Intrinsic anti-inflammatory properties in the serum of two species of deep-diving seal

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Summary Statement: This study identifies anti-inflammatory properties in serum of seals, which could protect these deep divers from negative downstream effects of lung collapse and bubble formation.

Abstract

Weddell and elephant seals are deep diving mammals, which rely on lung collapse to limit nitrogen absorption and prevent decompression injury. Repeated collapse and re-expansion exposes the lungs to multiple stressors, including ischemia/reperfusion, alveolar shear stress, and inflammation. There is no evidence, however, that diving damages pulmonary function in these species. To investigate potential protective strategies in deep-diving seals, we examined the inflammatory response of seal whole blood exposed to lipopolysaccharide (LPS), a potent endotoxin. IL6 cytokine production elicited by LPS exposure was 50-500× lower in blood of healthy northern elephant seals and Weddell seals compared to that of healthy human blood. In contrast to the ~6× increased production of IL6 protein from LPS-exposed Weddell seal whole blood, isolated Weddell seal peripheral blood mononuclear cells, under standard cell culture conditions using media supplemented with fetal bovine serum (FBS), produced a robust LPS response (~300×). Induction of *Il6* mRNA expression as well as production of IL6, IL8, IL10, KC-like and TNFα were reduced by substituting FBS with an equivalent amount of autologous seal serum. Weddell seal serum (WSS) also attenuated the inflammatory response of RAW 267.4 mouse macrophage cells exposed to LPS. Cortisol level and the addition of serum lipids did not impact the cytokine response in cultured cells. These data suggest that seal serum possesses anti-inflammatory properties, which may protect deep divers from naturally occurring inflammatory challenges such as dive-induced hypoxia-reoxygenation and lung collapse.

INTRODUCTION

Marine mammals such as pinnipeds (seals and sea lions) are highly specialized predators that pursue and capture prey while breath-holding. During these dives they draw down their body oxygen stores resulting in generalized hypoxemia and local tissue hypoperfusion and hypoxia (Guppy et al., 1986; McDonald and Ponganis, 2013; Meir et al., 2009). They have evolved a highly compliant, collapsible lung that accommodates the tremendous pressure changes that occur during deep dives. Further, lung collapse may prevent tissue nitrogen accumulation, narcosis, and decompression injury (Falke et al., 1985; Kooyman et al., 1971; McDonald and Ponganis, 2012; Ridgway and Howard, 1979). Remarkably, deep diving marine mammals tolerate this hypoxia, pressure-induced lung-collapse, and ischemia-reperfusion (IR) events without apparent harm. While the physiology and behavior of deep divers has been explored for decades (Butler and Jones, 1997; Costa and Sinervo, 2004; Kooyman et al., 1981; Ponganis et al., 2011), only recently have we begun to examine biochemical mechanisms of cell-level protection in these unique animals.

In contrast, humans typically suffer lung injury after IR (Cheng et al., 2006) and after cyclical collapse and re-expansion of alveoli (atelectrauma) (Leite et al., 2012; Lohser and Slinger, 2015). Rapid re-expansion of a previously collapsed human lung can also produce pulmonary oedema, a phenomenon that is, in part, mediated by inflammatory cytokines (Suzuki et al., 1992). Remarkably, despite repeated trips to depths that induce lung collapse and re-expansion, diving seals do not display evidence of significant lung injury (Kooyman and Ponganis, 1998). This may in part be due to having a pulmonary surfactant with low surface activity (Miller et al., 2006a) that acts as an anti-adhesive surfactant promoting alveolar opening upon lung re-expansion (Foot et al., 2006; Gutierrez et al., 2015; Miller et al., 2006b; Spragg et al., 2004). However, the mechanisms that protect against tissue injury and/or cytokine production following atalectrauma and IR remain to be investigated. Further, it is not known whether marine mammals avoid decompression sickness or whether they have some mechanism to tolerate bubble formation (Hooker et al., 2012). Hyperbaric injury leads to cytokine-mediated inflammation in animal models (Bigley et al., 2008; Ersson et al., 1998; Wang et al., 2015). Recent studies suggest that marine mammals are likely to experience some level of decompression sickness and thus one might expect some mechanism to tolerate associated bubble formation and mediate downstream effects. A reduced inflammatory response might provide such a protective mechanism.

