Full Title

CD43Lo classical monocytes participate in the cellular immune response to isolated primary blast lung injury

Running Title

CD43Lo classical monocytes in primary blast lung injury

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Background

Explosive blast trauma occurs by four mechanisms: primary injuries are due to the isolated effect of the blast wave on the human body, secondary injuries are due to damage from fragments energised from the explosion, tertiary injuries result from acceleration of the body against an object or through the explosion energising hard materials in a solid form (not fragments) and encroaching upon the person, and quaternary injuries describe other physical insults, including burns and smoke inhalation [1].

Gas-containing organs such as the lungs are particularly susceptible to barotrauma, and 'Primary Blast Lung Injury' (PBLI) is a commonly reported injury amongst blast casualties. A review of terrorist bombings worldwide found the incidence of PBLI in immediate fatalities to be as high as 47% [2]; whilst in a review of military blast casualties that survived to emergency admission, PBLI was found in 11.2% of 648 blast casualties. In this series, 16.2% of mounted and 17.1% of dismounted injuries developed PBLI, which was significantly associated with increased mortality [3,4].

Laboratory investigations of PBLI have sought to recapitulate the injury under experimental conditions. Studies investigating the subsequent immune response have been reported most commonly in rats [5-10], mice [11-15] and swine [16,17]. These studies have offered overwhelming evidence of the neutrophil response to PBLI in animal models, with increases in circulating and broncho-alveolar lavage fluid (BALF) neutrophils, increases in Myeloperoxidase (MPO) activity in the lung, and neutrophil staining by histology [9-12,14,18,19]. However, the immune response to tissue damage differs depending on the type of organ injured and their local physiology; it remains unclear what specific contribution blast injuries to different parts of the body make to the inflammatory response that ensues. Whilst most clinically blast injured patients are polytraumatised, discerning these interacting immune responses experimentally is necessary for translational studies seeking to better understand, monitor or attenuate the immune response in blast trauma.

Further, knowledge of the role played by other immune cells to PBLI is limited – no study has characterised the full repertoire of immune cells in PBLI; specifically, the involvement of monocytes in PBLI remains unclear. These cells are frequently recruited to sites of inflammation; in humans they are characterised by differential CD14 and CD16 expression [20], in mice by CCR2, CX3CR1 and Ly6C [21,22] and in rats by CD43 alone or in conjunction with His48 [23]. In rats, CD43 Hi and Lo monocytes are considered to be analogous to the Ly6C Lo (Non-classical) and Ly6C Hi (Classical) murine monocytes respectively. Classical Ly6C Hi monocytes are recruited to tissues in sterile [24] and infectious [25] models of inflammation. There, they can differentiate into inflammatory macrophages and dendritic cells, amplifying or resolving inflammation. In rats, circulating monocyte subsets are activated by inflammatory stimuli [26], respond differentially to sterile danger signals sub-acutely [27], and have been shown to migrate to the lung in LPS-induced pulmonary inflammation [23]. Moreover, there is growing evidence that inflammation to localised trauma may 'spill-over', causing immune effects at uninjured distal organs. This has been documented in models of blast limb trauma [28,29] and PBLI has been associated with immune changes in the spleen [15,30].

Accordingly, the aim of this study was to utilise a rodent model to investigate the cellular immune response elicited from an isolated primary blast lung injury alone. We hypothesized

that an isolated PBLI would elicit an acute monocytic - in addition to neutrophilic - response; we sought to delineate whether this monocyte response was subset selective; if it occurred in the blood and or lung, and if so at what acute time points; additionally, we examined the profile of immune cells in liver and splenic tissue 6hr after injury to investigate the sub-acute distal organ effects of PBLI.

Methods

Animals and Licence

Sprague-Dawley female rats (200-280g) were purchased from Charles River (Kent, UK) under Home Office licence. Food and water were supplied ad libitum. U.K. Home Office guidelines for animal welfare were strictly observed. This study was performed under full institutional and departmental licence with ethic committee and Home Office approval. Euthanasia was performed under license by overdose intraperitoneal (I.P.) injection of pentobarbitone.

Isolated PBLI Model

Blunt thoracic trauma was induced using a compressed air driven shock tube (Figure S1) as detailed in the supplementary. A purpose-built subject support (Figure S2) bolted onto the outlet flange of the shock tube was used to expose only the thorax to a single focal shock wave. No part of the rat was inside the shock tube at any time. In this study a burst pressure of 14 bar was utilised delivering a peak pressure at the chest of ~130kPa. Polyethylene inserts enabled driver volume to be reduced to 10% of maximum; producing a short-duration shock wave (Figure S3). Full shock tube characterisation and rig details are included in the supplementary.

Procedure and Physiological Measurements

Rat physiology was recorded using a MouseOx system (Starr Life Sciences Corp, USA) in accordance with manufacturer's instructions. Rats were anaesthetized with I.P. injection of 60 mg/kg ketamine and 0.20 mg/kg medetomidine and a single shock wave was applied to the chest. Shams did not receive a shock wave but were otherwise treated identically. After the procedure rats were injected with atipamezole I.P. to reverse sedation and were administered buprenorphine analgesia sub-cutaneously.

Following atipamezole injection animals regained consciousness in under 10 mins, with no difference in recovery time between sham and blasted rats. Physiological measurements were made 1 min and 5 min after anaesthesia, 1 min and 5 min after shock wave application, immediately after recovery and 3hr and 6hr thereafter.

Tissue collection

Unless otherwise stated, centrifugations were performed at 4ºC and media used throughout was Roswell Park Memorial Institute medium (RPMI) (Sigma-Aldrich) containing 10% Fetal Bovine Serum (FBS) (Invitrogen, USA). After pentobarbitone overdose, blood was collected in citrated tubes on ice from the right femoral vein and centrifuged at 1300g for 10 mins. Each sample was obtained from an individual subject, there were no repeat samplings of tissue or blood in this study. Plasma was collected and stored at -80ºC for further analysis. Whole lungs were lavaged twice using 3mL of ice cold RPMI each with a 30 second pause before collection, in order to gain a representative sample of the total lung environment, as performed previously [23]. Volume and duration of lavage were standardised across all samples to ensure consistent Broncho-alveolar lavage fluid (BALF) concentrations and lung tissue cytokine measurements. BALF was centrifuged at 800g for 5 mins, supernatant was stored at -80ºC and cells were resuspended on ice. For FCM tissue analysis: right inferior lung lobes, superior caudate liver lobes and spleen were collected in media on ice.

Tissue processing

Lung and liver lobes were cut by scissor into smaller fragments and suspended in media containing tissue digestion enzymes; DNAse I 5mg/mL, Collagenase D 30mg/mL and Dispase II 5mg/mL (all from Roche, Switzerland). Tissue was incubated in digestion buffer in a water bath with shaking at 37ºC for 30 minutes. After, liver cells were resuspended in 36% Percoll (Sigma-Aldrich) [31,23] and centrifuged for 10mins at 500g without break to separate leukocytes from fibrous tissue. Spleen was cut into smaller fragments but not subjected to enzymatic digestion. All tissues were strained using a 40μM nylon mesh (Fisher Scientific, USA). Red blood cells were lysed using Ammonium-Chloride-Potassium buffer as previously described [23]. Cell pellets were resuspended and viable cells counted using Trypan blue staining solution on a haemocytometer (Nikon, Tokyo Japan). Liver (Right Lateral Lobe) and lung (left lobe) were harvested and fixed for 24hrs in 10% Buffered Formalin. Paraffin-embedded sections (4 μm) were stained with hematoxylin and eosin (H&E). Images of the slides were captured using a light microscope (Leica, Germany). Cytospins were performed on glass covered slips stained with Wright-Geimsa stain (Sigma-Aldrich).

