Life in the slow lane: a multi-omics approach to molecular adaptations in hibernating Syrian hamster liver
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DOI:
10.33612/diss.837297894

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2023

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 5

Phase specific suppression of neutrophil function in hibernating hamster

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Developmental & Comparative Immunology Volume 119, June 2021, 104024
ABSTRACT

Here, we studied the effect of hibernation on neutrophil functionality, by analyzing pathogen binding, phagocytosis and oxidative burst in neutrophils from aroused and summer euthermic animals. Hibernation consists of periods of torpor, characterized by a profoundly lowered metabolism and almost complete clearance of circulating leukocytes with neutrophils residing marginated to the vessel wall, interspersed by brief periods of arousal with restoration of metabolism and numbers of circulating leukocytes to levels not different from summer euthermic levels. In addition to changes in neutrophil circulation, the function of the innate immune system is reduced during hibernation, although the effects on neutrophil function are hitherto undocumented. We demonstrate a profoundly reduced neutrophil function in early arousal in hibernating Syrian hamsters. Neutrophil function was fully restored by incubating cells in plasma from summer euthermic animals, signifying that inhibition of neutrophil function in early arousal is likely mediated by an inhibitory plasma factor. Our results expand fundamental knowledge about the hibernators’ immune system. Identification of the inhibiting factor may be of relevance for the modulation of neutrophil function in humans with relevance to both infections and auto-inflammatory diseases.
INTRODUCTION

Hibernation is an energy-conserving state that promotes survival during periods of food scarcity, which consists of periods of torpor with a low metabolism and a reduction in body temperature that are interspersed by brief periods of euthermia, termed arousal(1). Because these conditions are similar to ischemia reperfusion injury, one might stipulate torpor/arousal cycles to cause organ damage, yet no evidence of organ damage is found following arousal. One of the hibernation protective mechanisms is a profound change in the immune system(2). The reduction in body temperature during torpor governs a lowering of the number of circulating leukocytes(3,4), reduces lymphocyte proliferation(5) and inhibits antibody production(6,7). The reduced number of circulating neutrophils during torpor, is rapidly restored in arousal to slightly higher numbers than found in summer euthermic animals(8,9) Moreover, hibernation leads to a reduced innate immune function in torpor as illustrated by absence of a response to challenge with lipopolysaccharide (LPS) up until the next arousal(10).

The effect of hibernation on neutrophil function has not been documented. Neutrophil function is a double-edged sword: while neutrophils play a major role in controlling bacterial and fungal infections, they are also important effectors of organ injury due to the generation of reactive oxygen species, contributing to the induction of organ injury in ischemia/reperfusion and sepsis(11). Since neutrophils rapidly recirculate upon arousal after margination to the vessel wall during torpor (6), we hypothesized that neutrophil function in early arousal is inhibited to protect against organ damage during this vulnerable stage of hibernation. Here, we studied neutrophil function in LPS-induced sepsis in the Syrian hamster during summer euthermia, winter euthermia and arousal, all representing animals at euthermic body temperature.

MATERIALS AND METHODS

Animal experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University Medical Center Groningen. Male and female Syrian hamsters (Mesocricetus auratus; Envigo) were housed at an ambient temperature of 21°C and summer photoperiod (light:dark (L:D) cycle, 14:10 h). Hibernation was induced by shortening the L:D cycle to 8:16 h for 10 weeks, followed by housing at continuous dim light (<5 Lux) at an ambient temperature of 5°C. Animals that did not show signs of torpor were called “winter euthermic”. Torpor-arousal patterns were monitored by movement detectors(12). Hamsters were sacrificed during summer euthermia (SE), winter
euthermia (WE), torpor (>24 h torpor), early arousal (1.5 h after start of arousal; AE) or late arousal (>8 h after start of arousal; AL). To induce endotoxemia, hamsters were injected intraperitoneally with 300 µg/kg LPS in SE, WE, AE (1.5 h after arousal) or AL (5 h after arousal) and sacrificed 3 h later. All animals were euthanized by exsanguination under isoflurane anesthesia (5% in air). In addition to blood serum, EDTA-anticoagulated blood was collected to obtain plasma, neutrophils and cell count measurements using an automated hematocytometer (Sysmex).