Species differ in their responses to inflammatory stimuli, a phenomenon that may have arisen to facilitate survival in diverse ecological niches (Okin and Medzhitov, 2012). Mice, for example, have a blunted response to various types of inflammatory challenge compared to humans (Warren et al., 2010). We therefore hypothesized that the immune response is modified in deep-diving seals to allow them to repeatedly transit to great depths without invoking inflammatory injury to the lungs, or other cells and tissues. We were particularly interested in responses of the innate immune system, which presents a generalized, fast-acting response, rather than the adaptive immune system that confers long-lasting, antigen-specific protection. To investigate the nature of the innate immune response in diving seals, we measured responses to lipopolysaccharide (LPS) in seal whole blood, seal monocytes and seal serum. LPS, also termed endotoxin, is the principal component of the outer membrane of Gram-negative bacteria, and is a well-established experimental challenge that induces a strong immune response in vertebrates (reviewed in Rosenfeld and Shai, 2006). In this study, we found a significantly diminished *ex vivo* cytokine response to LPS in seal whole blood compared to the response in humans. Additional *in vitro* experiments using Weddell seal monocytes and a mouse macrophage cell line suggest that the differences in cytokine response to LPS in diving seals is derived from a yet-to-be defined component of seal serum.

MATERIALS AND METHODS

Sample collection

Blood samples from Weddell seals (*Leptonychotes weddellii*) (n=20, 11M, 9F) were collected during Oct-Dec 2015 and 2016 in Erebus Bay, Antarctica. Adults and weaned pups were visually in good health, and a basic blood panel (iStat 6+, Abaxis, Union City, CA, USA) confirmed that blood parameters (e.g. glucose, hemoglobin concentration, BUN) were within the normal ranges for this population (Mellish et al., 2011). Venous blood was collected from sedated adults (2mg/kg ketamine, 0.1mg/kg midazolam hydrochloride IM induction; 0.5mg/kg ketamine, 0.025mg/kg midazolam IV maintenance dosed as needed), and weaned pups restrained by headbag, both according to previously published protocols (Mellish et al., 2011). Samples were drawn into heparinized or EDTA-coated vacutainers and kept chilled during transport to laboratory facilities (45-90 min). Whole blood and isolated monocytes were processed immediately, and exposed to LPS on-site in Antarctica. Serum, as well as LPS-exposed cells and plasma were then stored at -80°C.

Human samples were collected from n=3 healthy volunteers (2M, 1F) under IRB authorization (2018P000004), following informed consent. Northern elephant seal (*Mirounga angustirostris*) blood samples were collected from adult females (n = 4, sampled February 15, 2017 late in the lactation period) sedated with telezol (1mg/100kg IM induction, 0.5mg IV maintenance dosed as needed; Hückstädt et al., 2012) at Año Nuevo, California. Mouse (*Mus musculus domesticus*) blood samples (n=12, all M) were obtained during terminal procedures as part of another project. Weddell seal samples were collected under National Marine Fisheries Service (#19439) and Antarctic Conservation Act (#2016-005) scientific permits.

Elephant seals were handled under NMFS permit 19108. All animal procedures were authorized under Massachusetts General Hospital and the University of California Santa Cruz Institutional Animal Care and Use Committees (IACUC).