Flow cytometry

Flow cytometry was conducted based on staining protocols and methods reported previously [23]. Briefly, cells were stained with antibodies as detailed in Table S1; washed and stained with Live/Dead dye (eBioscience, USA) in PBS, before blocking with anti-CD32 and staining with antibodies in buffer containing PBS, Bovine Serum Albumin (1%) (BSA) and Sodium Azide (0.1%). Cells were stained with antibodies at concentrations stated in Table S1, incubated at 4ºC for 30mins then washed and fixed (BD Cell Fix) before analysis using a multi-parameter flow cytometer (Fortessa LSR BD Biosciences, USA). Compensation was performed using fluorescent beads (OneComp eBeads, eBioscience).

ELISA

BALF was thawed and analysed using a multiplex ELISA (MesoScaleDiscovery Maryland, USA) according to the manufacturer's instructions for IL-6, TNF-α, IL-1β , IFN-y and CXCL-1. BALF Albumin and MCP-1 were both analysed by ELISA (Bethly Laboratories, USA and eBioscience respectively) according to manufacturer's instructions. In both instances, ELISA plates were allowed to develop and absorbance measured on a spectrophotometer (Tecan, Switzerland).

Data & Statistics

In this study, a total of 39 rats were used across the sham and blast groups (Table 1). Where duplicate measures were taken, their means were first calculated and then included as a single measurement in further analysis (technical replicate); all data were collected from 2-3 separate individual experiments (biological replicate) and - unless otherwise stated - expressed as median ± 25% and 75% quartiles. Flow cytometric data were analysed using FlowJo v7.6.5 (Tree Star Inc, USA). Unless otherwise stated, statistical data analysed using a non-parametric Mann Whitney t-test in GraphPad Prism v5 (San Diego, USA), *$p<0.05$, **$p<0.01$. Except for physiological data, individual animals were assayed for each time point.

Results

Physiology

Subject survival was 100%. As shown in Figure 1, rats subjected to a single shock wave experienced a transient bradycardia at 1 min (Figure 1A) (258bpm ± 10.30, $p<0.05$) which had returned to sham levels by 5 min. Rats recovered oxygenation (Figure 1B) by 5mins after PBLI and showed a mildly increased respiratory rate (Figure 1C) compared to shams throughout the study period ($p>0.05$).

Pulmonary barotrauma and inflammation

Histological hallmarks of pulmonary barotrauma were observed including free erythrocytes as a result of alveolar haemorrhage together with evidence of neutrophil and monocyte cell infiltration into the tissue (Figure 2A,B). There was no evidence of abdominal trauma in subjects with PBLI by liver histology (data not shown). To assess whether an inflammatory response had occurred in the lung and to support the validity of the PBLI initiated, BALF proteins were measured. Rises were observed in CXCL-1, IL-6 and TNF-α in the BALF at 3hr ($p>0.05$) (Table 2). We did not detect any significant increases in IFN-γ or IL-1β cytokines in the BALF or plasma; we observed a three-fold and two-fold increase in plasma high mobility group box 1 protein (HMGB1) and Heat-shock protein 70 (HSP70) respectively at 6hrs ($p=0.2$) (data not shown).

Cellular immune response

An increase in circulating neutrophils was observed in rats with PBLI (Figure 3A). Compared to shams, at 1hr this increase was three-fold ($p<0.01$) rising to almost five-fold ($p<0.05$) by 6hr. Likewise significant increases were seen in CD43Lo/His48Hi monocytes in the blood at all time points (Figure 3A); additionally, we saw a non-significant rise in total circulating leukocytes across the time course in rats with PBLI. This was most pronounced at 1hr ($p<0.01$) (as illustrated by representative FCM plots in Figure 3B) and remained raised at 3hr ($p<0.05$), beginning to subside by 6hr ($p<0.01$). No significant differences in CD43Hi/His48Int-Lo monocytes, NK, B or T Cells were observed in the blood at any time point following PBLI (Table 3); though we did observe a gradual recovery in the number of circulating lymphocytes by 6hr.

A marked increase in neutrophils in the lung was observed following PBLI (Figure 4A). This increase was three-fold at 1hr ($p<0.01$) as illustrated in Figure 4B and two-fold at 3hr ($p<0.05$) as compared to levels in sham controls at each of these time points. By 6hr it had almost returned to sham levels. Additionally, significant increases were seen in CD43Lo/His48Hi monocyte-macrophages (Figure 4A) which were six-fold higher at 1hr ($p<0.01$) and two-folder higher at 3hr ($p<0.05$) before subsiding to levels comparable to sham by 6hr. No significant differences were found in CD43Hi/His48Int-Lo monocyte-macrophages, NK, B or T Cells throughout the time course (Table 3). A significant increase in BALF neutrophils was seen at all time points post-injury, peaking at 3hr (Figure 5A). We examined whether any delayed changes in BALF monocyte-macrophages occurred at 3 or 6hr; a significant increase in CD43Lo/His48Hi monocyte-macrophages was seen at 3hr (Figure 5B) but neither monocyte-macrophage subset was raised at 6hr (Figure 5B). A significant increase was also observed in BALF MCP-1 concentration at 3hr ($p<0.05$) and 6hr ($p<0.01$) (Figure 5C).

Distal inflammation

No significant differences were seen in immune cell proportions in the liver at 6hr (Figure 6A). However, we observed a trend towards lower neutrophils and NK Cells, and higher CD43Lo/His48Hi monocyte-macrophages and B Cells ($p>0.05$). In the spleen, significant increases in both monocyte-macrophage populations ($p<0.05$) and decreases in CD4 T Cells was observed ($p<0.05$); no significant differences were seen in neutrophil, NK or B Cells. No significant difference was observed in absolute numbers of cells in the spleen between PBLI and sham (data not shown).

Discussion

Model

The aim of this study was to discern the immune response to isolated PBLI - in the absence of other blast injury mechanisms or distal organ insult. In this study we utilised a compressed air driven shock tube and custom designed rig to induce PBLI through reproducible shock wave loading of the chest alone (Figure S2, S3 and Table S3) and not the head and abdomen. Notably, we measured the loading at the chest as well as shock tube outlet; a measure lacking from previous reports of PBLI [32]. Preliminary experiments established that loading the chest with 138 kPa for ~2ms induced a consistently survivable injury. Female rats were used in this study, their immune cell biology is highly comparable to male rats, and demonstrates homology to murine and human immune systems – a prerequisite for further translational research.

