

# Differential immune response generation in rats acutely exposed to hypobaric hypoxia and determination of susceptibility and tolerance

## Abstract

Hypobaric hypoxia is a stressful condition at high altitude and becomes progressively more severe with increasing altitude. Hypobaric hypoxia condition may invoke systemic immune response. In the present study, we wished to identify the immune factors contributing in mediating a differential tolerance to acute hypobaric hypoxic stress. Adult Sprague-Dawley rats were exposed to simulated hypoxia equivalent to 223mmHg pressure in decompression chamber. Screened on the basis of time taken for onset of gasping, animals were categorized as susceptible (<10mins), normal (10–25 min) or tolerant (>25mins) while unexposed animals were considered as controls. The tolerant animals displayed significantly high macrophage functionality through increased phagocytic activity as well as iNOS expression and nitric oxide level. Further, hypoxia tolerant animals showed significantly increased Toll-like receptor-4 (TLR-4) expression, proinflammatory cytokines (TNF- $\alpha$ , IL-6, IL-1 $\beta$  and MCP-1) and NF- $\kappa$ B expression while susceptible animals showed increased ROS generation and HIF-1 $\alpha$  expression. Apparently the neutrophil marker  $\beta$ 2 integrin (CD18) was also significantly higher in tolerant animals as compared to other groups. This study suggest that a prompt immune response under hypoxic stress as indicated by macrophage (NO generation) and neutrophils ( $\beta$ 2 integrin expression) activity could be promising factors in screening individuals' tolerance to acute sub lethal hypoxia and contributing substantially to withstand it for longer duration.

**Keywords:** Hypobaric hypoxia, innate immune system, Macrophage, NF- $\kappa$ B, Nitric oxide,  $\beta$ -2 integrin, heterodimer, unacclimatized, unacclimatized, hyperventilatory, adaptive immune system, adaptive immune system

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**Abbreviations** DAMP: Damage-Associated Molecular Pattern; DCFH2-DA:2',7'- dichloro-dihydro-fluorescein diacetate; ELISA: Enzyme Linked Immunosorbent Assay; FACS: Fluorescence-activated cell sorting; HIF1 $\alpha$ : Hypoxia-inducible factor 1-alpha; iNOS: Inducible Nitric oxide Synthase; MFI: Median Fluorescence Intensity; NO: Nitric Oxide; ROS: Reactive Oxygen Species; TLR: Toll-like receptors; TNF- $\alpha$ : Tumor Necrosis Factor Alpha; CD18: Cluster of Differentiation 18.

## Introduction

Hypobaric hypoxia, a unique and major stress present at high altitude, is an environmental condition which may modulate the immune responses of an individual. Hypoxia challenge usually puts unacclimatized individuals at risk of acute mountain sickness that endanger the life at high altitudes. The low partial pressure of oxygen at high altitude affects the functioning of the immune system.<sup>1</sup> The organisms can induce a series of adaptive processes to cope with reduced oxygen level. The lack of oxygen stimulates many hypoxia inducible genes which play an important role in facilitating adaptation to hypoxia by regulating activation of a heterodimer transcriptional factor hypoxia inducible factor-1 (HIF-1) which modulate the response through various downstream signaling pathways to regulate homeostasis at cellular level.<sup>2</sup> Hypoxia has been demonstrated to regulate another transcription factor Nuclear Factor- $\kappa$ B (NF- $\kappa$ B) via HIF-1 $\alpha$ .<sup>3,4</sup> The NF- $\kappa$ B, in turn trans-activates number of proinflammatory, apoptotic and oncogenic genes that collectively

function to foster cellular adaptation to stress.<sup>5</sup> Also under hypoxic conditions, lower availability of oxygen acts as an electron sink in the mitochondrial electron transport system resulting in generation of superoxide radicals that subsequently generate hydroxyl and peroxynitrite radicals.<sup>6,7</sup> This free radical production being a chain reaction leads to oxidative stress.

Indeed, some of the human studies to examine immune function at altitude have indicated that natural killer cell activity and humoral immunity are either unaffected or enhanced.<sup>8-10</sup> While the innate immune response due to hypobaric hypoxia has not been completely defined, both neutrophils and macrophages sense the hypoxic stress in the body and adapt their metabolic activity in a number of ways when exposed to hypoxia. Upon stimulation, they activate other cells of the innate or adaptive immune system which help the body to cope up low oxygen tension.<sup>11-13</sup> It has been observed that some individuals are more susceptible to hypobaric hypoxia than others when exposed to same conditions suggesting a possible predisposition of certain factors. Hence, it is assumed that organisms possibly have mechanism to compensate for low oxygen levels at high altitude. A comparison of the difference in the innate immune responses to hypoxic stress may reveal essential facts about possible mechanisms underlying the hypoxic tolerance. This study brings to light for the first time the involvement of innate immune factors in hypoxic tolerance. Our study highlights the significance of the innate immune system as evident by macrophages and neutrophils' response in increasing the sustenance under acute environmental stress through disparity in CD

18 expression and inflammatory signaling pathways which could be the possible indicators for hypoxia tolerance.

## Materials and Methods

### Reagents and chemicals

All the chemicals used were of the analytical grade and procured from Sigma Chemicals unless mentioned otherwise. The anti rat-CD18 FITC and TLR-4 FITC were purchased from BD Biosciences (BD India Pvt Ltd, India). Zymosan A-FITC, rat monoclonal anti- $\beta$ -actin, anti-HIF-1 $\alpha$ , anti- Phosphorylated I- $\kappa$ B anti-NF- $\kappa$ B and anti-iNOS antibodies, and anti-mouse and anti-rabbit IgG Ab conjugated with HRP/ AP were purchased from Sigma-Aldrich (St. Louis, MO). TNF- $\alpha$ , IL-6, IL-1 $\beta$ , TGF-  $\beta$  and MCP-1 ELISA kits were purchased from eBiosciences (Minneapolis, MN). IgA, IgG and IgM ELISA kits were purchased from ICL, Inc (OR, USA).

### Experimental Animals

Male Sprague Dawley rats (150 $\pm$ 10g), maintained in the institute's animal house, were used for all experiments. Animals had access to food and water ad libitum and were maintained at a constant temperature of 24°C  $\pm$  2°C with 12 h each light-dark cycle. The study was approved by the Animal Ethical Committee of the institute in accordance with Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) of the government of India. All efforts were made to minimize the number of animals and their suffering for experimentation purpose.