**Analysis of gene expression**

First, mRNA was isolated according to the protocol of Nucleospin II (Macherey-Nagel). The following oligonucleotide sequences were used (Biolegio, the Netherlands): TNFα: CCGCATGTGGTTGCTCTACG (forward), TTGACCTCGGCAGCTG (reverse); IL-6: TCTGCAACTCC GGT GAT AC (forward), TGGTGCTCTGAATGACTCTG (reverse); IL-1B: CGGCAGGTGTCAGCTC (forward), GCAGCATAGCCACAGTCAG (reverse); Complement C3: CTGCTGCTATGTCAGCTTC (forward), CCACGGTGCTGCTGCTG (reverse); TLR2: GAGGAAGCCAAGAAAGGCTCC (forward), GAGCTGAAGTGGGAGGTCC (reverse); TLR4: CTGGCTGTCTACTCTTCAAC (forward), GCACATAACAGGTGGCATTC (reverse); LBP: TTGACAGCTCCACTCG (forward), CCCAGGAGAGACTCAG (reverse); CD14: AAGCCGACCTGGGAGGTTC (forward), AGCGTGTTGCTGTTACCTC (reverse). Real-Time PCR was performed using AbsoluteTM qPCR SYBR® Green ROX Mix (Westburg, Leusden, Netherlands). Quantitative real-time PCR was performed at 95 °C for 15 min followed by 40 cycles of denaturing at 95 °C for 15 sec and annealing/ extending at 60 °C for 1 min using Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, CA, USA). Oligonucleotide primers (Sigma Aldrich) were designed using SEcentral, Primer3 and NCBI primerblast and validated by assessing the efficiency, melting- and temperature curves using qPCR. The specificity of the primers was verified by the Standard Nucleotide BLAST on NCBI. All reactions were carried out in duplicate and obtained threshold cycles (Ct) values were averaged.

**Neutrophil isolation**

EDTA anticoagulated blood was centrifuged for 10 min at 1600xg. Plasma was collected, flash frozen in liquid nitrogen and stored at -80°C until further analysis. The remainder of the blood was diluted 1:1 with RPMI medium and pipetted carefully on a Ficoll layer (1/3th of the diluted blood volume; Ficoll-Paque Plus GE Healthcare). After 30 min of centrifugation at 500xg, the upper layers were discarded and the layer containing granulocytes and erythrocytes was collected. Erythrocytes were lysed by cold hypotonic lysis buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA) and
after two times washing in HBSS, the granulocyte fraction containing ±95% neutrophils was resuspended in HBSS. Cell viability was >90% as assessed by Trypan Blue staining.

**Analysis of neutrophil phagocytosis and oxidative burst**

Isolated neutrophils (1x10^7 cells/ml) were incubated in HBSS for 15 min at 37°C with either 10% pooled plasma from SE or AE animals. Subsequently, neutrophils were centrifuged at 60xg for 5 min and supernatant was replaced by *Escherichia coli* (*E.coli*) particles labeled with pHrodo (Thermo Fisher Scientific) in the presence of 10% plasma as described in the results section and incubated at 37°C for 2 h. Fluorescence was measured at an excitation of 560 nm and emission 585 nm. Subsequently, neutrophils were collected and MDA was measured according to the manufacturer’s protocol (Oxiselect TBARS Assay Kit, STA-330, Cell Biolabs).

**Analysis of pathogen binding**

Isolated neutrophils (1x10^7 cells/ml) were incubated in HBSS for 15 min at 37°C with either 10% pooled plasma from SE or AE animals, followed by incubation with 10 µM cytochalasin D for 30 min at 37°C. Then, samples were incubated with 1 µg/ml LPS-FITC for 30 min at 37°C. All samples were fixed in 2% PFA and kept at 4°C protected from light until analysis. Analysis of LPS-FITS binding to neutrophils was performed by flow cytometry on a FACSVerse flow cytometer (BD Biosciences) and analyzed by FlowJo software version 10.4.2 for Windows workstations. Debris and residual erythrocytes were excluded by forward and side scatter patterns.

**Measurement of LPS binding protein and immunoglobulin G in plasma**

Circulating levels of immunoglobulin G (IgG; Abcam) and LPS binding protein (LBP; MyBiosource) were measured by an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocol. In short, 50 µl of diluted sample was incubated with 50 µl antibody cocktail for 1 hour. After washing, samples were incubated with TMB substrate for 5 min. After addition of stop solution absorbance was measured at 450 nm.