Hallmarks of pulmonary barotrauma

PBLI is characterised by several features, including, histological and physiological changes in the cardiorespiratory system. A transient bradycardia (as observed in Figure 1) is frequently reportedly in PBLI and is due to a vagal nerve mediated reflex, which in the absence of further insult resolves rapidly [7,8]. We saw a modest but sustained increase in respiratory rate after PBLI, which may have been compensatory as a result of pulmonary damage and reduced gas-exchange, thereby maintaining peripheral oxygenation in subjects [33] throughout the study period. PBLI causes damage to lung tissue that promotes cellular infiltration, through the destructive effects of stress and shear waves at air-fluid tissue boundaries. In PBLI, studies have shown neutrophil infiltration histologically or by assaying activation markers such as MPO in BALF or lung homogenates [9-12,14,18,19,34]. Our histological data showed marked cellular infiltration and free erythrocytes due to alveolar haemorrhage consistent with previous human and rodent reports [11,35]. Furthermore and in line with previous studies [18,36], we observed increased BALF levels of CXCL-1, IL-6, TNF-α and albumin – an indicator of endothelial and epithelial barrier damage. Collectively these data support the validity of the injury generated in this system resulting in PBLI.

Cellular inflammatory response

This study is the first to directly enumerate7 immune cells using FCM in a rodent model of PBLI. An acute cellular inflammation was observed driven by neutrophils and classical monocytes in the blood and lung, together with increases in BALF neutrophils and monocyte-macrophages.

Neutrophils

PBLI is known to elicit mobilisation of neutrophils from the bone marrow into the blood [9,11,12] and promote their activation [19,37]. We saw significant increases in neutrophils in the blood across all time points, which may in part be driven by circulating chemokines and complement products previously reported to be elevated in PBLI [12,19,36,37]. Additionally, we observed a rapid increase in neutrophils in the lung at 1 and 3hr, returning to baseline by 6hr. Blast damage to blood vessels leads to their rupture and leakage of vascular contents into the parenchyma as seen in this study; it has been shown that neutrophils can attach to these red blood cells in haemorrhagic foci [38].

Monocyte-Macrophages

Understanding of the role of other immune cells in PBLI is limited, with few studies examining the monocyte response. Those which have show isolated monocytes 4hr post-injury express higher levels of CCR2 mRNA compared to sham, and exhibit reduced migratory potential *ex-vivo* to chemoattractants [18]. Another study by the same authors, using a combined model of PBLI and haemorrhage showed reduced pro-inflammatory cytokine production from peripheral blood mononuclear cells (PBMCs) *ex-vivo* after combined insult at 20hr [15], whilst another study at 2hr showed increased cytokine production from peripheral blood mononuclear cells [39].

To determine the still unclear nature of the monocyte response to PBLI *in vivo*, we used FCM to examine changes in the two circulating rat monocyte populations. To the best of our knowledge, we report for the first time a significant and sustained response of CD43Lo/His48Hi (classical) circulating monocytes to PBLI in the rat. These cells were raised as early as 1hr after insult and only began to subside by 6hr. Interestingly, this increase was not seen amongst the CD43Hi/His48Int-Lo (non-classical) monocyte subset. We found increases in plasma MCP-1 in PBLI rats compared to shams at all time-points, however this only reached significance at 6hr (data not shown). In a model of primary blast limb injury, CD43Lo but not CD43Hi monocytes were raised in the blood of rats subjected to prolonged duration blast waves [40].

A study of PBLI in mice suggested increases in BALF mononuclear cells as evidence for monocyte migration to the lung [18]. Alongside marked changes in circulating cells, we found a rapid and significant increase in CD43Lo/His48Hi monocyte-macrophages at 1hr in the lung compared to shams (Figure 4A, B), which had all but subsided by 6hr. This increase could be in part due to an accelerated recruitment by cytokines and chemokines such as CINC-1, MIP-2 and enhanced ICAM-1 expression [18,34,36,37] or damage products [41], which we found raised in the plasma at 6hr of PBLI rats (data not shown). There was a significant increase in CD43Lo/His48Hi monocyte-macrophages at 3hr in the BALF supporting our correlate findings in the lung, together with significant increases at 3 and 6hr in the monocyte-macrophage chemoattractant MCP-1.

A study of PBLI in mice found no ameliorating effect of neutrophil depletion on acute lung injury and suggested monocytes (without subset discrimination) – which were raised in the blood - may be implicated in PBLI pathogenesis [37]. The elevation of CD43Lo monocytes in the blood and CD43Lo monocyte-macrophages in the lung and BALF seen in this study suggest they – like their murine Ly6+ equivalents - may play key effector rolls in responding to sterile as well as infectious inflammatory stimuli; indeed CD43Lo monocyte-macrophages have been reported in significantly increased numbers in the lung at 3 and 24hr in LPS-induced pulmonary inflammation [23] and participate from the luminal side of the alveolar-capillary boundary as well as from within the parenchyma during lung allograft rejection [42].

Other immune cells

Because of the lack of data on other immune cell types in PBLI, and evidence to suggest NK Cell [43], B Cell [44], CD4+ [45] and CD8+ [46] T Cell involvement in trauma, we examined their changes in the circulation and lung in our model. There were no significant changes in the blood or lungs of these cells, supporting the view that PBLI releases damage mediators with specific- rather than pan-immune cell effects. Notably, these data highlight the importance of enumerating individual immune cell populations in blast injury studies; as lymphocytes

predominate the circulating immune cell pool in rats[23], assaying of total leukocytes numbers in the blood may not always rise significantly, even in the presence of significant circulating neutrophil or monocyte responses. Moreover, direct enumeration guards against misinterpretation of inflammation assays such as MPO – which is highly expressed by classical monocytes [47] as well as neutrophils.

Distal organ immune cells

Evidence suggests distal organs may undergo delayed inflammatory changes following PBLI. Hepatic Kupffer cells are thought to become primed to release pro-inflammatory cytokines shortly after PBLI [36]. In a combined model of PBLI and femoral fracture, significant increases in lung and hepatic neutrophil infiltration were observed, peaking at 6hr [48]. In rodent models of PBLI, splenic immune cells demonstrate supressed cytokine release [30] which may be responsible for diminished survival following a septic 'second-hit' [39]. Yet, another study of PBLI found no effect on splenocyte cytokine release unless in the combined presence of neutrophil depletion [37], suggesting a neutrophil specific role for splenic suppression.

In light of these varied and conflicting findings we sought to characterise *in vivo* the respective proportions of immune cells in the liver and spleen 6hr post-PBLI (Figure 6). In the liver we saw no significant changes in immune cells. In the spleen we saw significant increases in the proportion of CD43Lo/His48Hi and CD43Hi/His48Int-Lo monocyte-macrophages, and significant decreases in CD3+CD4+ T cells. Collectively these data demonstrate for the first time the immune cell environment of two major distal organs in PBLI. This splenic data is timely given recent studies in mice show the spleen can directly give rise to Ly-6C+ monocytes (analogous to CD43Lo rat monocytes) that respond to sterile inflammation [49]. Further understanding of the contribution of different tissues and organs to the immune response in PBLI is needed.

Clinical implications and limitations

Blast exposed patients may present with myriad clinical injuries or symptoms, many of which could appear benign and thereby complicate simple and rapid triage during mass-casualty events. Anecdotal surrogates for PBLI such as tympanic membrane perforation are not supported by evidence [50]. It is important to identify patients that have suffered PBLI even if the injury doesn't appear immediately life threatening. This study offers evidence to suggest CD43 Lo classical monocytes – in addition to neutrophils – can be detected in the blood or BALF acutely after PBLI; and could offer clinicians an additional triage tool to identify those at potential risk of deleterious systemic immune activation or in need of further monitoring. Importantly, the immune response in a real-world clinical environment will be additionally impacted by interacting blast injury mechanisms and damage to distal organs, as well as the immune response to therapeutic intervention such as blood transfusion. A limitation of this study is that it does not examine quantitatively whether circulating or BALF immune cells correlate with injury severity; this should be a focus of further research.