### Hypobaric Hypoxia Exposure

Hypobaric hypoxic tolerance was determined by measuring the time taken for the onset of gasping (GT). Rats were exposed, one at a time, to acute hypobaric hypoxia equivalent to 32,000ft (9754m;223 mmHg) in a simulated decompression chamber attached to a mercury barometer, by gradually increasing the altitude at a rate of 2000ft/min, at 32°C. The airflow in the chamber was at the rate of 2 L/min to prevent accumulation of exhaled air, while the relative humidity was maintained at 40% to 50%. The time taken for appearance of the first sign of gasping, a characteristic hyperventilatory response, was recorded using an electronic stopwatch. Accordingly, the animals (n=10) were divided into following groups:

- I. Unexposed control group – Maintained in the normoxic condition within the same laboratory condition (C)
- II. Hypobaric hypoxia Susceptible – Gasping time <10 min (HHS)
- III. Hypobaric hypoxia Normal – Gasping time between 10-25 min (HHN)
- IV. Hypobaric hypoxia Tolerant – Gasping time >25 min (HHT).

Following an interval of 1 week, the animals were again exposed to the same altitude of 9754m to evaluate their tolerance or susceptibility to acute hypoxia. In total, the animals were given three consecutive exposures once/week under similar conditions with seven days for recovery in between. Again when the animals showed the onset of the characteristic hyperventilatory response, depending on their tolerance or susceptibility to hypoxic stress, they were brought down to normoxic levels at the normal rate of decent (2000ft/min).<sup>14-17</sup> Immediately after third exposure, animals were anaesthetized under sodium pentobarbital and samples collected. Retro-orbital blood samples were collected in two parts, one part in heparinized tube for cell surface marker's study and another part was allowed to clot

for collection of sera and stored at -80°C for further use. Peritoneal macrophages were collected from the peritoneal cavity for molecular studies.

### Isolation of peritoneal elicited macrophage

To isolate peritoneal macrophages, rats were administered 4% starch solution in the peritoneal cavity 48 hours prior to sacrifice. After the hypoxia exposure, macrophages were isolated from the peritoneal lavage as described elsewhere.<sup>18</sup> Briefly, the peritoneum was cleaned with 70% ethanol and then injected with approximately 40 ml ice-cold autoclaved 1X PBS followed by gentle massage to allow the distribution of lavage fluid throughout the peritoneal cavity. The lavage fluid was then withdrawn and re-flushed at least 5 times using a 10 ml syringe. Finally cells were washed twice in RPMI 1640 and resuspended in complete RPMI media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 $\mu$ g/ml). Macrophages were identified by adherence to tissue culture plate (1 h, 37°C) and morphology characteristics under light microscopy. Cell viability was confirmed by exclusion of trypan blue.

### Phagocytic Assay

To quantify the extent of phagocytosis, the ratio of engulfed opsonised zymosan-A FITC was determined in peritoneal macrophages of hypoxia exposed and control animals. Briefly, FITC tagged Zymosan-A particles were opsonized using unexposed control rat sera and incubated at 37°C for 10min. The opsonized Zymosan-A-FITC (20 $\mu$ g/ml) were then added to one million cells/ml peritoneal macrophages and incubated for 45min at 37°C. After incubation, the cells were washed with 1X PBS to remove unbound particles and resuspended in 500 $\mu$ l 1X PBS. Phagocytic activity measurement was carried out on a FACS Calibur, with Cell Quest Pro software. Total 10000 events were analyzed to assess the effect of hypobaric hypoxia exposure on peritoneal macrophages.

### ROS determination

Intracellular ROS generation during hypobaric hypoxia exposure was assessed and compared with unexposed control animals using 2',7'- dichlorodihydrofluorescein diacetate (DCFH2-DA) as described elsewhere.<sup>19</sup> Briefly, peritoneal macrophages of exposed and control animals at a concentration of one million cells/ml were incubated for 15min after addition of DCFH2-DA (10 $\mu$ M) at 37°C. DCFH2-DA is a compound that enters cells and fluoresces only when it is oxidized by reactive oxygen species, particularly hydrogen peroxide and hydroxyl radicals. ROS generation was assessed by measuring the formation of dichlorofluorescein (DCF) fluorescence with excitation at 495 nm and emission at 529nm on FACS Calibur with Cell Quest Pro software. Total 10000 events were analyzed to assess the effect of hypobaric hypoxia exposure on peritoneal macrophages.

### Toll-like receptor-4 expression analysis

TLR-4 expression in hypoxia exposed and control animals, was measured in peritoneal macrophages. Briefly, peritoneal macrophages at a concentration of one million cells/ml were suspended in 300 $\mu$ l RPMI 1640. In FACS tubes, the cells were incubated with anti-TLR-4 FITC Ab for 45 minutes. After incubation, cells were washed twice with 1X PBS and resuspended in 500 $\mu$ l PBS and analyzed on a FACS Calibur with Cell Quest Pro software. Total 10000 events were analyzed to assess the effect of hypobaric hypoxia exposure on TLR-4 expression in peritoneal macrophages.

## Nitrite and TNF- $\alpha$ Analysis

Nitrite, a biological metabolite of NO was determined in the cell supernatant of peritoneal macrophages of hypoxia exposed and control animals using Griess reagent.<sup>20</sup> Briefly, peritoneal macrophages were adjusted at a concentration of 0.5 million/ml and plated in 96-well plate for 24 h. Nitrite concentration was determined by mixing 100 $\mu$ l of cell-free supernatants with equal volume of Griess reagent. A chromophoric azo-derivative molecule was produced in the reaction between nitrite and the reagent containing sulfanilic acid and N-(1-naphthyl) ethylene diamine that absorbed light at 540 nm in a microplate ELISA reader (Biotek, USA). Sodium nitrite (1.65-100 $\mu$ M) was used to generate a standard curve with each assay. Pro-inflammatory cytokine TNF- $\alpha$  was estimated in cell supernatant of peritoneal macrophages of exposed and control animals by solid phase enzyme immunoassay using ELISA kit based on multiple Ab sandwich principle and as per manufacturer's instructions.