**Data analysis**

Statistical analyses were performed using SPSS Statistics 26 (IBM). After verifying normal distribution of the data, a Student’s t-test (two-tailed) was used to analyze the effect of the treatments as compared to control, when two groups were compared. In case of multiple comparisons, a one-way ANOVA with post-hoc Bonferroni was used. A value
of $p < 0.05$ was considered to be significantly different. Data are expressed as mean ± standard error of the mean (SEM). Figures were made using GraphPad Prism 8. The graphical abstract was created with BioRender.com.

**RESULTS AND DISCUSSION**

The immune response to endotoxin is inhibited in the hibernation season

To investigate the functionality of the innate immune system, we injected LPS (300 μg/kg) or saline in Syrian hamsters during different euthermic phases of hibernation (AE, AL), with euthermic (WE, SE) animals serving as control. Injection of LPS similarly increased body temperature in all groups (Fig. 1A, black bars) and reduced the number of circulating leukocytes in SE and AL (Fig. 1B, black bars). Administration of LPS profoundly increased liver expression of TNF-α and IL-1β in all groups, whereas expression of IL-6 was mainly increased in SE, WE and AL, but only minimally in AE. As expected, saline injection caused no differences in body temperature, number of circulating leukocytes or levels of pro-inflammatory cytokines in any group (Fig. 1C–E).

![Fig. 1. Effects of hibernation on endotoxemia. A: Body temperature; B: Circulating leukocyte numbers; C–E: liver mRNA levels of pro-inflammatory cytokines TNF-α (C), IL-1β (D) and IL-6 (E). Bars represent mean ± SEM; one-way ANOVA with post-hoc Bonferroni test. a: $p < 0.05$ compared to corresponding saline control; b: $p < 0.05$ compared to endotoxemia in SE. SE: summer euthermic, WE: winter euthermic, AE: arousal early, AL: arousal late.](image-url)
Under normal conditions, after LPS is recognized by innate immune cells through pathogen recognition receptors (PRRs), signalling pathways such as NF-KB upregulate the transcription, translation and release of inflammatory cytokines IL-6, TNF-α and IL-1β. In turn, these cytokines activate cyclooxygenase 2 (COX2) in peripheral cells, leading to production of prostaglandin E2 (PGE2), which induce fever by stimulating hypothalamic EP3 receptors(13). Our data show a highly differential response to LPS across the groups. LPS provokes a febrile response all groups, suggesting intact pathogen recognition with subsequent production of prostaglandins. Yet, there is a differential regulation of the decrease in the number of circulating leukocytes (absent in WE and AE) and liver expression of IL-6 (inhibited in AE), TNF-α (inhibited throughout winter conditions) and IL-1β (not affected). Inhibition of increases in TNF-α and IL-6 might explain the absence of LPS-induced leukopenia in WE and AE, as they regulate leukocyte recruitment and direct the transition from neutrophil to mononuclear leukocyte infiltration(14). All three cytokines are mainly expressed by Kupffer cells in the liver, but their dynamics differ: while TNF-α and IL-1β are primary response genes, IL-6 is a secondary response gene. Delayed expression of secondary response genes is governed by epigenetic regulation, as these genes have more densely packaged chromatin and require ATP-dependent nucleosome remodelling complexes for their transcription(15). Potentially, this epigenetic regulation underlies the reduced IL-6 expression in response to LPS in AE hamsters. In contrast, primary response genes like TNF-α and IL-1β do not require nucleosome remodelling complexes for their transcription and hence epigenetic regulation is unlikely to explain the reduced TNF-α expression in winter conditions (WE, AE, AL). Lower TNF-α expression in winter conditions implies that modulation of some LPS effects during hibernation are caused by adaptation to winter conditions, rather than being provoked by body temperature or hibernation phase (i.e. torpor, arousal) per se. This is in line with the observation that fever and sickness behaviour are reduced in Syrian hamsters housed under short-day conditions(16,17).

Further, activation of NF-KB induces early, transient and high expression of TNF-α and IL-1β within 4 h. In the case of IL-1β, this early phase is followed by a second phase of relatively low continuous expression (up to 24 h) induced by HIF-1α, while TNF-α does not have a second expression phase(18). The highly upregulated expression of HIF-1α in winter could explain the associated increase in IL-1β expression(19,20).