Conclusion

In summary, this study shows that isolated PBLI leads to a rapid increase in circulating neutrophils and – for the first time - CD43Lo/His48Hi monocytes. These cells are also elevated in the lung at 1 and 3hrs but return to baseline levels by 6hrs. We show specific monocyte-

macrophage immune cell changes in the spleen but not liver 6hr after injury. The methodology employed in this study adds clarity to the varied roles played by immune cells in PBLI across several tissues. Neutrophils and classical monocytes may serve as tools in translation studies to assess blast injured subjects or patients, including those considered susceptible to systemic immune activation or 'second-hits' such as infection.

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Statement of authorship
ABV and SR designed the research; ABV, AS, TE, HA conducted the experiments; HA, WM, AB, SR offered technical experimental guidance and assistance; ABV, TE, HA, AS analysed the data; ABV and SR wrote the paper with input from AS, TE, HA, WM, AB.

Figure 1 Physiological effects of shock wave induced PBLI

Legend

Physiological measurements after sham (——) or PBLI (------). Rats were anesthetized and subjected to a single shock wave or sham procedure. An infra-red neck collar Sensor was used to measure heart rate (A), arterial oxygen saturation (B) and respiratory rate (C). Recordings were taken at 1 min and 5 min after induction of anaesthesia, 1 min and 5 min after PBLI, after recovery when rats were awake, and 3hr and 6hr thereafter. Peri-anaesthesia n= 9-19; at 3hrs n=6-11; at 6hrs n=3 from 2-3 independent experiments presented mean ± SEM using unpaired t-test where *$p<0.05$.

Figure 2 Histological changes in the lung after injury

Legend

Histological imaging of lungs after sham or PBLI. Left lung lobes were fixed in formaldehyde, embedded in wax, cut and stained with H&E. Images are representative obtained from a light microscope at 10X (A), 40X or 100X (B) magnification. Images are representative.

Figure 3 Neutrophils and CD43Lo/His48Hi Monocytes are elevated in the blood after PBLI

Legend

Total cells, neutrophils and CD43Lo/His48Hi monocyte levels (A), representative flow cytometry (FCM) images (B) in the blood at 1, 3 and 6hr after sham (-) or PBLI (+). Cells were distinguished and enumerated by 9-colour FCM. Data are: at 1hr n=7-8, at 3hr n=6-7, at 6hr n=5, in each group from 2-3 independent experiments presented as median ± 25^{th} and 75^{th} percentile where *$p<0.05$,**$p<0.01$.

Figure 4 Rapid recruitment of Neutrophils and CD43Lo/His48Hi Monocyte-Macrophages to the lung

Legend

Fold changes in total neutrophils and CD43Lo/His48Hi monocyte-macrophages (A), representative flow cytometry (FCM) images (B) in the lung at 1, 3 and 6hr all compared to time point specific shams. Cells were distinguished and enumerated by 9-colour FCM. Data are: at 1hr n=7-8, at 3hr n=6-7, at 6hr n=5, in each group from 2-3 independent experiments, presented as median fold change over sham-specific time point group ± 25th and 75th percentile where *$p<0.05$,**$p<0.01$.

Figure 5 Cellular BALF changes after PBLI

Legend

Total neutrophils and neutrophils as percentage of total cells (A) in BALF at 1, 3 and 6hr; total CD43Lo/His48Hi monocyte-macrophages (B) 3 and 6hr; concentration of MCP-1 in the BALF at 1, 3 and 6hr (C) after sham (-) or PBLI (+). Cells were distinguished and enumerated by FCM. Cellular data are: total cells in each group at 1hr n=5, 3hr n=6-7, 6hr n=5-6; neutrophils in each group at 1hr n=7-8, at 3hr n=6-7, at 6hr n=5-6; CD43Lo and CD43Hi monocytes in each group at 3hr n=6, at 6hrs n=2-3. MCP-1 ELISA data in each group are: 1hr n=3-5, 3hr n=5, 6hr n=5 from 2-3 independent experiments run in duplicate. All data presented as median ± 25th and 75th percentile where *$p<0.05$, **$p<0.01$. Summary error measures are not presented where $n<=3$.

Figure 6 Immune cells in distal tissues at 6hr

Legend

Proportions of neutrophils, CD43Lo/His48Hi monocyte-macrophages, NK, B and T cells of total cells in Liver (A) and Spleen (B) at 6hr after sham (-) or PBLI (+). Cells were distinguished and enumerated by FCM. Liver data in each group are n=5-6; spleen data in each group are n=4-6; both from 2-3 independent experiments presented as median ± 25th and 75th percentile where *$p<0.05$, **$p<0.01$.

Table 1: Study groups and subjects

Legend

Subjects included in this study at each time point.

Table 2 Pulmonary inflammatory changes in the BALF

Legend

CXCL-1, IL-6 and TNF-α cytokines and albumin in the BALF at 1, 3 and 6hr after sham or PBLI. Multiplex ELISA data in each group: CXCL-1, IL-6 and TNF-a all n=4 in duplicate from 2-3 independent experiments at all time points; Albumin data in each group: at 1hr n=5, 3hr n=6-7, 6hr n=5-6, all in duplicate from 2-3 independent experiments. Data presented as median ± 25th and 75th percentile where *$p<0.05$,**$p<0.01$.

Table 3 Immune cells in the blood and lung

Legend

CD43Hi/His48Lo monocyte-macrophages, NK, B and T cells at 1, 3 and 6hrs in the blood and lung. Data are: at 1hr n=7-8, at 3hr n=6-7, at 6hr n=5, in each group from 2-3 independent experiments presented as median ± 25^{th} and 75^{th} percentile where *$p<0.05$,**$p<0.01$. Lung data is shown as a ratio to time-specific sham groups. CD8 data were unobtainable in sufficient numbers at 6hrs and are not included.