## Expression of cytoplasmic and nuclear proteins

To investigate expression of various cytoplasmic and nuclear proteins after hypobaric hypoxia exposure, macrophages were lysed using cytoplasmic extraction buffer (10mM HEPES, 1.5mM MgCl<sub>2</sub>, 10mM KCl, 200mM Sucrose, 0.5mM DTT, 0.25% Nonidet P-40, 0.1% protease inhibitor cocktail) and nuclear extraction buffer (20mM HEPES, 420mM NaCl, 0.2mM EDTA, 1.5mM MgCl<sub>2</sub>, 0.5mM DTT, 25% Glycerol, 0.1% protease inhibitor cocktail). The protein concentrations were determined by BCA method<sup>21</sup>, and equal amounts of protein (50 $\mu$ g) were loaded on a SDS-PAGE. After electrophoretic separation, the proteins were transferred and fixed on to a PVDF membrane (0.45  $\mu$ m), in transfer buffer (25 mM Tris, 180 mM glycine, 20% methanol, pH 8.3). Equal loading and transfer efficiency were verified by staining with 2% Ponceau S. The blots were blocked in 1X PBS containing 3% BSA for 90 min at room temperature, washed and then probed for two hours at room temperature or overnight at 4°C, with respective monoclonal antibodies viz.  $\beta$ -actin, Phosphorylated I- $\kappa$ B and iNOS for cytoplasmic extract. Nuclear extract blots were probed with monoclonal antibodies directed against NF- $\kappa$ B and HIF-1 $\alpha$ . The membranes were washed with 1X PBS-Tween20 (0.05%) (PBST) and incubated with anti-mouse and anti-rabbit IgG conjugated with HRP/AP specific against the primary monoclonal antibodies. For detection of protein expression, either chemiluminescence substrate for HRP or BCIP/NBT for AP was used.

## Cytokine and chemokine estimation in sera

The levels of cytokines and chemokine viz TNF- $\alpha$ , IL-6, IL-1 $\beta$ , TGF- $\beta$ , and MCP-1 in the sera of control and hypoxia exposed animals were measured by ELISA as per manufacturer's instructions. Briefly, the cytokine estimation was carried out on the principle of solid phase enzyme immunoassay. Multiple Ab sandwich assay was performed using commercially available ELISA kit (eBiosciences, Minneapolis, MN). The ELISA plates were coated with respective coating antibodies specific for rat TNF- $\alpha$ , IL-6, IL-1 $\beta$ , TGF- $\beta$  and MCP-1 to capture the specific cytokines present in the standard, and serum. The anti-TNF- $\alpha$ , anti-IL-6, anti-IL-1 $\beta$ , anti-TGF- $\beta$  and anti-MCP-1 rabbit polyclonal Ab conjugated to biotin was added to each well respectively, followed by streptavidin/ Horseradish peroxidase (HRP) incubation for 20min. At the end, color was developed using peroxide and a chromogenic substrate, 3',3',5,5'-tetramethylbenzidine (TMB) solution. The substrate initiated a peroxidase catalyzed color change,

which was stopped within 15 min by acidification with stop solution (2N H<sub>2</sub>SO<sub>4</sub>). Absorbance was measured by ELISA reader (Biotek, USA) at 450 nm. The quantities of the cytokines in samples were measured from the standard curve constructed from the standards.

## Immunoglobulin estimation in sera

Immunoglobulins IgA, IgG and IgM were measured by double Ab sandwich ELISA method as per manufacturer's instructions. Briefly IgA, IgG and IgM were measured in serum by capture ELISA using rat immunoglobulin reference (ICL, Inc, OR, USA). After adding samples to the pre-coated plates, goat anti-rat IgA, IgG and IgM conjugated to HRP was added. Following incubation and washing steps, the enzyme bound to the immunosorbent was assayed by addition of TMB solution. After twenty minutes of incubation at room temperature, stop solution was added. The absorbance was measured at 450 nm in ELISA Reader. The quantity of the Ig's in test samples were interpolated from the standard curve constructed from the standards and correlated for sample dilution.

## Flow cytometric evaluation of expression of cell surface and activation markers

To determine the expression of  $\beta$ -2 integrin on neutrophils, 100  $\mu$ l of whole blood from each control and hypoxia exposed rats was incubated with anti-CD18 FITC for 45 minutes. After incubation, RBCs were lysed using 1X FACS lysing solution (BD Pharmingen, USA), and washed twice with 1X PBS. Cells were resuspended in 500  $\mu$ l 1X PBS and analyzed using FACS Calibur (Becton Dickinson, San Jose, CA, USA) with Cell Quest Pro software.

## Statistical Analysis

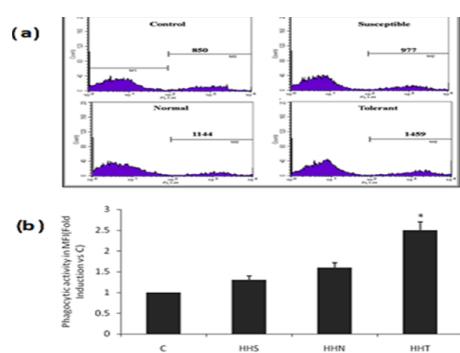
All the experiments were performed on a minimum of three different occasions and data are presented as mean  $\pm$  E. The significance of difference between unexposed control, susceptible, normal and tolerant groups were analyzed using one-way ANOVA. Differences were considered significant at  $p < 0.05$ . Data were analyzed using SPSS-16 software.

## Results

Due to hypoxia, after reaching the desired altitude, the respiratory network in animals responds with an augmentation of respiratory activity and generation of sighs which is followed by a respiratory depression and finally generation of gasps. All the exposed animals were differentiated and categorized on the basis of onset of this characteristic hyperventilatory response, gasping time

## Phagocytic activity

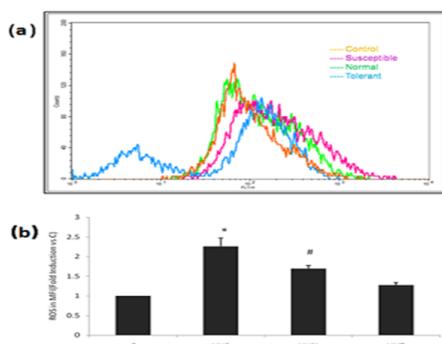
To determine the effect of hypobaric hypoxia on macrophage functionality, phagocytic assays were performed in elicited peritoneal macrophages of hypoxia exposed and control animals using opsonised Zymosan-A FITC. The phagocytic activity of exposed animals was increased significantly as compared to controls. Almost 2.5 fold increase in phagocytosis was observed in tolerant group ( $2.5 \pm 0.2$ ;  $p < 0.001$  vs C) when normalized against control. The increase in phagocytic activity of tolerant group with respect to susceptible ( $1.30 \pm 0.097$ ) and normal group ( $1.59 \pm 0.12$ ;  $p < 0.01$  vs C) was also significant ( $p < 0.05$ ) indicating better adaptive response to stress condition (Figure. 1a,1b).



