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Phase specific suppression of neutrophil function in hibernating Syrian hamster

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ABSTRACT

Hibernation consists of alternating periods of reduced metabolism (torpor) with brief periods of metabolism similar to summer euthermia (arousal). The function of the innate immune system is reduced during hibernation, of which the underlying mechanisms are incompletely understood. Here, we studied neutrophil functionality during hibernation in Syrian hamsters. The inflammatory response to LPS-induced endotoxemia is inhibited in hibernation, partly mediated by reduced IL-6 production in early arousal. Furthermore, neutrophil pathogen binding, phagocytosis and oxidative burst is profoundly reduced in early arousal. Functionality of both summer and early arousal neutrophils was repressed in plasma from early arousal and mixed plasma from early arousal and summer euthermic, but restored by summer euthermic plasma, signifying that a plasma factor in early arousal inhibits TLR-recognition. Identification of the inhibiting factor may offer a target to modulate neutrophil function with relevance to (auto-)inflammatory diseases.

1. Introduction

Hibernation 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 (Jastroch et al., 2016). Because torpor/arousal conditions share similarities with ischemia/reperfusion, one might stipulate torpor/arousal cycles 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 (Bouma et al., 2010a). The reduction in body temperature during torpor governs a lowering of the number of circulating leukocytes (Bouma et al., 2010b, 2011), reduces lymphocyte proliferation (Maniero, 2000) and inhibits antibody production (Bouma et al., 2013a; Larsen, 1971). The reduced number of circulating neutrophils during torpor is rapidly restored in arousal to slightly higher numbers than found in summer euthermic animals (Havenstein et al., 2016; Sergiel et al., 2015). Moreover, torpor features a reduced innate immune function as illustrated by absence of a response to challenge with lipopolysaccharide (LPS) up until the next arousal (Prendergast et al., 2002).

The effect of hibernation on neutrophil function has not been documented thus far. 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 (Souto et al., 2011). Since neutrophils rapidly recirculate upon arousal after margination to the vessel wall during torpor (Bouma et al., 2013b), 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 endotoxemia in the Syrian hamster during summer euthermia, winter euthermia and arousal, all representing animals at euthermic body temperature.

2. Materials and methods

2.1. Animal experiments

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

Abbreviations: AE, early arousal; AL, late arousal; L:D cycle, light:dark cycle; SE, summer euthermia; WE, winter euthermia.

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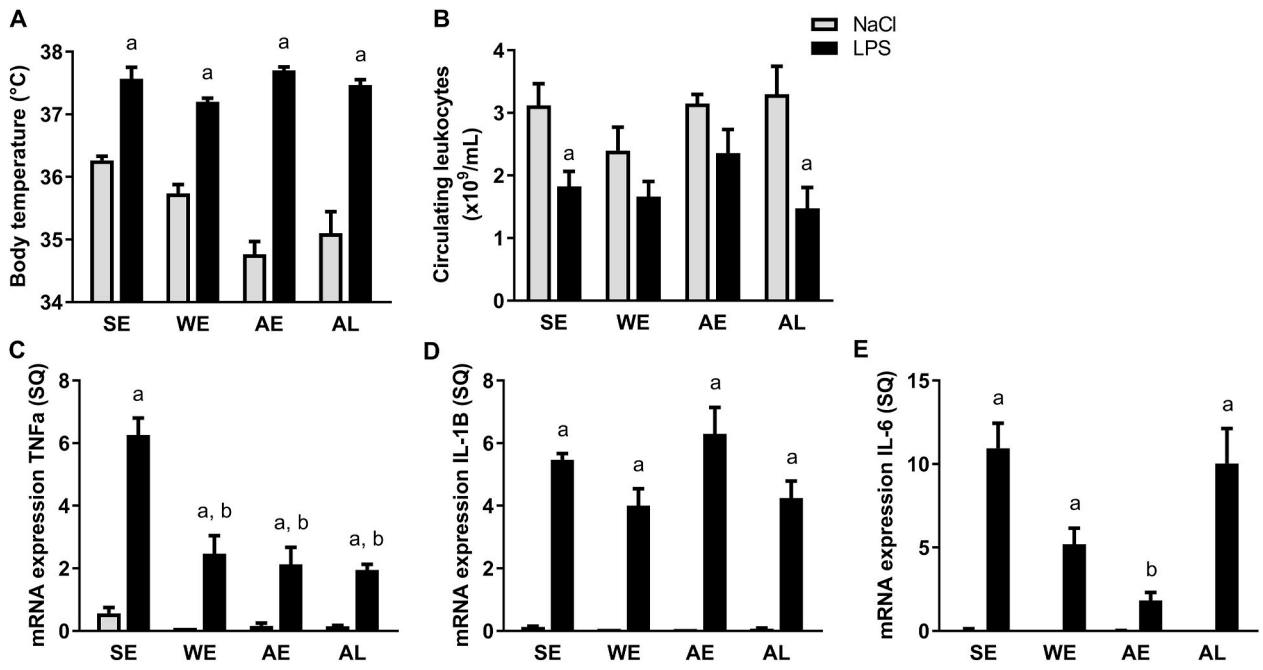

