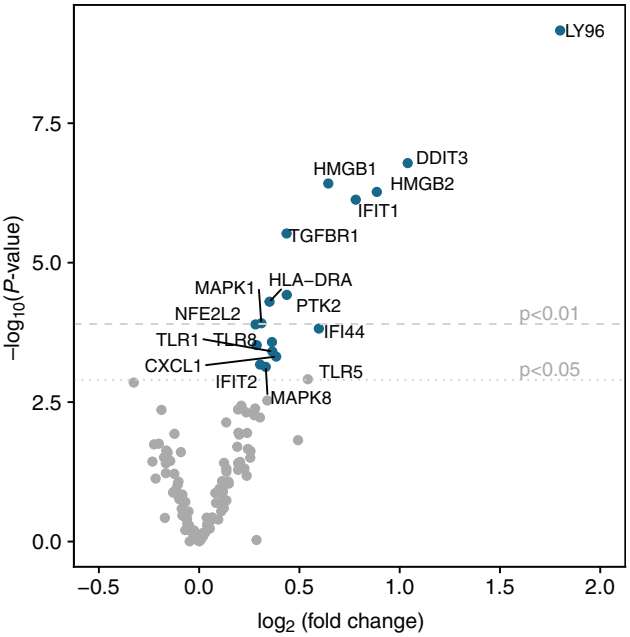

Figure 5. Volcano plot from NanoString data
Blue points represent significantly differentially expressed genes. Values are plotted with raw $\log_{10}$ *P*-values on the *y*-axis, with dotted grey lines indicating adjusted *P*-value thresholds. [Colour figure can be viewed at wileyonlinelibrary.com]

after cell death or active secretion, extracellular HMGB1 can act as a pro-inflammatory mediator by binding to other pro-inflammatory molecules, such as LPS or interleukin-1β, to activate TLR4 receptors and initiate downstream inflammatory signalling (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 signalling and induces production of cytokines and chemokines, such as tumour necrosis factor (TNF; Yang et al., 2015, 2020). In neutrophils, HMGB1–TLR4 signalling increases production of reactive oxygen species through activated neutrophil NADPH oxidase activity (Billiar et al., 2021; Fan et al., 2010; Yang et al., 2020).

In addition to *HMGB1*, *S100A8* was also significantly upregulated after 1 day of acute high-altitude exposure (Fig. 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 and in phagocytes in inflammatory conditions, such as inflammatory bowel and lung diseases (Rugtveit et al., 1994; Zwadlo et al., 1988). The S100A8/S100A9 heterodimer has previously been found to be an endogenous activator of TLR4 (Ehrchen et al., 2009; Foell et al., 2007; Ma et al., 2017; Vogl et al., 2007). Furthermore, S100A8 has been found to interact directly with the TLR4–MD-2 complex to initiate downstream signalling (Vogl et al., 2007). Previous research has also found that S100A8/S100A9 significantly increases secretion of pro-inflammatory cytokines, such as TNF-α and interleukin-6, in cultured BV-2 microglial cells. When TLR4 was inhibited, the pro-inflammatory cytokine secretion was blunted after S100A8/S100A9 stimulation