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Figure 1

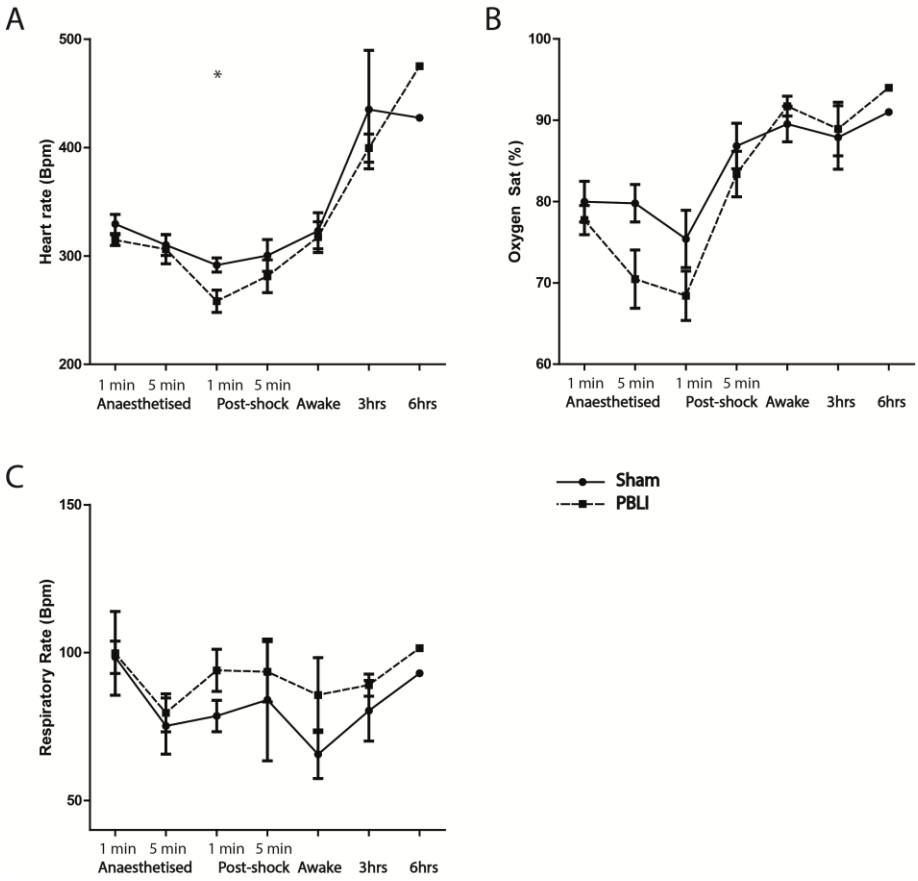

A
Heart rate (Bpm)
500
400
300
200
*
1 min
5 min
1 min
5 min
Anaesthetised
Post-shock
Awake
3hrs
6hrs
B
Oxygen Sat (%)
100
90
80
70
60
1 min
5 min
1 min
5 min
Anaesthetised
Post-shock
Awake
3hrs
6hrs
C
Respiratory Rate (Bpm)
150
100
50
1 min
5 min
1 min
5 min
Anaesthetised
Post-shock
Awake
3hrs
6hrs
Sham
PBLI