**Figure 1** Hypobaric hypoxia induced differential Phagocytic activity. Peritoneal macrophages isolated from animals exposed to hypobaric hypoxia or unexposed control were incubated with opsonized ZymosanA-FITC and analyzed by flowcytometry. (a) Representative histograms illustrating change in phagocytic activity of Susceptible, Normal and Tolerant animals, (b) Graph indicating values as Median Fluorescence Intensity (MFI) relative to the unexposed control  $\pm$  S.E. (\*p< 0.05 vs C; #p<0.01 vs C, HHT); (n=10)

### Oxidative stress due to hypoxia exposure

Intracellular ROS content was examined to determine the extent of oxidative stress due to hypobaric hypoxia exposure in exposed and control animals. The superoxide generation increased in hypoxia exposed animals when compared to unexposed controls. All the median fluorescence intensity (MFI) values were normalized against control by taking ratio of hypoxia exposed/ control cells. The susceptible group ( $2.26 \pm 0.20$ ;  $p<0.05$  vs C, HHT) and normal group ( $1.69 \pm 0.077$ ;  $p<0.01$  vs C, HHT) showed significantly higher level of intracellular ROS followed by tolerant group ( $1.26 \pm 0.065$ )(Figure 2a, 2b).

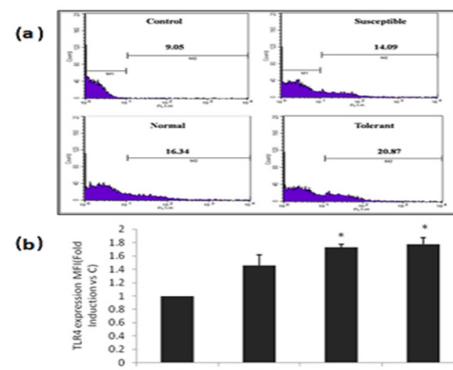


**Figure 2** Hypobaric hypoxia induced Oxidative stress. ROS were determined by incubating peritoneal macrophage cells with DCFH-DA ( $10\mu\text{M}$ ) isolated from animals exposed to hypobaric hypoxia or unexposed control. The production of ROS was monitored by changes in DCFH-DA fluorescence using flowcytometry. (a) Representative overlay of histograms demonstrating change in Superoxide level of Susceptible, Normal and Tolerant animals; (b) Graph indicating values as Median Fluorescence Intensity (MFI) relative to the unexposed control  $\pm$  S.E. (\*p< 0.05 vs C, HHT; #p<0.01 vs C, HHT); (n=10)

### TLR-4 expression analysis

The presence of surface receptor TLR-4 on macrophages plays an important role in the innate immunity. The contribution of TLR-4 during hypobaric hypoxia was investigated for early danger signals. The flowcytometric analysis revealed increased TLR-4 expression in hypoxia exposed animals in comparison to control. The tolerant group

( $1.77 \pm 0.095$ ;  $p<0.01$  vs C) and normal group ( $1.72 \pm 0.05$ ;  $p<0.01$  vs C) animals showed significantly higher TLR-4 expression followed by susceptible group ( $1.45 \pm 0.16$ ) (Figure 3a,3b).



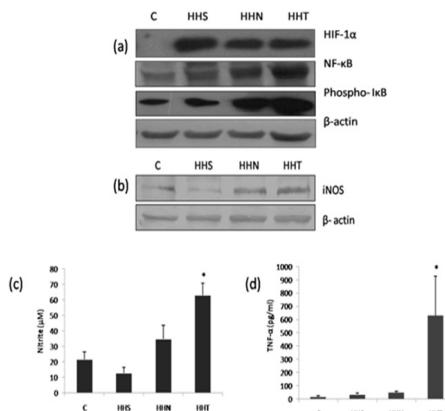
**Figure 3** Hypobaric hypoxia differentially upregulated TLR4 expression. Peritoneal macrophages were isolated from animals exposed to hypobaric hypoxia or unexposed control. Surface protein expression of TLR4 was analysed by flow cytometry after macrophages were stained with FITC-conjugated anti-TLR4 Ab. (a) Representative histograms illustrating change in TLR4 expression of Susceptible, Normal and Tolerant animals; (b) Graph indicating values as Median Fluorescence Intensity (MFI) of protein levels relative to the unexposed control  $\pm$  S.E. (\*p< 0.01 vs C); (n=10)

### HIF-1 $\alpha$ and NF- $\kappa$ B expression

To evaluate the role of transcription factors in facilitating animals to withstand hypobaric hypoxia, expression of two transcription factors HIF-1 $\alpha$  and NF- $\kappa$ B were assessed in peritoneal macrophages. Macrophages of exposed and control groups were lysed to extract nuclear proteins. Equal amount of protein was subjected onto SDS-PAGE followed by immunoblotting, to estimate the above mentioned nuclear factors. The expression of nuclear factors was apparent in peritoneal macrophages of hypoxia exposed animals as compared to control. Amongst the exposed groups, the NF- $\kappa$ B expression markedly increased with increase in tolerance to hypoxia while HIF-1 $\alpha$  expression decreased.  $\beta$ -actin was used as loading control (Figure 4a).