LPS ex vivo exposures and IL6 protein detection

Whole blood samples from all species were diluted in 3 parts RPMI medium 1640 to prevent hemolysis (Gibco, Grand Island, NY, USA #11835-030, containing 1% HEPES, 1% Na pyruvate, 1% non-essential amino acids, and 1% penicillin/streptomycin), and were maintained in a CO_2 incubator for 4 h at 37°C with 1 to 1000 ng/mL LPS. *Escherichia coli* lipopolysaccharide (O55:B5) was purchased from List Biologicals (Campbell, CA, USA), and prepared in phosphate-buffered saline. Plasma was separated from incubated samples by centrifugation at 4°C (3000*g*), then snap-frozen. Interleukin-6 (IL6), a sensitive indicator of acute innate immune activation by LPS, was measured in the plasma of each species with the most appropriate assay kit (Quantikine ELISA, R&D Systems, Minneapolis, MN, USA, human #D6050, mouse #M6000B, canine #CA6000 for Weddell and elephant seals). Weddell seal IL6 has a high (99%) amino acid sequence similarity with another Monachine seal (Hawaiian monk seal, *Neomonachus schauinslandi*), and cytokines have been reported to be similar among seals generally, including elephant seals (Khudyakov et al., 2017), supporting comparisons between seal species using the same Quantikine ELISA. Previous work has also validated cytokine assays for elephant seals specifically (Peck et al., 2016), supporting the use of cross-reactive commercial kits to study cytokines in seals. To expand cyto- and chemokine detection to 13 substances in Weddell seal plasma (n=8 adults, n=7 pups), we used a bead-based, multiplex canine panel on the Bio-Plex 200 platform (Millipore Billerica, MA, USA #CCYTOMAG-90K), previously documented to cross-react with pinnipeds (Levin et al., 2014).

Monocyte isolation and LPS in vitro exposures

Monocytes were isolated from Weddell seal buffy coats by density-dependent centrifugation through a column of Histopaque 1077 (30 min, 400*g* at RT). The layer containing peripheral blood mononuclear cells (PBMCs) was washed, then resuspended and plated in serum-free media (OptiMem, Gibco #31985070, USA) and placed in a 37°C CO_2 incubator to allow the monocytes to adhere (~4 h). These plates were washed to remove non-adherent cells (primarily lymphocytes), and the remaining adherent cells were stimulated with increasing concentrations of LPS (1-1000 ng/mL), provided in DMEM (Gibco #11965118, 1% penicillin/streptomycin) with 10% serum (fetal bovine serum, FBS, Corning, Manassas, VA, USA #35-015-CV, <20 EU/mL endotoxin, not heat-inactivated) for 12 h (n=6 adults and n=6 pups, each with 3 technical replicates per dose, were initially used to determine the dose-response). Cell cultures were routinely tested for mycoplasma.

We used several manipulations of the cell culture medium in conjunction with LPS stimulation to evaluate the mechanisms of anti-inflammatory action of seal serum. We first compared the inflammatory response of Weddell seal monocytes cultured in standard conditions (DMEM, 1% penicillin/streptomycin), with commercially available serum (10% FBS) to media supplemented with the seal's autologous serum (10% serum, n=8 additional animals, 3 technical replicates in each dose×serum treatment). To address the possibility that baseline differences in serum cortisol between our wild population of Weddell seals and the controlled conditions of cell culture media could affect responses, this experiment was repeated to supplement FBS with 10-10,000 ng/ml hydrocortisone for 18-24 h prior to stimulation with a single LPS dose (100 ng/mL, n=11 seals x 3 technical replicates per seal). These hydrocortisone doses were selected to span and exceed the range of serum cortisol levels that would exist in any marine mammals, and that have been reported in Weddell seals (Barrell and Montgomery, 1989; Bartsh et al., 1992; Liggins et al., 1979; Shero et al., 2015). Cells from all technical replicates were harvested in lysis buffer or Trizol (see below) for gene expression analyses, and a subset of samples from n=6 seals (1 supernatant sample from each serum condition treatment at 0, 1, and 100 ng/mL LPS exposures) were processed to measure cytokine production using the canine multiplex assay.

Murine RAW cell validation experiments

To test the anti-inflammatory potential of Weddell seal serum (WSS), we tested its effect, compared to FBS, on the LPS-response of a mouse monocytic cell line (RAW 264.7 cells, tested mycoplasma-free). Cells were grown overnight in 6 well plates, then washed in DMEM without serum, and incubated for 6 h with (a) regular medium (10% FBS), or (b) DMEM with 10% pooled, decomplemented WSS. Cells were then stimulated with varying concentrations of LPS (3 technical replicates per dose×serum treatment). A single batch of decomplemented WSS was used in this, and all subsequent experiments, created by pooling archived serum from free-ranging, healthy adult Weddell seals (n=16 males and females, NMFS authorization #18662) and abolishing protein complement activity by heat-inactivation (56ºC for 30 min).