Figure 2

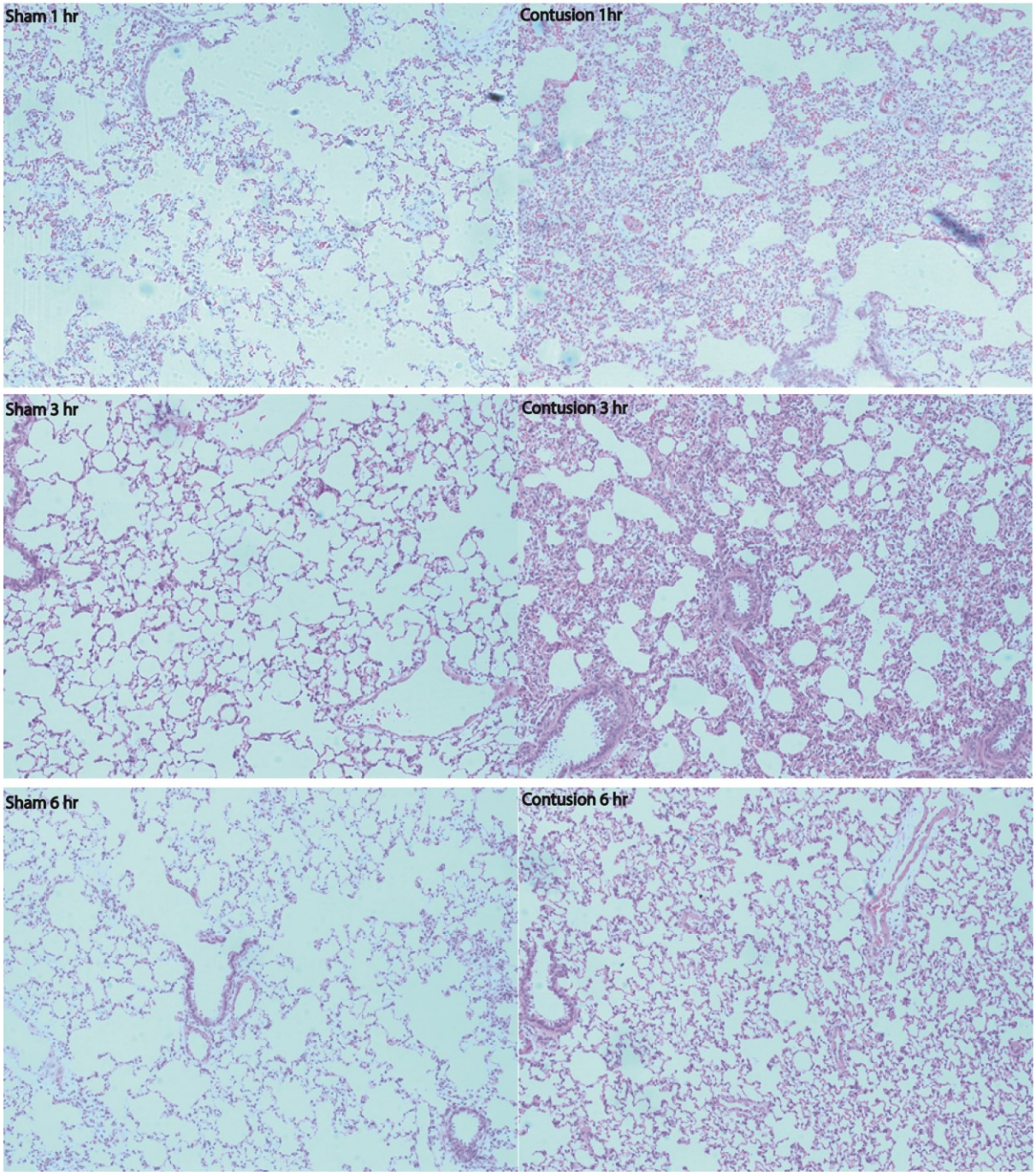

Sham 1 hr
Contusion 1hr
Sham 3 hr
Contusion 3 hr
Sham 6 hr
Contusion 6 hr

Figure 2b

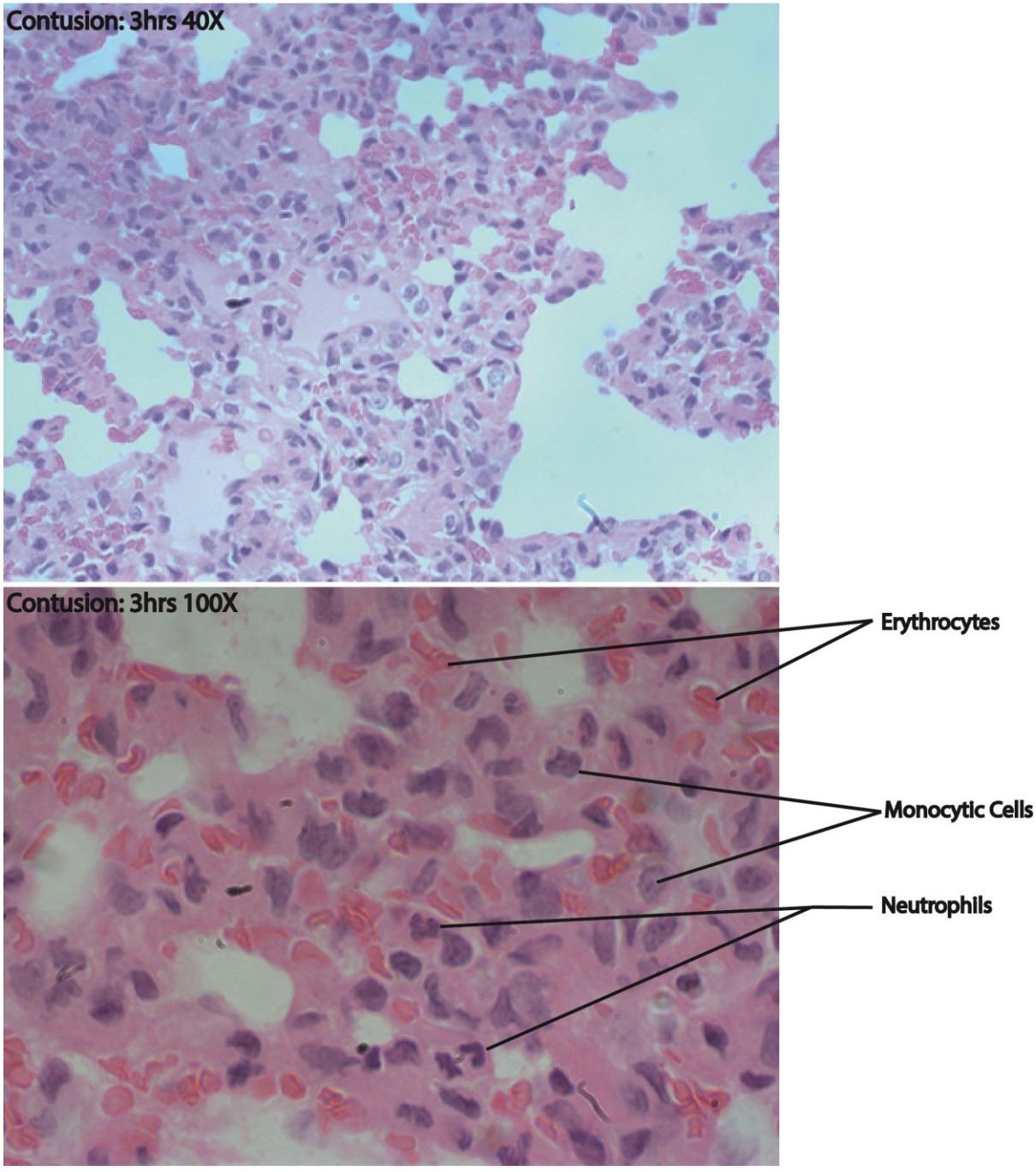
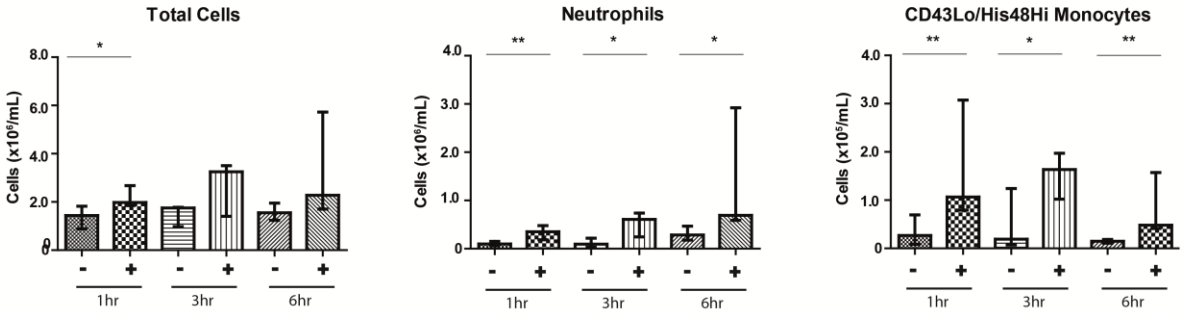

Contusion: 3hrs 40X
Contusion: 3hrs 100X
Erythrocytes
Monocytic Cells
Neutrophils

Figure 3

A


Total Cells
Neutrophils
CD43Lo/His48Hi Monocytes
Cells (x10⁶/mL)
1hr
3hr
6hr

B

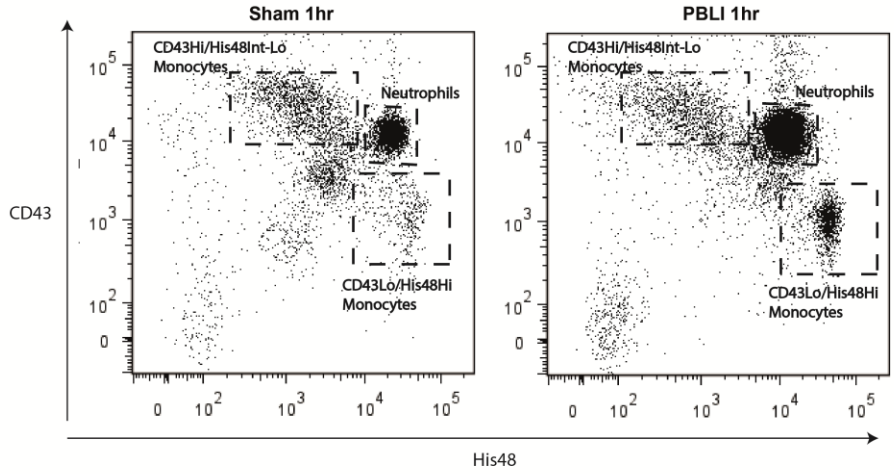
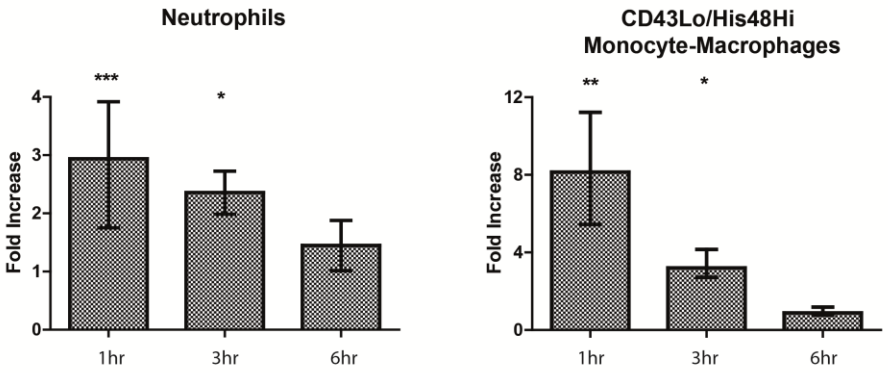

Sham 1hr
PBLI 1hr
CD43Hi/His48Int-Lo Monocytes
Neutrophils
CD43Lo/His48Hi Monocytes
CD43
His48

Figure 4

A


Neutrophils
CD43Lo/His48Hi
Monocyte-Macrophages
Fold Increase
***
*
**
*
1hr
3hr
6hr