Thus, the reduced inflammatory response to LPS-induced endotoxemia in hibernation was partly mediated by adaptations to winter conditions, possibly due to epigenetic regulation, and partly regulated by an unknown regulatory pathway that reduced IL-6 production in AE.
Early arousal neutrophils have strongly impaired phagocytic and oxidative burst capacity

To assess changes in neutrophil functionality, we measured their phagocytic capacity using *E. coli* conjugated to a pH-sensitive fluorochrome (pHrodo) and assessed the subsequent oxidative burst by measuring malondialdehyde (MDA) levels in the presence of autologous plasma. AE animals showed a profoundly lowered neutrophil phagocytic capacity and oxidative burst as compared to SE, WE and AL (Fig. 2A and B). Suppression of both phagocytosis and oxidative burst in AE, which are both induced by pathogen binding to a toll-like receptor (TLR) (21), suggests a reduced pathogen recognition, rather than defective intracellular pathogen processing or signaling.

**Fig. 2. Neutrophil functionality is profoundly reduced during arousal.** A: Neutrophil phagocytosis of *E. coli* conjugated to fluorescent-labeled pHrodo, B: Oxidative burst of neutrophils stimulated by *E. coli* measured by malondialdehyde (MDA). Bars represent mean ± SEM; statistical analysis by Student’s t-test (two-tailed). a: p < 0.05 compared to SE; b: p < 0.05 compared to WE. SE: summer euthermic, WE: winter euthermic, AE: arousal early, AL: arousal late.

Recognition of LPS involves binding to LPS binding protein (LBP), an acute-phase protein produced in the liver, and subsequent binding to pattern recognition receptors (PRRs; *i.e.* CD14, TLR-2, TLR-4 and MD2) on the neutrophil membrane (22). MD2 is a small protein that interacts with the ectodomain of TLR4 to form the heterodimer MD2-TLR4, representing the functional LPS-receptor(23). Binding of LPS to LBP augments recognition of LPS almost 1000-fold(24), while further pathogen recognition is facilitated by opsonins, such as immunoglobulins and complement. Binding of LPS to LBP facilitates the transfer of LPS to MD2-TLR4 via CD14(23). To study whether changes in pathogen recognition factors governs the reduced neutrophil function in AE, we measured levels of circulating LBP, major opsonizing factors (*i.e.* IgG, C3) and the expression of CD14, TLR-2 and TLR-4 in spleen, which were not different between the groups (Fig. 3A–F). The normal complement C3 levels in plasma during hibernating phases suggests that the complement system remains functional during the entire hibernation season in hamster, as observed previously in ground squirrel(5) and in bear(25).
Phase specific suppression of neutrophil function in hibernating hamster

In short, AE animals show a profound ‘neutrophil paralysis’ with both *E. coli* and LPS being unable to induce phagocytosis or oxidative burst in neutrophils, which could not be explained by changes in LBP, major opsonizing factors or expression of PRRs.

**Neutrophil function is modulated by a humoral factor in AE plasma**

Next, we assessed whether the reduced pathogen recognition is mediated by neutrophil-intrinsic changes or by a humoral factor in plasma. First, we blocked phagocytosis using cytochalasin D, an inhibitor of actin-filament formation(26), to allow measurement of neutrophil recognition and binding of FITC-conjugated *E. coli*. These experiments were subsequently repeated using neutrophils from either SE or AE hamsters incubated in pooled plasma from either SE or AE hamsters. Incubating SE neutrophils in AE plasma lowered their binding of LPS three-fold (Fig. 4A) and reduced *E. coli* phagocytosis by almost 50% (Fig. 4B), as compared to SE neutrophils in SE plasma. Vice versa, incubation of AE neutrophils in SE plasma augmented their phagocytosis function as compared to AE neutrophils in AE plasma (Fig. 4B). Thus, these data demonstrate that SE plasma rescues the functionality of AE neutrophils. Conversely, AE plasma inhibits *E. coli* binding to and phagocytosis by SE neutrophils, implying that the ‘neutrophil paralysis’ in AE is mediated by a humoral plasma factor, which is either necessary for or inhibiting pathogen recognition by neutrophils.
Fig. 4. Neutrophil functionality is reduced by factors in plasma from early arousal. A: Flow cytometry analysis of binding of FITC-conjugated E.coli by SE-neutrophils incubated in SE- or AE-plasma while phagocytosis is blocked by cytochalasin; B–C: Neutrophil phagocytosis of pHrodo labeled E.coli measured by fluorescence in SE- (black bars) and AE-neutrophils (grey bars) incubated in SE- or AE-plasma (B) or an equal mixture of SE- and AE-plasma (untreated, heat-inactivated, pronase) or cell culture medium (RPMI) (C). Bars represent mean ± SEM; statistical analysis by Student’s t-test (two-tailed). a: p < 0.05 compared to SE neutrophils in SE plasma; b: p < 0.05 compared to AE neutrophils in SE plasma. SE: summer euthermic, AE: arousal early, h.i. AE: heat inactivated AE plasma, pron. AE: pronase-treated AE plasma.