### NO and iNOS Expression and TNF- $\alpha$

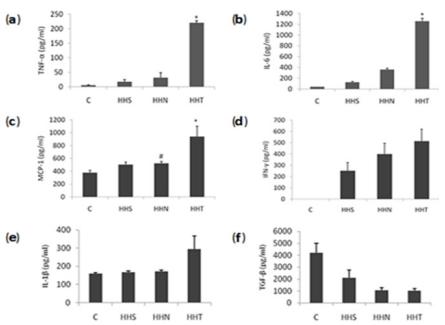
In animals exposed to hypobaric hypoxia to differentiate them for sustenance under stressful condition, generation of NO in macrophage cell supernatants increased with increase in tolerance as compared to control. The NO production was maximum in peritoneal macrophages of tolerant animals ( $63 \pm 7\mu\text{M}$ ;  $p<0.01$  vs C,  $p<0.001$  vs HHS), followed by normal ( $34.59 \pm 9\mu\text{M}$ ) and susceptible ( $12.63 \pm 3.9\mu\text{M}$ ), when compared to controls ( $21.5 \pm 4.97\mu\text{M}$ ) (Figure 4c). This was further manifested by increased iNOS expression in cytoplasmic extract of peritoneal macrophages subject to SDS-PAGE and immunoblotting while  $\beta$ -actin was used as loading control (Figure 4b). TNF $\alpha$  also has vasodilatory effect and contribute to vasodilation of blood capillaries. The amount of TNF- $\alpha$ , in macrophage cells supernatant of control and hypoxia exposed animals was tested by ELISA. The TNF- $\alpha$  level was found to be significantly higher in tolerant ( $629.1 \pm 299$  pg/ml;  $p<0.05$  vs C, HHS and HHN) as compared to control ( $16.35 \pm 9.6$  pg/ml), susceptible ( $34.43 \pm 11.7$  pg/ml) and normal ( $49.58 \pm 8.2$  pg/ml) (Figure 4d).



**Figure 4** Hypobaric hypoxia activates inflammatory pathway. A total of 5 X 106 cells isolated from hypobaric hypoxia exposed and unexposed control animals were taken. At the end of incubation, supernatants were collected and cytoplasmic and nuclear proteins were extracted. The proteins were separated on SDS-PAGE and transferred on to PVDF membrane for probing with anti-rat monoclonal antibodies. (a) Immunoblot analysis of transcription factors HIF-1 $\alpha$  and NF- $\kappa$ B of hypobaric hypoxia exposed and control animals. (b) Expression of iNOS validated by immunoblotting.  $\beta$ -actin was used as the loading control. Immunoblot results are representative of three separate experiments. (c) Determination of Nitric oxide levels ( $\mu$ M  $\pm$  SE) using Griess reagent in peritoneal macrophages supernatant; (n=10) (\*p<0.005 vs C, HHS). (d) Determination of TNF- $\alpha$  secretion in peritoneal macrophages supernatant of hypoxia exposed and unexposed control animals; (n=10). (\*p<0.05 vs C, HHS, HHN)

#### Cytokines and chemokine estimation in serum

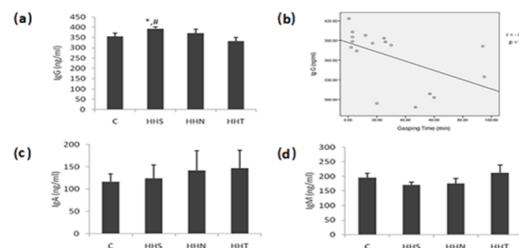
The levels of pro and anti-inflammatory cytokines like TNF- $\alpha$ , IL-6, IL-1 $\beta$ , IFN- $\gamma$ , MCP-1 and TGF- $\beta$  were determined in sera of hypoxia exposed and control animals. There was a considerable increase in pro-inflammatory cytokine and chemokine levels in exposed animals as compared to control animals. The tolerant animals secreted significantly higher levels of TNF- $\alpha$ , IL-6 and MCP-1 (p<0.05) as compared to control, normal and susceptible animals (Figure 5a-5c). The level of IL-1 $\beta$  and IFN- $\gamma$  were also marginally high in tolerant animals but it was not significant (Figure 5d,5e). Interestingly, the anti-inflammatory cytokine TGF- $\beta$  level in susceptible animals was lower than control animals but higher than normal and tolerant animals (Figure 5f). These finding further confirmed the earlier results of the study, where the level of TNF- $\alpha$  in macrophage cells supernatant was found to be significantly higher in tolerant animals.



**Figure 5** Effect of hypobaric hypoxia on cytokines and chemokine. (a) TNF- $\alpha$ , (b) IL-6, (c) MCP-1, (d) IFN- $\gamma$ , (e) IL-1 $\beta$ , and (f) TGF- $\beta$  production determined in sera samples of animals exposed to hypobaric hypoxia or unexposed control by ELISA. Data presented are the mean  $\pm$  S.E. (\*p<0.05 vs C, HHS, HHN; #p<0.05 vs C); (n=10)

#### Immunoglobulins estimation in serum

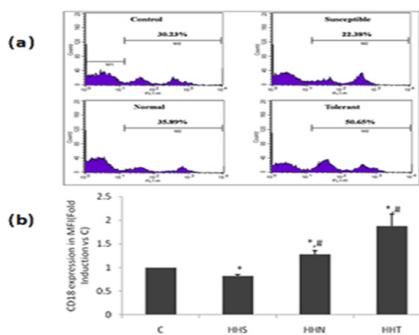
To investigate humoral response to hypoxia the IgG, IgA and IgM levels were estimated in sera samples of exposed and control animals. The Ig levels in hypoxia exposed animals indicated variations as compared to control animals. The susceptible animals showed significantly higher level of IgG in sera as compared to control (p<0.05) and tolerant animals (p< 0.001). Also IgG levels showed a negative correlation (r = -0.515; p<0.03) with gasping time of exposed animals at this altitude (Figure 6a,6b). An increase in IgA levels with increase in tolerance to hypoxia was observed while IgM levels were not altered significantly (Figure 6c,6d).



**Figure 6** Effect of hypobaric hypoxia on Immunoglobulins level. (a) IgG, (c) IgA and (d) IgM levels measured in sera samples of animals exposed to hypobaric hypoxia or unexposed control by ELISA. Data presented are the mean  $\pm$  S.E. (\*p<0.05 vs C; #p<0.05 vs HHT). (b) Correlation of serum IgG and gasping time (Pearson correlation coefficient, r = -0.515; p<0.03); (n=10)

#### Expression of $\beta$ 2 integrin

For determination of neutrophil activity, anti- CD18 antibody was added in heparinised blood of hypoxia exposed and control animals and analyzed by flowcytometry. A significantly variable expression of CD18 was observed in hypoxia exposed animals when evaluated by normalizing (taking ratio) against controls (p< 0.05). Tolerant rats showed significantly enhanced CD18 expression (1.87 $\pm$ 0.258), normal range animals showed intermediate expression (1.27 $\pm$ 0.075), while susceptible rats demonstrated significantly diminished expression (0.81 $\pm$ 0.033; p< 0.001 vs HHN; p< 0.01 vs HHT) in comparison to control (Figure 7a,7b).