Fig. 1. Effects of hibernation on endotoxemia. A: Body temperature; B: Circulating leukocyte numbers; C–E: liver mRNA levels of pro-inflammatory cytokines TNF-α (C), IL-1β (D) and IL-6 (E). Bars represent mean ± SEM; one-way ANOVA with post-hoc Bonferroni test. a: $p < 0.05$ compared to corresponding saline control; b: $p < 0.05$ compared to endotoxemia in SE. SE: summer euthermic, WE: winter euthermic, AE: arousal early, AL: arousal late.

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 5 °C. Hibernation patterns were monitored by movement detectors (Oklejewicz et al., 2001). A representative example of body temperature changes during hibernation is shown in Supplemental Fig. 1. Non-hibernating animals were called winter euthermic. Hamsters were sacrificed during summer (SE; Tb 35.8 ± 0.35 °C) or winter euthermia (WE; Tb 36.8 ± 0.65 °C), early arousal (1.5 h after start of arousal (AE); Tb 34.8 ± 2.33) or late arousal (>8 h after start of arousal (AL); Tb 34.8 ± 2.72 °C). Endotoxemia was induced by i.p. injection of 300 μg/kg LPS (1 ml/kg) or saline (1 ml/kg). Animals were sacrificed 3 h later by exsanguination under isoflurane anesthesia. Blood was collected to obtain cell counts using an automated hematocytometer (Sysmex), plasma and neutrophils.

2.2. Analysis of gene expression

MRNA was isolated according to the protocol of Nucleospin II (Macherey-Nagel). For oligonucleotide sequences (Biolegio, the Netherlands) see Supplemental Table 1. RT-PCR was performed using AbsoluteTM qPCR SYBR® Green ROX Mix (Westburg, Netherlands). Quantitative RT-PCR was performed at 95 °C for 15 min followed by 40 cycles of denaturing at 95 °C for 15 s and annealing/extending at 60 °C for 1 min using Bio-Rad CFX384 RT-PCR Detection System (Bio-Rad Laboratories, 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. Specificity of primers was verified by Standard Nucleotide BLAST on NCBI.

2.3. Neutrophil isolation

EDTA anticoagulated blood was centrifuged for 10 min at 1,600xg. Plasma was flash frozen in liquid nitrogen and stored at −80 °C. Cells were diluted 1:1 in RPMI, layered on Ficoll (Ficoll-Paque Plus, GE Healthcare) and centrifuged for 30 min at 500xg. 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). The granulocyte fraction containing ±95% neutrophils was two times washed and then resuspended in HBSS. Verification of neutrophil isolation was determined microscopically after Giemsa staining of a smear of the isolated cells. Cell viability was >90% (Trypan Blue staining).

2.4. Analysis of neutrophil phagocytosis and oxidative burst

Neutrophils (1×10^7 cells/ml) were incubated in HBSS for 15 min at 37 °C with 10% pooled plasma from SE or AE. After centrifugation at 60xg for 5 min, supernatant was replaced by *Escherichia coli (E.coli)* particles labeled with pHrodo (Thermo Fisher Scientific) in 10% plasma and incubated at 37 °C for 2 h. Fluorescence was measured at an excitation of 560 nm and emission 585 nm. Neutrophils were collected and MDA was measured (Oxiselect TBARS Assay Kit, Cell Biolabs).

2.5. Analysis of pathogen binding

Isolated neutrophils (1×10^7 cells/ml) were incubated in HBSS for 15 min at 37 °C with 10% pooled plasma from SE or AE, followed by incubation with 10 μM cytochalasin D for 30 min at 37 °C and subsequent incubation with 1 μg/ml LPS-FITC for 30 min at 37 °C. Samples were fixed in 2% PFA and kept at 4 °C protected from light until analysis. Neutrophil binding of LPS-FITC was measured by a FACSVerse flow cytometer (BD Biosciences) and analyzed by FlowJo version 10.4.2.

2.6. Measurement of plasma LBP and IgG

Plasma levels of immunoglobulin G (IgG; Abcam) and LPS binding protein (LBP; MyBiosource) were measured by ELISA according to the manufacturer's protocol.