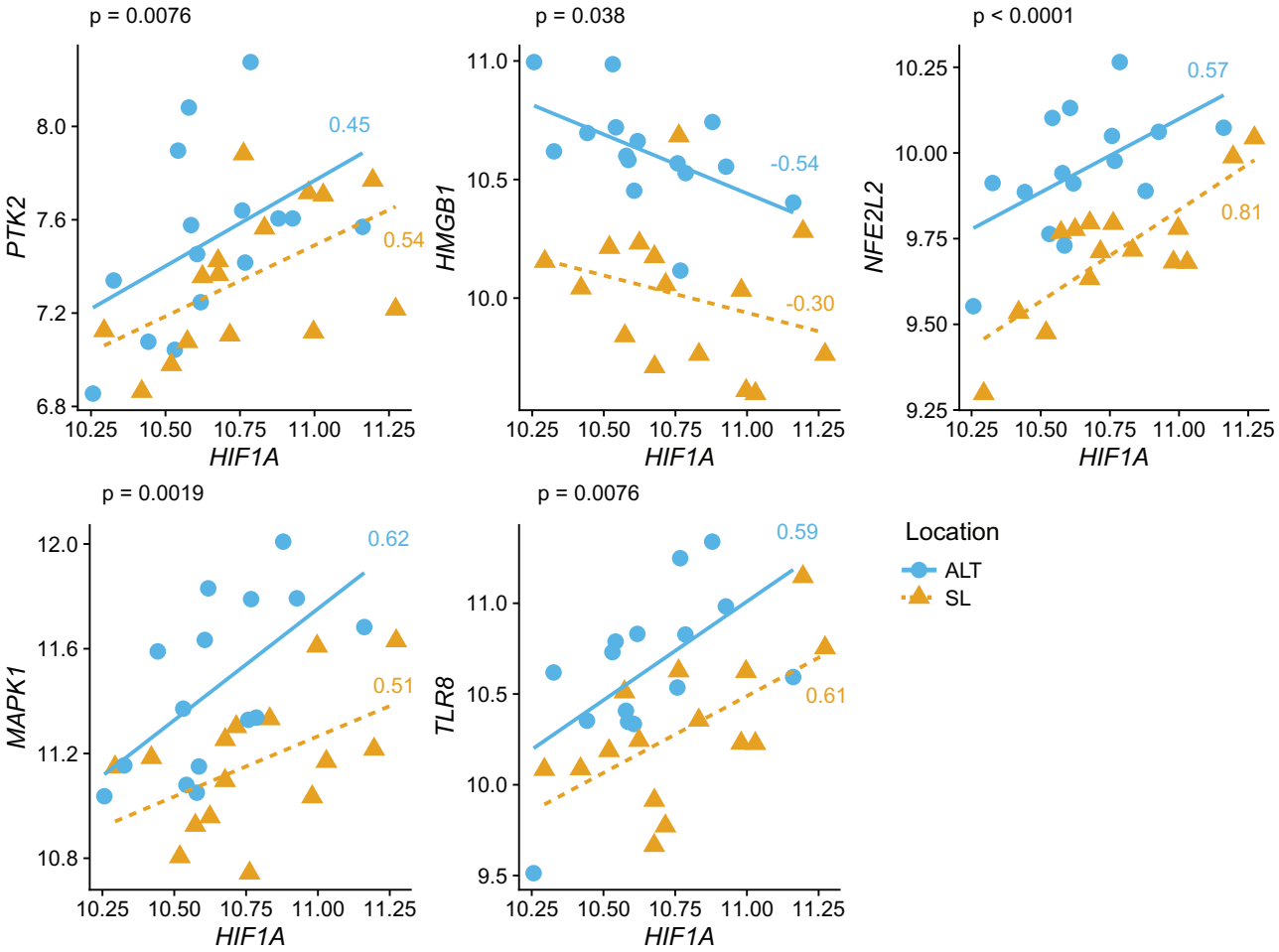

Figure 6. Relationships between *HIF1A* expression and upregulated inflammatory response genes at high altitude
Expression levels are reported as the normalized $\log_2$ counts. Pearson correlation coefficients for sea level (SL) and high altitude (ALT) are provided independently. Orange items (triangles and dotted lines) represent sea-level expression levels, and blue items (dots and continuous lines) represent expression levels on the first day at high altitude. [Colour figure can be viewed at wileyonlinelibrary.com]

(Ma et al., 2017). Additionally, in a mouse model that lacked the S100A8/S100A9 complex, these mice were protected from endotoxin-induced lethal sepsis (Vogl et al., 2007). Together, these data support the hypothesis that the TLR4 signalling pathway is sensitized to subsequent inflammatory stimuli following systemic hypoxic stress, at least after 1−3 days of high-altitude exposure.