To evaluate the potential for species differences in serum lipid level to interfere with the action of LPS, we conducted a separate experiment in RAW cells (3 replicates per condition×dose) using four serum conditions (a) 10% FBS (0.37 mmol/L triglycerides), (b) 10% FBS with lipid supplementation (0.43 mmol/L), (c) 10% WSS (0.45 mmol/L), and (d) 10% delipidated WSS (0.32 mmol/L). Lipids were added to FBS based on cell culture conditions appropriate for Weddell seal primary cells (2.5% Lipid Mix 1 #L0288, Sigma, St. Louis, MO, USA; our Unpublished Observations; De Miranda et al., 2012), and lipids were removed from WSS by reserving the bottom, lipid-depleted layer after centrifugation at 13,000g for

20 min (Fu et al., 2007). Triglyceride levels were confirmed in the four conditions (Enzychrom, San Francisco, CA, USA, #ETGA-200).

Hyperlipidemic mouse model

To examine the possibility that high lipid levels could affect inflammatory responses *in vivo*, we conducted an *ex vivo* LPS exposure experiment in whole blood samples from control (n=4) and hyperlipidemic mice (n=8). LDLR knock-out mice (lacking a low-density lipoprotein receptor making them susceptible to hypercholesterolemia, C57BL/6J background) were maintained on either a regular diet (control mice, Prolab Isopro RMH 3000, LabDiet, St. Louis, MO, USA) or a high fat diet (hyperlipidemic mice, 40% lipid, Research Diets Inc., New Brunswick, NJ, USA). After 20 weeks, blood samples were collected, then treated with LPS as per the *ex vivo* exposure protocol described above.

Propidium iodide staining to detect cell viability

Viability of RAW cells cultured with FBS and WSS was examined to confirm that any observed differences in inflammatory output were not related simply to differences in cell survival. RAW cells were prepared at a constant density in 6 well plates, then incubated with DMEM supplemented with either 10% FBS or 10% WSS for 6 h. The cells were then mechanically detached and stained with propidium iodide (0.5 μg/ml), a nuclear stain that is excluded by viable cells. The proportion of propidium iodide negative cells (i.e. viable cells) was recorded by flow cytometry. Flow cytometry was performed using a FACS Aria III machine (BD Biosciences, San Jose, CA, USA), and the results were analyzed using FlowJo software (TreeStar, Ashland, OR, USA). In all cases, the gating parameters were set to exclude doublets.

qPCR to detect IL6 and inflammatory gene expression

Total RNA was isolated from frozen, lysed monocytes using the RNeasy mini kit (#74104, Qiagen, Germantown, MD, USA) according to the manufacturer's protocol, or Trizol with chloroform/isopropanol extraction. cDNA was produced from n=3 separate RNA preparations for each condition (#4368813, Applied Biosystems, Foster City, CA, USA), and evaluated by real-time PCR. Weddell seal mRNA was assayed with Fast SYBR® Green Master Mix (LifeTechnologies, Carlsbad, CA, USA). Seal *Il6* was amplified with: 5'- ACAAGTGCGAAGACAGCAAG and 5'- CCCTCATAGTTGGCCTGGAT forward and reverse primers, respectively, and expression level was normalized to a reference gene (β-actin: forward 5'- GGAAATCGTGCGTGACATCA, reverse 5'- CAGGAAGGAAGGCTGGAAGA) for each sample using the ΔCT method. Gene expression in mouse monocytes was quantified with a Taqman qPCR system, with target genes normalized to 18S ribosomal RNA (Hs03003631_g1, ThermoFisher, Waltham, MA, USA). Commercially available primers for mouse cells are as follows: *Il6* (Mm00446190_m1); *TNFα* (Mm00443258_m1); *Il-1β* (Mm00434228_m1); and *Il10* (Mm01288386_m1).

Statistical Analyses

2-way ANOVA with repeated measures and multiple test correction was used to compare experimental treatments across the LPS dose-response curve (response of whole blood to *ex vivo* stimulation between species, inflammatory response of both seal and mouse monocytes in FBS versus WSS, IL6 production in normal versus hyperlipidemic mice). Sidak pairwise post-hoc comparisons were used to examine any interaction between factors (across the dose-response curve) when global F-tests for the interaction term in the 2-way models were significant. An effect of hydrocortisone treatment on a constant LPS exposure in seal monocyte *Il6* production was tested using a 1-way ANOVA with repeated measures. Analyses were conducted in Prism 7 (GraphPad Software, Inc., La Jolla, CA, USA). All tests were two-tailed. Data are reported as mean ± standard deviation.