B

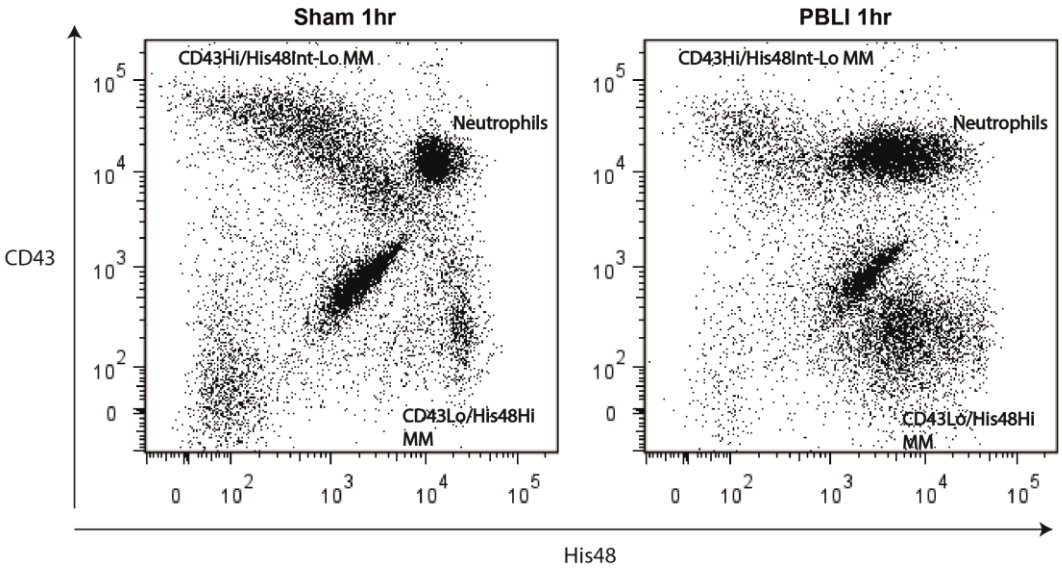

Sham 1hr
PBLI 1hr
CD43Hi/His48Int-Lo MM
Neutrophils
CD43Lo/His48Hi
MM
CD43
His48

Figure 5

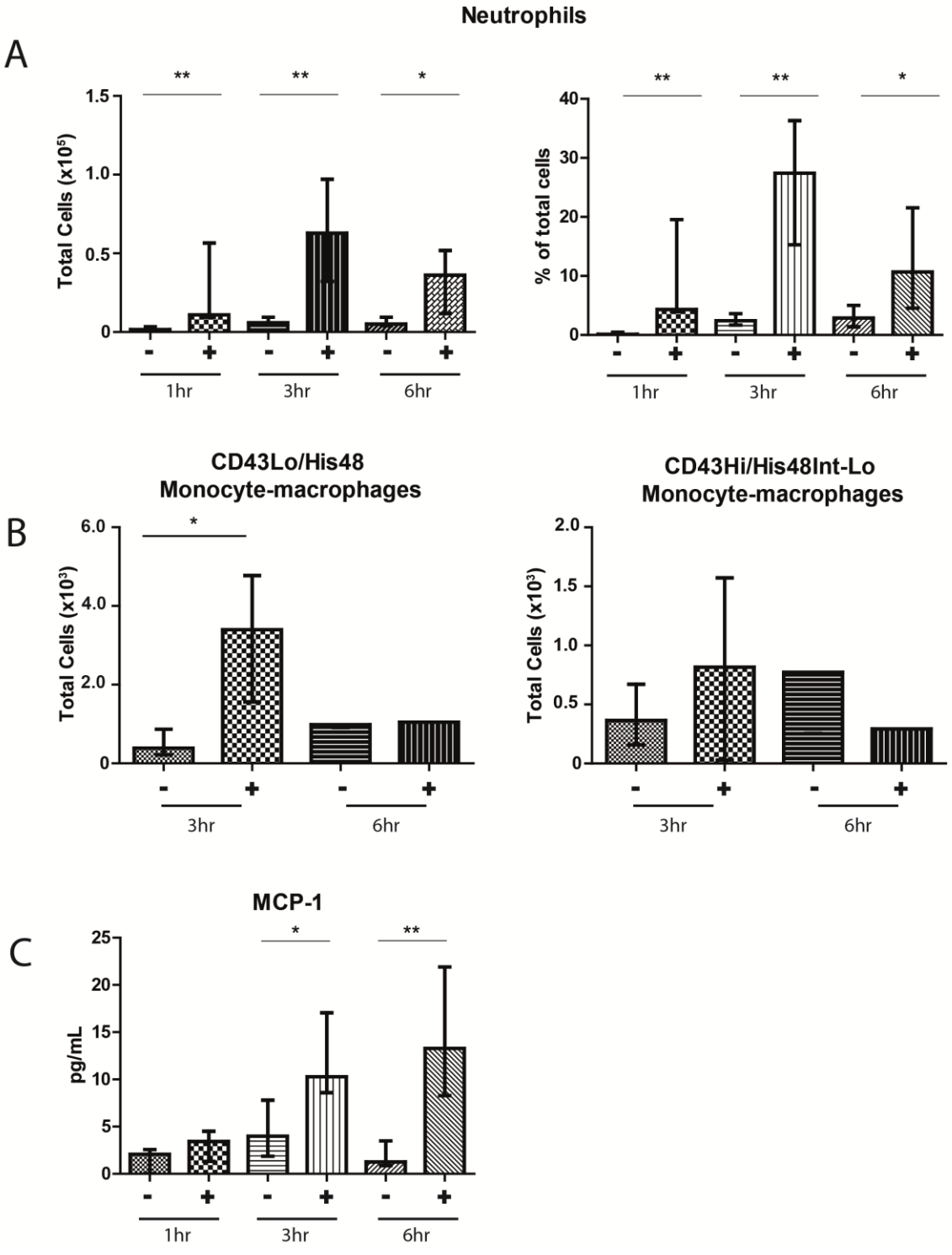

A
Neutrophils
Total Cells (x10⁵)
% of total cells
1hr
3hr
6hr
B
CD43Lo/His48
Monocyte-macrophages
CD43Hi/His48Int-Lo
Monocyte-macrophages
Total Cells (x10³)
C
MCP-1
pg/mL