Activation of TLR4 might also contribute to the release of IL8, a chemotactic cytokine that recruits and mobilizes neutrophils to sites of infection. Previous studies have shown 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 revealed that *IL8* mRNA expression is significantly upregulated on both the first and third days at high altitude (Fig. 3) and approaches significance for a negative association with S_{pO_2} (Table 4). This falls in line with previous research in ARDS, where Hirani et al. (2001) found that IL8 levels were negatively correlated with arterial oxygen saturation in patients with severe ARDS and that *in vitro* hypoxic stimuli significantly upregulated 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 might 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), whereby 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 hypoxaemia 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; Chakraborty & Burns, 2022; Pugin, 2007), which 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, in which patients have an increase in both pro- and anti-inflammatory mediators in circulation (Bone, 1992; Chakraborty & Burns, 2022; Dinarello et al., 1993). 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 previously been used as biomarkers for risk of death in septic shock patients (Dubois et al., 2019; Karakike 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 (Fig. 1), although this requires further study. Interestingly, one previous report finds that pancreatitis patients who also had high-altitude polycythaemia (and associated baseline hypoxaemia) developed more severe cases of SIRS compared with patients who had only acute pancreatitis (Zhu et al., 2020). This indicates that patients with hypoxaemia might be at higher risk for an exaggerated systemic inflammatory response.

Acute mountain sickness and inflammatory marker expression

Acute mountain sickness commonly manifests in sojourners to high altitude (>2500 m; Gallagher & Hackett, 2004; Hackett, 2000; Julian et al., 2011; Luks et al., 2017). Most individuals affected by AMS 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, hypoxaemia, vasogenic oedema and acidosis) in addition to 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 development of AMS. For example, multiple groups report that in individuals acutely exposed to high altitude select pro-inflammatory cytokine and inflammatory marker (most notably C-reactive protein, interleukin-1β and interleukin-6) concentrations are increased compared with their sea-level concentrations (Hartmann et al., 2000; Song et al., 2016). Some groups have also found associations between the incidence of AMS and expression of these candidate inflammatory markers (Boos et al., 2016; Kammerer et al., 2020; Klausen et al., 1997; Liu et al., 2017; Malacrida et al., 2019; Song et al., 2016). Accordingly, Dumont et al. (2000) showed a reduction in AMS incidence and severity with anti-inflammatory drug treatment. Both steroids and non-steroidal anti-inflammatory drugs reduced the incidence of AMS, despite their different mechanisms of action (Gertsch et al., 2010, 2012; Kanaan et al., 2017; Lipman et al., 2012; Nepal et al., 2020; Rock et al., 1989; Tang et al., 2014; Zheng et al., 2014). However, other studies demonstrated no significant association between pro-inflammatory cytokine concentration and AMS incidence (Lundeberg et al., 2018; Swenson et al., 1997). As a result, questions remain about how high-altitude-induced inflammation might 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, because many studies have shown that inflammatory markers, such as interleukin-6 and TNF-α, resolve rapidly after expression. Previous studies have also reported that although the protein expressions of interleukin-6 and TNF-α in plasma increased significantly after acute vigorous exercise, there was no change in mRNA expression in peripheral blood mononuclear cells (Bernecker et al., 2013; Ostrowski et al., 1998, 1999). Thus, although protein levels can remain elevated after 24 h of exposure, mRNA expression levels might have resolved by this time. Nonetheless, we identified other significant components of inflammatory signalling 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 S_{pO_2} ($R = 0.68$; $P < 0.01$; Table 4). Although most of the participants in our study had mild AMS symptoms, this might indicate that *FASLG* and the FAS/FASLG pathway might 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 crucial role in protection against autoimmunity in addition to tightly regulating immune system activation by activation-induced cell death (Brunner et al., 2003; Griffith et al., 1995; Nagata & Golstein, 1995; 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 with their baseline SL expression. Furthermore, we also found that the FAS signalling pathway was enriched with significantly differentially expressed genes after the first day of high-altitude exposure (Table 3). This might have multiple implications. First, evidence suggests that endothelial cells produce soluble Fas ligand in hypoxia, which protects them from activation-induced cell death (Mogi et al., 2001). Second, pro-inflammatory cytokines (such as TNF-α) can induce expression of Fas ligand 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, protein levels of pro-inflammatory cytokines have been found to be elevated in plasma upon hypoxic exposure (Boos et al., 2016; Julian et al., 2011; Song et al., 2016). Thus, this initial elevation in pro-inflammatory cytokine concentration upon high altitude exposure might induce Fas ligand expression on tissue cells and cause apoptosis in activated T cells to dampen the immune response. However, given that the majority of our RNA sample should be derived from peripheral blood mononuclear cells, this might not explain the increased *FASLG* expression we observed. A final interpretation is that elevated *FASLG* expression might be reflective of T cells inducing mutual activation-induced cell death amongst the T-cell population (Brunner et al., 2003).