**Figure 7** Hypobaric hypoxia induced differential expression of cell surface marker CD18. Surface protein expression of CD18 was analyzed in whole blood of animals exposed to hypobaric hypoxia or unexposed control by flowcytometry. One million cells were collected in FACS tubes and stained with anti- CD 18 FITC conjugated Ab. (a) Representative histograms indicating variation in CD18 expression of Susceptible, Normal and Tolerant animals; (b) Graph indicating CD18 expression. Values shown as gated % of Median Fluorescence Intensity (MFI) of protein levels relative to the unexposed control  $\pm$  S.E. (\*p< 0.05 vs C; #p< 0.01 vs HHS); (n=10)

## Discussion

In the present study, the exposure of animals to acute hypobaric hypoxia, equivalent to 32,000ft showed a difference in the onset of gasping time. Rats are a good model to study the effect of extreme hypobaric hypoxia as smaller animals have higher capillary density in tissues, hence show hypoxia related characteristics at a much higher altitude.<sup>22</sup> Severe hypoxia had been known to directly enhance the excitability of neurons within the 'gasping centre' or 'pre-Botzinger complex' in brain, resulting in augmentation and then a decline in the rhythmic bursts, characteristic of gasping.<sup>23</sup> Hence, gasping time was considered as an indicator of tolerance to hypobaric hypoxia. During this study, most of the animals began gasping within 10-25min, some animals' sustained hypobaric hypoxia for much longer duration (> 25 min), whereas other animals started gasping within 10min of exposure. The time of onset of gasping clearly indicated the response of animals to hypoxia with respect to their susceptibility or tolerance.

It was interesting to understand the contribution of immunological response to indicate hypobaric hypoxic stress. So we evaluated the cellular and molecular mechanisms of innate immune system to understand the signaling pathway in hypobaric hypoxia susceptible, normal and tolerant animals. The innate immune system provides immediate defense against any danger signal by activating and recruiting macrophages and neutrophils as the first line of responders.

The macrophages are the key players of innate immune system, being highly versatile cell type with an impressive repertoire of functions to detect danger signals and represent the systemic effect of stress on animals.<sup>24</sup> We focused on the functions of peritoneal macrophages which are the most responsive cell types in the presence of stressful stimulus.<sup>11,25</sup> With the purpose of understanding the cellular and molecular mechanism, interestingly, our study indicated that the tolerance to hypobaric hypoxia relied on efficiency of macrophages response to the stress signals. Amongst the exposed animals, the phagocytic activity increased with increase in tolerance to hypoxia in comparison to control animals indicating enhanced activation of macrophages. Phagocytic efficiency has also been reported to be increased by hypoxia in many other studies.<sup>12,13</sup> It is a well-known fact that during hypoxia in response to metabolic stress, a burst of intracellular ROS is generated, via mitochondrial dependent signaling process<sup>26</sup> Studies also suggest that ROS regulates HIF-1 $\alpha$  stabilization.<sup>27-29</sup>, has suggested that oxidative stress during systemic hypoxia results due to change in ROS-NO balance as the increased ROS during hypoxia deplete tissue NO levels.<sup>29</sup> Our findings showed higher ROS and HIF-1 indicating higher oxidative stress in susceptible animals as compared to normal or tolerant animals, which is well supported by the above mentioned studies. Although, in contrast to these studies<sup>30</sup>, suggested that HIF-1 functions as a ROS sensor to prevent the overproduction of mitochondrial ROS.<sup>30</sup>

The Toll-like receptors play a critical role in early innate immunity by recognizing pathogen-associated molecular pattern (PAMP), or endogenous molecules released by damaged tissues called damage-associated molecular pattern (DAMP).<sup>31</sup> These DAMPs appear to accentuate innate immunity by engaging cell surface receptors such as TLR-4. In the present work, the tolerant animals expressed maximum TLR-4 receptors as compared to normal or susceptible animals, indicating thereby a better and early recognition of danger associated damage. Hence fortifying the cells by activating the downstream pathway to reprimand the hypoxic damage and withstand the stress.

It is evident that under hypoxic conditions, the transcription factor NF- $\kappa$ B gets activated, which mediates inflammatory responses

like up-regulation of iNOS & NO production and TNF- $\alpha$  secretion. Interestingly the present study proposes that the NF- $\kappa$ B expression increased with tolerance to hypoxia amongst the exposed animals, thereby mediating increased production of NO and TNF- $\alpha$ . Furthermore, the promoter region of iNOS has hypoxia responsive enhancer (HRE) site, which is induced by hypoxia, leading to iNOS activation and NO production.<sup>32</sup> NO has vasodilatory property<sup>33,34</sup> and in our study, the level of nitrite as an indicator of NO in the supernatant of peritoneal macrophages, clearly indicated and supported higher iNOS expression in tolerant animals as compared to other groups. Thus NO could help the tolerant animals to sustain the hypoxic condition by providing vasodilation of blood capillaries. Previous studies have also revealed that the iNOS derived NO regulates and diminishes HIF-1 $\alpha$  stabilization,<sup>35,36</sup> probably by a negative feedback loop in the HIF-1 $\rightarrow$ iNOS cascade<sup>37</sup> Our study is in good agreement with the above mentioned findings as the NO production increased with increase in hypoxia tolerance while HIF-1 $\alpha$  expression decreased. Inferring that, during initial phase of exposure, the HIF-1 $\alpha$  expression increased to regulate hypoxia responsive genes including iNOS which on activation produced NO. This increased NO, by negative feedback loop, then led to suppression of HIF-1 expression as evident in tolerant animals. However studies have also shown an effect of NO on HIF-1 $\alpha$  stabilization and accumulation, depending on NO donors, dose and time.<sup>38,39</sup>

A number of cytokines and cell adhesion molecules are secreted and expressed by immune cells in the circulation under stressful conditions. Hence, it was mandatory to study the spontaneous secretion of number of inflammatory mediators in the sera samples of exposed animals, to get the better understanding of the animal responses to hypobaric hypoxia stimulant. Our results revealed significant increase in the levels of pro-inflammatory cytokines and chemokine like TNF- $\alpha$ , IL-6 and MCP-1 and a trend of increased IL-1 $\beta$  and IFN- $\gamma$  in tolerant animals, while anti-inflammatory cytokine TGF- $\beta$  was higher in susceptible animals. In fact, another study has indicated that immunomodulator increased sustenance of susceptible animals as observed by increase in NO, TNF- $\alpha$  and  $\beta$ -2 integrin level which in turn facilitated better vasodilation and hence better oxygenation to organs and finally increase in gasping time (Unpublished data). Liu et al reported that immune cell invasion and increased expression of inflammatory cytokines in rat carotid body is a conditional requirement for the development of chronic hypoxia-induced chemosensory adaptation.<sup>40</sup> Studies have also shown TNF- $\alpha$  to have both endothelium-dependent and -independent vasodilator properties<sup>41</sup> which attributed to activation of NOS, generation of NO, and relaxation of vascular smooth muscle.<sup>42</sup> In agreement to the above findings, our study indicates that pro-inflammatory cytokines especially TNF- $\alpha$  helped in vasodilation and facilitated tolerant animals to sustain hypoxia for longer duration as compared to susceptible animals.