Figure 6a

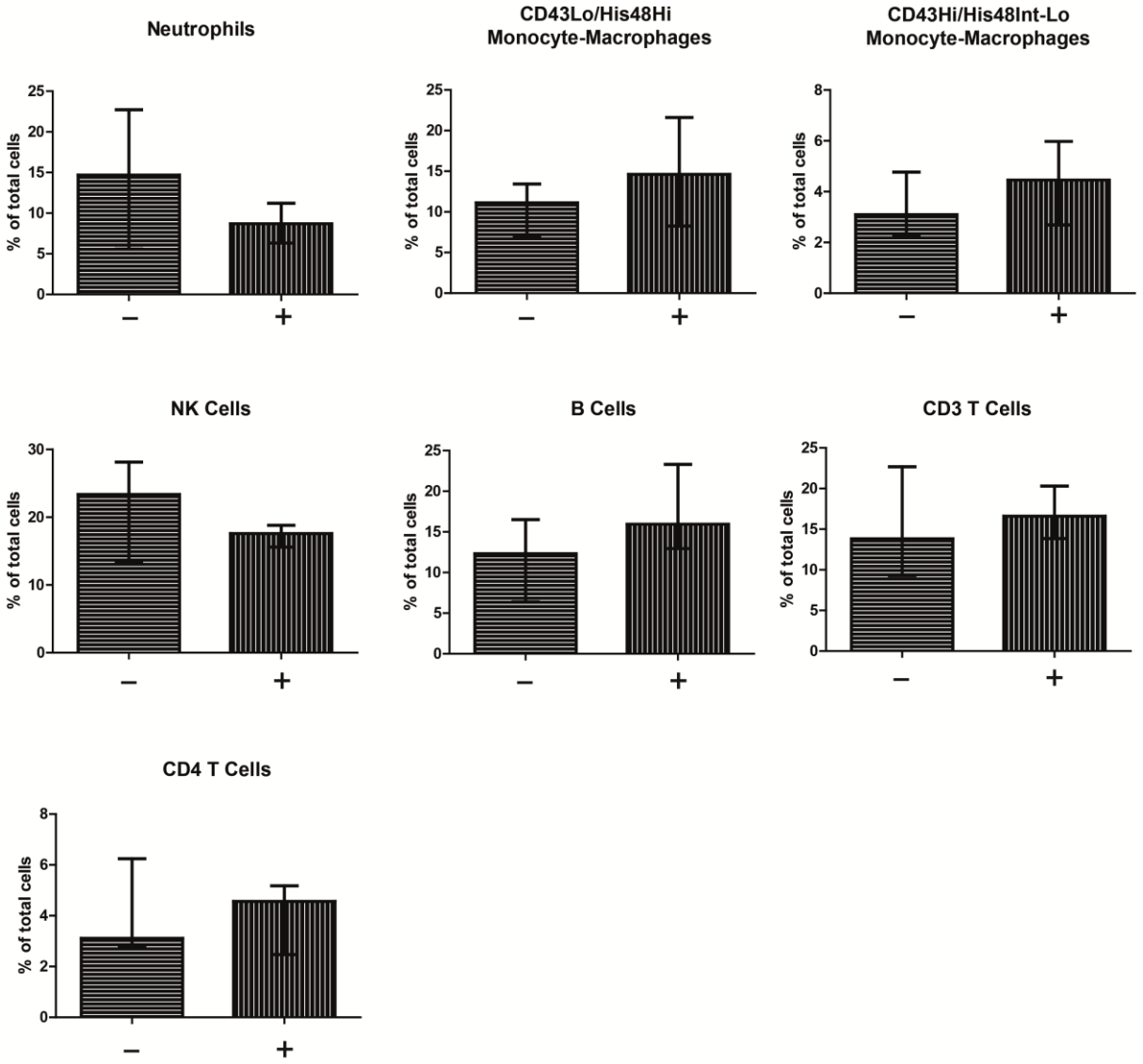
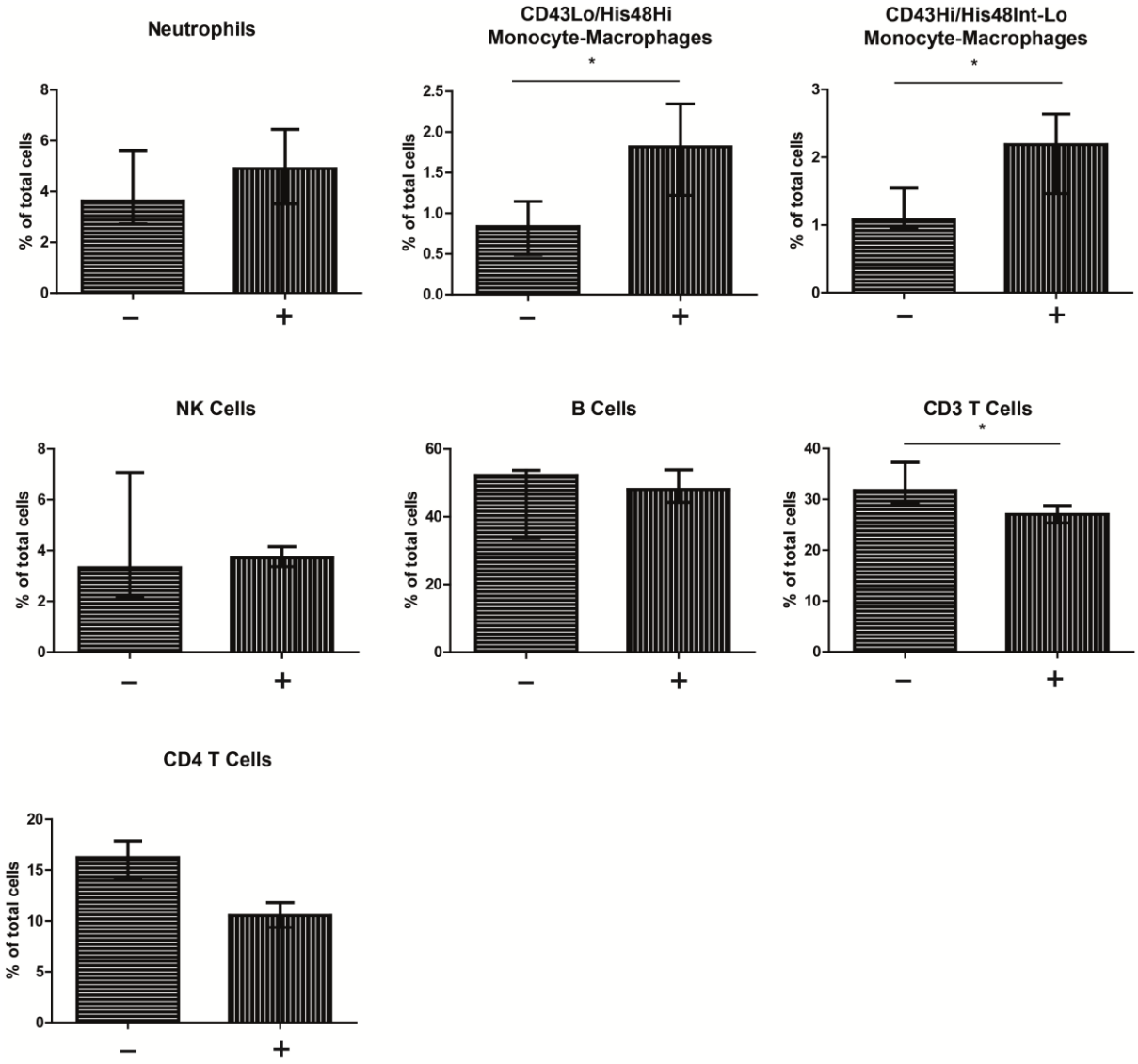

Neutrophils
CD43Lo/His48Hi Monocyte-Macrophages
CD43Hi/His48Int-Lo Monocyte-Macrophages
NK Cells
B Cells
CD3 T Cells
CD4 T Cells
% of total cells
–
+

Figure 6b


Neutrophils
% of total cells
0
2
4
6
8
–
+
CD43Lo/His48Hi
Monocyte-Macrophages
*
% of total cells
0.0
0.5
1.0
1.5
2.0
2.5
–
+
CD43Hi/His48Int-Lo
Monocyte-Macrophages
*
% of total cells
0
1
2
3
–
+
NK Cells
% of total cells
0
2
4
6
8
–
+
B Cells
% of total cells
0
20
40
60
–
+
CD3 T Cells
*
% of total cells
0
10
20
30
40
–
+
CD4 T Cells
% of total cells
0
5
10
15
20
–
+