2.7. Data analysis

Statistical analyses were performed using SPSS Statistics 26 (IBM),

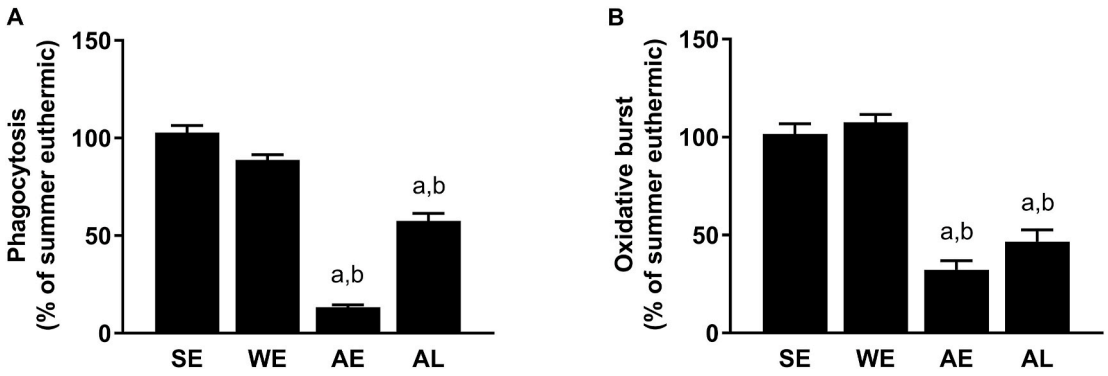

Fig. 2. Neutrophil functionality is profoundly reduced during arousal. A: Neutrophil phagocytosis of *E.coli* conjugated to fluorescent-labeled pHrodo, B: Oxidative burst of neutrophils stimulated by *E.coli* measured by malondialdehyde (MDA). Bars represent mean ± SEM; statistical analysis by Student's 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.

with p-value < 0.05. After confirming normal distribution of the data, data were analyzed by Student's t-test (two-tailed) for single comparisons or one-way ANOVA (post-hoc Bonferroni) for multiple comparisons. Figures were created with GraphPad Prism 8, the graphical abstract was produced with Biorender (www.biorender.com).

3. Results and discussion

3.1. 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).

Under normal conditions, after LPS is recognized by innate immune cells through pathogen recognition receptors (PRRs), signalling pathways such as NF-ΚB upregulate the transcription, translation and release of inflammatory cytokines IL-6, TNF-α and IL-1β (RW.ERROR - Unable to find reference:doc:5f79e16fe4b09ea1a09f7bc5). 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 (Pakai et al., 2018). 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 (McLoughlin et al., 2004). 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 (Ramirez-Carrozzi et al., 2009). 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 (Ashley et al., 2012; Prendergast et al., 2008).

Further, activation of NF-ΚB 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 (Pulugulla et al., 2016). The highly upregulated expression of HIF-1α in winter could explain the associated increase in IL-1β expression (Biggar et al., 2015; Maistrovski et al., 2012).

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.

3.2. Early arousal neutrophils have strongly impaired phagocytotic 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) (Segal et al., 1980), suggests a reduced pathogen recognition, rather than defective intracellular pathogen processing or signaling.

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 (Palsson-McDermott and O'Neill, 2004). MD2 is a small protein that interacts with the ectodomain of TLR4 to form the heterodimer MD2-TLR4, representing the functional LPS-receptor (Fitzgerald and Kagan, 2020). Binding of LPS to LBP augments recognition of LPS almost 1000-fold (Wurfel et al., 1997), 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 (Fitzgerald and Kagan, 2020). To study whether changes in pathogen recognition factors governs the reduced neutrophil function in AE, we measured levels of circulating