We also desired to contemplate the degree of change in major Immunoglobulin (IgG, IgA and IgM) levels and find the modulation of humoral immune response under the stressful conditions of acute hypobaric hypoxia and their relationship with hypoxic tolerance. Our study for the first time analyzed the endogenous humoral immune response, where we found a negative correlation between IgG level and gasping time. Although no significant change in the IgA and IgM levels was observed.

Apart from macrophages, the innate immune system recruits Neutrophils as the pioneer immune cells to respond under stressful conditions. Hence, in quest for identifying the role of neutrophils

and granulocytes under hypoxic condition, cell activation marker,  $\beta 2$  integrin (CD18) expression was also considered. For the first time in our study, the role of  $\beta 2$  integrin in determining tolerance against hypoxia was considered and tolerant animals showed significantly enhanced  $\beta 2$ -integrin expression, which enhances neutrophils function, while susceptible animals showed significantly diminished expression as compared to control. Under stressful conditions,  $\beta 2$  integrin receptor binds with adhesion molecule ICAM-1 on the surface of endothelial cells leading to strong adherence and influx of neutrophils at the site of action.<sup>43</sup> Under hypoxic condition, CD18 is induced and increases avidity of endothelial –leukocyte adhesion by increasing expression of several cell selectins and influence myeloid functioning.<sup>44</sup> also showed hypoxia induced leukocyte  $\beta 2$  integrin expression and function by transcriptional mechanisms dependent upon HIF-1.<sup>45</sup> Thus  $\beta 2$  integrin plays major role in response against hypoxic stress.

## Conclusions

Our studies suggests that the animals exhibiting prompt innate immune response, and were able to show elevated macrophage and neutrophil activity indicated by higher level of NO, TNF- $\alpha$  and  $\beta 2$  integrin could tolerate hypobaric hypoxic conditions for longer duration. This may be attributed to the fact that NO, TNF- $\alpha$  and IL-6 are all well-known vasodilators and help blood capillaries to carry more blood and hence more oxygen to the tissues leading to longer sustenance to low oxygen availability. On the other hand, animals susceptible to hypobaric hypoxia showed low levels of NO and TNF- $\alpha$ , but high levels of superoxide in their peritoneal macrophages indicating oxidative stress. Thus, the tolerant animals demonstrated better innate immune system and utilized the inflammatory response via NF- $\kappa$ B regulated signaling pathway by biased production of proinflammatory and vasodilatory molecules like TNF- $\alpha$  and NO, as well as neutrophil activation against hypoxia exposure for sustenance in harsh hypoxic conditions. In conclusion, our study revealed that on exposure to hypobaric hypoxia, a screening of innate immune response in terms of  $\beta 2$  integrin and NO could be potential factors for determining hypoxia tolerance.

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## Conflict of Interest Disclosures

The authors have no financial conflicts of interest.

## References

1. Oliver SJ, Macdonald JH, Smith ADH, et al. High Altitude Impairs In Vivo Immunity in Humans. *High Alt Med Biol* 14. 2013;(2):144–149.
2. Wang GL, Jiang BH, Semenza GL. Effect of altered redox states on expression and DNA-binding activity of hypoxia-inducible factor 1. *Biochem Biophys Res Commun*. 1995;212(2):550–556.
3. Rius J, Guma M, Schachtrup C, et al. NF- $\kappa$ B links innate immunity to the hypoxic response through transcriptional regulation of HIF-1a. *Nature*. 2008;453(7196):807–811.
4. Nakayama K. cAMP-response Element-binding Protein (CREB) and NF- $\kappa$ B Transcription Factors Are Activated during Prolonged Hypoxia and Cooperatively Regulate the Induction of Matrix Metalloproteinase MMP1. *J Biol Chem* . 2013;288(31):22584–22595.
5. Pahl HL. Activators and target genes of Rel/NF- $\kappa$ B transcription factors. *Oncogene*. 1999;18(49):6853–6866.
6. Drose S, Brandt U. Molecular Mechanisms of Superoxide Production by the Mitochondrial Respiratory Chain. Mitochondrial Oxidative Phosphorylation. *Adv Exp Med Biol*. 2012;748:145–169.
7. Waypa GB, Marks JD, Guzy RD, et al. Superoxide Generated at Mitochondrial Complex III Triggers Acute Responses to Hypoxia in the Pulmonary Circulation. *Am J Respir Crit Care Med*. 2013;187(4):424–432.
8. Biselli R, Moli SL, Matricardi PM, et al. The effects of hypobaric hypoxia on specific B cell responses following immunization in mice and humans. *Aviat Space Environ Med*. 1991;62(9 Pt 1):870–874.
9. Chohan IS, Singh I, Balakrishnan K, et al. Immune response in human subjects at high altitude. *Int J Biometeorol*. 1975;19(3):137–143.
10. Facco M, Zilli C, Siviero M, et al. Modulation of immune response by the acute and chronic exposure to high altitude. *Med Sci Sports Exerc*. 2005;37(5):768–774.
11. Cramer T, Yamanishi Y, Clausen BE, et al. HIF-1alpha is essential for myeloid cell-mediated inflammation. *Cell*. 2003;112(5):645–657.
12. Peyssonnaux C, Datta V, Cramer T, et al. HIF-1 $\alpha$  expression regulates the bactericidal capacity of phagocytes. *J Clin Invest*. 2005;115(7):1806–1815.
13. Nizet V, Johnson RS. Interdependence of hypoxic and innate immune responses. *Nat Rev Immunol*. 2009;9(9):609–617.
14. Jain K, Suryakumar G, Prasad R, et al. Upregulation of cytoprotective defense mechanisms and hypoxia-responsive proteins imparts tolerance to acute hypobaric hypoxia. *High Alt Med Biol*. 2013; 14(1):65–77.
15. Jain K, Suryakumar G, Prasad R, et al. Differential activation of myocardial ER stress response: a possible role in hypoxic tolerance. *Int J Cardiol*. 2013;168(5):4667–4677.
16. Padhy G, Sethy NK, Ganju L, et al. Abundance of plasma antioxidant proteins confers tolerance to acute hypobaric hypoxia exposure. *High Alt Med Biol*. 2013;14(3):289–297.
17. Jain K, Suryakumar G, Ganju L, et al. Differential hypoxic tolerance is mediated by activation of heat shock response and nitric oxide pathway. *Cell Stress Chaperones*. 2014;19(6):801–812.
18. Brisseau GF, Grinstein S, Hackam DJ, et al. Interleukin-1 Increases Vacuolar-type H1-ATPase Activity in Murine Peritoneal Macrophages. *J Biol Chem*. 1996;271(4):2005–2011.
19. Bass DA, Parce JW, Dechatelet LR, et al. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. *J Immunol*. 1983;130(4):1910–1917.
20. Griess P. Bemerkungen zu der Abhandlung der HH. Weselsky und Benedikt Ueber einige Azoverbindungen. *Ber Deutsch Chem Ges*. 1879;12(1):426.
21. Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using biinchoninic acid. *Anal Biochem*. 1985;150(1):76–85.
22. Olson EB, Jr. Dempsey JA. Rat as a model for humanlike ventilatory adaptation to chronic hypoxia. *J Appl Physiol Respir Environ Exerc Physiol*. 1978;44(5):763–769.
23. St John WM. Medullary regions for neurogenesis of gasping: noeud vital or noeuds vitals? *J Appl Physiol*. 1996;81(5):1865–1877.
24. Lewis JS, Lee JA, Underwood JCE, et al. Macrophage responses to hypoxia: relevance to disease mechanisms. *J Leukoc Biol*. 1999;66(6):889–900.
25. Murdoch C, Muthana M, Lewis CE, et al. Hypoxia Regulates Macrophage Functions in Inflammation. *J Immunol*. 2005;175(10):6257–6263.