Our data also show that *FASLG* mRNA expression is also positively correlated with S_{pO_2}. Mogi et al. (2001) showed 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 S_{pO_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) showed that there was a significant reduction of circulating Fas ligand in sojourners to high altitude and in native highlanders. It is important to note that circulating plasma protein can come from multiple sources, such as endothelial cells, and we were measuring gene expression in immune cells. Additionally, it was shown that highlanders with pulmonary hypertension had a significantly lower circulating levels of Fas ligand in plasma compared with lowlander control subjects (Kosanovic et al., 2019; Sydykov et al., 2021). Overall, these results indicate a potential role of FAS signalling in high-altitude acclimatization and adaptation and highlight the importance of immune system regulation in hypoxia. These promising new findings warrant future study because 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; Fig. 5). Previous studies clearly illustrate effects of hypobaric hypoxia exposure on production of reactive oxygen species and biomarkers of oxidative damage (Chandel et al., 1998; Malacrida et al., 2019; Waypa & Schumacker, 2002). One downstream gene target of the NRF2 pathway is haem oxygenase-1 (*HO-1*), a cytoprotective rate-limiting enzyme that is crucial for degradation of haem into equimolar amounts of

Fe^{2+}, biliverdin and CO, all of which play roles as anti-oxidants and regulators of inflammation, apoptosis and angiogenesis (Tift et al., 2020). Our study shows a non-significant trend for increased end-tidal CO after 1 day at high altitude (Table 1). Given that the only known source of endogenous CO is the haem oxygenase pathway, increased CO upon acute high-altitude exposure might indicate increased haem degradation. Furthermore, elevated CO levels might provide tissue-protective effects, such as those described in models of acute inflammation in ischaemia–reperfusion injury, vascular injury or disease, and sepsis (Knauert et al., 2013; Minamino et al., 2001; 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, although our study group included both men and women, we did not examine sex-specific changes in inflammatory gene expression patterns owing 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 after acute high-altitude exposure. Therefore, although 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 used in our study were collected from peripheral whole blood; therefore, our data are 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.

Conclusion

In conclusion, we demonstrate that acute exposure to high-altitude hypoxia triggers significant changes in inflammation-related gene expression. Specifically, our analysis has led to the identification of several inflammatory-related genes that might be involved in immune system sensitization, such as components of the TLR4 signalling pathway. Clearly, hypoxaemia and high-altitude exposure have significant impacts on inflammatory signalling, 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 might exacerbate pro-inflammatory cytokine production in peripheral blood mononuclear cells *in vitro*. Additionally, investigating the potential role of *LY96* and *HMGB1* in immune system sensitization might 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 hypoxaemia modulates inflammatory responses in critical and chronic illnesses, such as ARDS and coronavirus disease 2019.

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Additional information

Data availability statement

The data supporting these findings is available in the Dryad data repository at: https://datadryad.org/stash/dataset/doi:10.6086/D1XM45.

Competing interests

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 E.C.H. conceived and designed the research. K. Pham, S.F., N.P., B.O. and E.C.H. assisted in sample collection. K. Pham, K. Parikh and E.C.H. analysed data, interpreted the results of experiments and prepared figures. K. Pham drafted the manuscript. K. Pham, K. Parikh and E.C.H. edited and revised 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.

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Keywords

acute mountain sickness, high altitude, hypoxia, inflammation

Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

Statistical Summary Document
Peer Review History