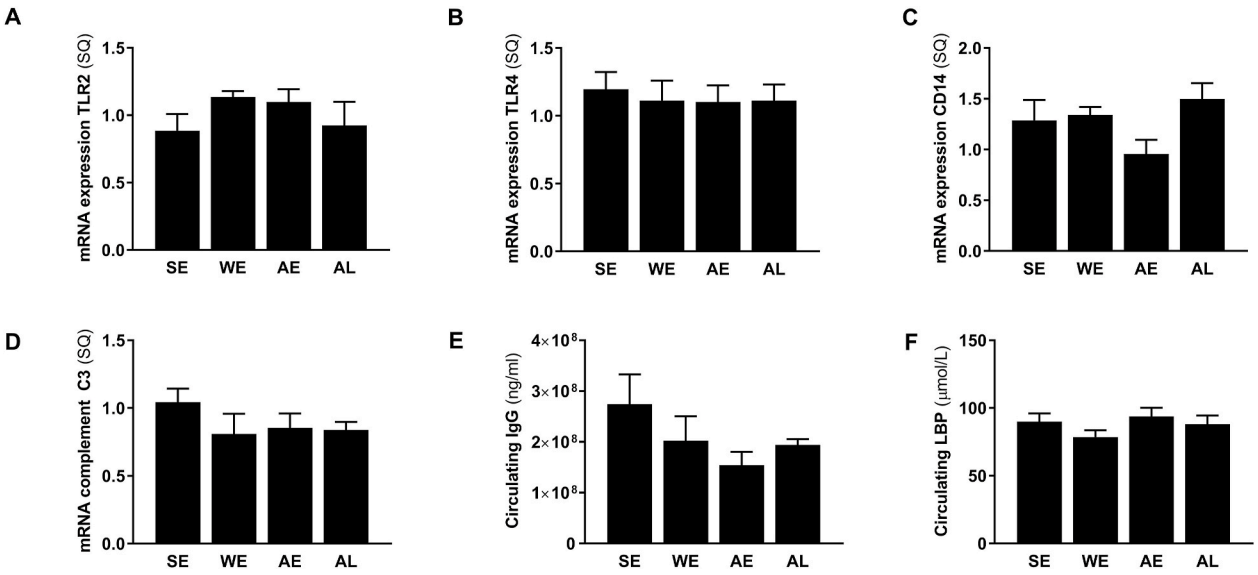

Fig. 3. Hibernation does not influence commonly known pathogen recognition factors. A–C: liver mRNA expression of pattern recognition receptors TLR-4 (A), TLR-2 (B) and CD14 (C). D: liver MRNA expression of complement C3, E: circulating IgG measured by ELISA, F: circulating LPS binding protein (LBP) measured by ELISA. 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.

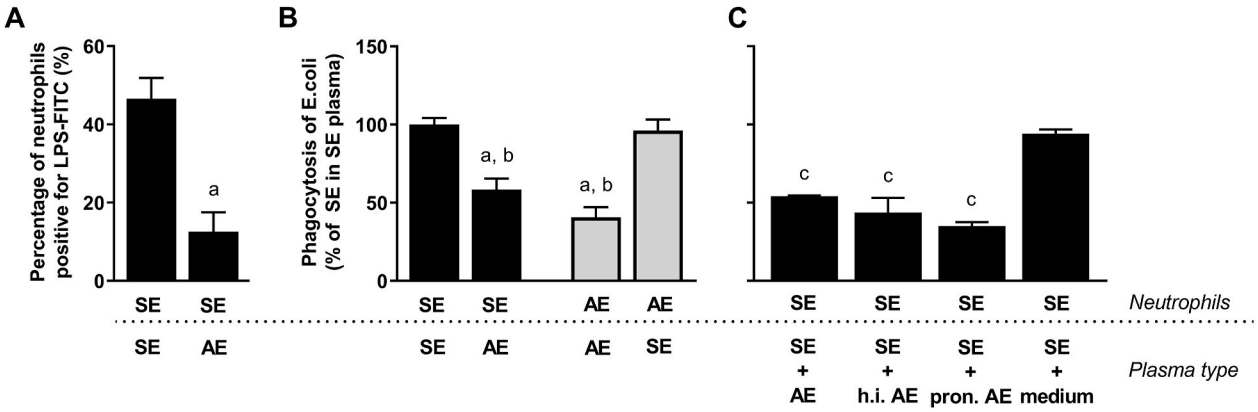

Fig. 4. Neutrophil functionality is reduced by factors in plasma from early arousal. A: Flow cytometry analysis of binding of FITC-conjugated *E.coli* by SE-neutrophils incubated in SE- or AE-plasma while phagocytosis is blocked by cytochalasin; B–C: Neutrophil phagocytosis of pHrodo labeled *E.coli* 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.

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 (Maniero, 2002) and in bear (Chow et al., 2013).

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.

3.3. 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 (Mortensen and Larsson, 2003), 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.

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* (Figs. 2 and 4B) and LPS (Fig. 4A), the humoral factor affects pathogen recognition of TLR4 (detecting the *E.coli* component LPS) (Fitzgerald and Kagan, 2020). 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) (Vaure and Liu, 2014). 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 (Maniero, 2005). 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 (Fitzgerald and Kagan, 2020). 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 (Wu et al., 2019). 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 (Troelstra et al., 1997). Thus, potentially, the humoral factor in arousal inhibits sCD14, resulting in a reduced neutrophil response to LPS while maintaining macrophage functionality.

4. 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.

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Disclosures

The authors declare no conflict of interests.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2021.104024.

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