26. Chandel NS, McClinton DS, Feliciano CE, et al. Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O<sub>2</sub> sensing. *J Biol Chem*. 2000;275:25130–25138.
27. Bonello S, Zahringer C, BelAiba RS, et al. Reactive oxygen species activate the HIF-1alpha promoter via a functional NFκB site. *Arterioscler Thromb Vasc Biol*. 2007;27(4):755–761.
28. Jung SN, Yang WK, Kim J, et al. Reactive oxygen species stabilize hypoxia-inducible factor-1 alpha protein and stimulate transcriptional activity via AMP-activated protein kinase in DU145 human prostate cancer cells. *Carcinogenesis*. 2008;29(4):713–721.
29. Steiner DR, Gonzalez NC, Wood JG. Interaction between reactive oxygen species and nitric oxide in the microvascular response to systemic hypoxia. *J Appl Physiol*. 2002;93(4):411–418.
30. Kirito K, Yongzhen H, Komatsu N. HIF-1 prevents the overproduction of mitochondrial ROS after cytokine stimulation through induction of PDK-1. *Cell Cycle*. 2009;8(17):2844–2849.
31. Akira S, Takeda K. Toll-like receptor signaling. *Nat Rev Immunol*. 2004;4:499–511.
32. Melillo G, Taylor LS, Brooks A, et al. Regulation of inducible nitric oxide synthase expression in IFN- $\gamma$  treated murine macrophages cultured under hypoxic conditions. *J Immunol*. 1996;157(6):2638–2644.
33. Kourembanas S, McQuillan LP, Leung GK, et al. Nitric oxide regulates the expression of vasoconstrictors and growth factors by vascular endothelium under both normoxia and hypoxia. *J Clin Invest*. 1993;92(1):99–104.
34. Allen JD, Gow AJ. Nitrite, NO and hypoxic vasodilation. *Br J Pharmacol*. 2009;158(7):1653–1654.
35. Agani FH, Puchowicz M, Chavez JC, et al. Role of nitric oxide in the regulation of HIF-1 expression during hypoxia. *Am J Physiol Cell Physiol*. 2002; 283(1):C178–C186.
36. Diez I, Calatayud S, Hernandez C, et al. Nitric oxide, derived from inducible nitric oxide synthase, decreases hypoxia inducible factor-1 $\alpha$  in macrophages during aspirin-induced mesenteric inflammation. *Br J Pharmacol*. 2010;159(8):1636–1645.
37. Yin JH, Yang DI, Ku G, et al. iNOS Expression Inhibits Hypoxia-Inducible Factor-1 Activity. *Biochem Biophys Res Commun*. 2000;279(1):30–34.
38. Kimura H, Weisz A, Kurashima Y, et al. Hypoxia response element of the human vascular endothelial growth factor gene mediates transcriptional regulation by nitric oxide: control of hypoxia-inducible factor-1 activity by nitric oxide. *Blood*. 2000;95(1):189–197.
39. Sandau KB, Fandrey J, Brune B. Accumulation of HIF-1 $\alpha$  under the influence of nitric oxide. *Blood*. 2001;97(4):1009–15.
40. Liu X, He L, Stensaas L, et al. Adaptation to chronic hypoxia involves immune cell invasion and increased expression of inflammatory cytokines in rat carotid body. *Am J Physiol Lung Cell Mol Physiol*. 2009;296(2):L158–L166.
41. Hollenberg SM, Cunnion RE, Parrillo JE. The effect of tumor necrosis factor on vascular smooth muscle. In vitro studies using rat aortic rings. *Chest*. 1991;100(4):1133–1137.
42. Baudry N, Vicaut E. Role of nitric oxide in effects of tumor necrosis factor-alpha on microcirculation in rat. *J Appl Physiol*. 1985; 75(6):2392–2399.
43. Wright HL, Moots RJ, Bucknall RC, et al. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology (Oxford)*. 2010;49(9):1618–1631.
44. Baudry N, Danialou G, Boczkowski J, et al. In vivo study of the effect of systemic hypoxia on leukocyte-endothelium interactions. *Am J Respir Crit Care Med*. 1988;158(2):477–483.
45. Kong T, Eltzschig HK, Karhausen J, et al. Leukocyte adhesion during hypoxia is mediated by HIF-1-dependent induction of beta2 integrin gene expression. *Proc Natl Acad Sci*. 2004;101(28):10440–10445.