Finally, we characterized the AE plasma factor paralyzing neutrophils. To identify whether AE plasma is deficient in an opsonizing factor or contains an inhibitory factor, we incubated SE neutrophils in mixed SE and AE plasma (1:1, Fig. 4C). A mixture of SE and AE plasma lowered phagocytosis capacity of SE neutrophils to the same level as AE plasma alone did. This observation suggests the presence of an inhibitory factor in AE plasma as absence of an opsonizing factor in AE plasma would have been supplemented by the SE plasma in this strategy. In line with this, mixing SE plasma with cell culture medium (1:1) did not lower phagocytosis of SE neutrophils. Next, we attempted to identify the inhibitory factor by pre-treating AE plasma with heat-inactivation and with a protease mixture (pronase). Pre-treatment of AE plasma still reduced phagocytic capacity of SE neutrophils to a similar level as found with AE plasma only (Fig. 4C).

Collectively, these data demonstrate that AE plasma contains a humoral, heat-stable and protease-resistant factor, for instance a thermostable lipid or multi-dimeric protein, that inhibits pattern recognition and subsequent neutrophil activation. Since we found a reduced neutrophils response both to *E.coli* (Fig. 2, Fig. 4B) and LPS (Fig. 4A), the humoral factor affects pathogen recognition of TLR4 (detecting the *E.coli* component LPS)(23). TLR4 is highly expressed in myeloid cells (a.o. monocytes, macrophages, neutrophils) but also in smaller amounts throughout the body (a.o. brain, kidney, liver, intestine, fat) (27). Therefore, the humoral factor in hibernation could have a broad impact on arousal physiology. Interestingly, in ground squirrel splenic macrophages effectively bind LPS-FITC throughout the hibernation season(28). An important difference in TLR4-recognition of LPS in macrophages compared to neutrophils is the dependence on CD14. CD14 is essential for efficient transfer of LPS-LBP to MD2-TLR4(23). CD14 can either exist in the outer plasma membrane (mCD14 present in myeloid cells), or as a soluble extracellular
protein (sCD14) to ensure LPS-responsiveness of cells not expressing CD14 (29). The mCD14 is present abundantly in monocytes, macrophages and dendritic cells, and at a 10-fold lower concentration in neutrophils. While mCD14 is most efficient in the LPS-response of neutrophils, sCD14 is also able to elicit a neutrophil response (30). Thus, potentially, the humoral factor in arousal inhibits sCD14, resulting in a reduced neutrophil response to LPS while maintaining macrophage functionality.

CONCLUSION

In conclusion, despite rapid restoration of numbers of circulating neutrophils upon arousal to summer euthermic values, neutrophil functionality remains profoundly reduced by a humoral inhibitor of pathogen recognition. Inhibition of pathogen recognition in AE also explains the absence of IL-6 expression in LPS-induced endotoxemia. Neutrophil paralysis in early arousal might well contribute to the absence of organ injury upon arousal from torpor. Our results are critical to understand the suppressed innate immune system in hibernation. Identifying the specific factor involved may aid the disclosure of a novel immunomodulating mechanism, which may be of relevance for the treatment of (auto-)inflammatory disorders.

FUNDING

Financial support was received from the MD/PhD grant (13-29) by the Junior Scientific Masterclass, University of Groningen, the Netherlands; granted to VAR and Talent PhD scholarship, University of Groningen, the Netherlands; granted to MMO.

DISCLOSURES

The authors declare no conflict of interests.

ACKNOWLEDGEMENTS

V.A.R., R.H.H. and H.R.B. designed the experiments, supervised the experiments and wrote the manuscript. V.A.R. and M.M.O. performed the experiments, collected the samples and analyzed the data. All authors accept full responsibility and accountability for the contents of this article.
Chapter 5

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