RESULTS

Reduced whole blood responses to ex vivo LPS exposure in seals

In both species of deep-diving seal (Weddell and northern elephant seals), the cytokine response of whole blood exposed to LPS *ex vivo* was lower compared to human blood exposed to LPS under the same conditions (Sidak posthoc $p<0.0001$ for both seals versus human; Fig. 1A). While IL6 protein content measured in human plasma increased >1000× following LPS stimulation, it increased only 10 and 100× in plasma from Weddell and elephant seals, respectively. IL6 production between the two seal species did not significantly differ (Sidak posthoc $p=0.9936$). IL6 production in Weddell and elephant seal blood was relatively consistent across all experimental LPS doses (with a plateau beyond 1ng/mL LPS) compared to humans, who demonstrate increasing IL6 production at each increase in LPS. Consistent with the scope of IL6 response in the ELISA, Weddell seal IL6 levels measured by bead-based multiplex cytokine panel, increased ~6× from a baseline of 2.5 ± 5.9 pg/mL plasma, when blood was stimulated with 100 ng/mL LPS (Fig. 1B). Of 13 cyto- and chemokines quantified by the multiplex assay, only five were above the detection limit in Weddell seal plasma (IL6, TNFα, IL10, IL18 and KC-like). Pro-inflammatory cytokines IL6 (Fig. 1B) and TNFα, as well as anti-inflammatory cytokine IL10 exhibited some degree of LPS dose-response, while IL18 and KC-like (keratinocyte chemoattractant) were detectable in all samples, but remained constant across level of LPS exposure (Fig. 2).

Isolated monocyte responses

In contrast to the limited ability of Weddell seal blood to generate IL6 protein upon *ex vivo* LPS exposure (~10× increase), mRNA expression of *Il6* increased robustly in isolated Weddell seal monocytes. Under standard cell culture conditions, *in vitro* LPS treatment dose-dependently increased *Il6* expression (>300× at 1000 ng/mL LPS; Fig 3A). To tease apart the different cytokine responses between the two experiments (isolated cells versus a whole blood scenario), we exposed monocytes to LPS as before, but added back autologous seal serum to the cell culture media. Replacement of FBS with autologous WSS conferred an anti-inflammatory benefit by decreasing *Il6* expression overall across the LPS dose-response curve ($F_{1,6}$=8.476; p=0.027; Fig. 3B). There was no significant interaction between LPS dose and experimental serum conditions in the 2-way ANOVA ($F_{4,24}$=0.34, p=0.85), indicating that the response to LPS does not differ with dose level.

The supernatant of isolated monocytes in these experiments likewise displayed a globally reduced cyto- and chemokine production in WSS compared to FBS (Fig. 4). Of the 7 chemo/cytokines that were detected in monocyte culture medium (supernatant) by the multiplex panel (Fig. 4A), IL6 ($F_{1,5}$=13.77, p=0.014), IL8 ($F_{1,5}$=463.8, p<0.0001), IL10 ($F_{1,5}$=14.5, p=0.013), KC-like ($F_{1,5}$=18.18, p=0.008), and TNFα ($F_{1,5}$=7.369, p=0.042) were significantly lower after LPS exposure in seal serum versus FBS (Fig. 4B).

Mouse macrophage responses

We next tested whether seal serum confers anti-inflammatory protection from LPS exposure in other systems. mRNA expression of *Il6* ($F_{1,2}$=246.9, p=0.004), *Tnf*α ($F_{1,2}$=292.2, p=0.003), *IL1*β ($F_{1,2}$=130.2, p=0.008) and *Il10* ($F_{1,2}$=385.4, p=0.003) was lower in murine RAW cells exposed to LPS in the presence of WSS, than in cells exposed to LPS in the presence of FBS (Fig. 5). Neither FBS or WSS affected RAW cell viability (Figure S1), suggesting that the lower inflammatory readout from cells cultured in WSS was not related to a difference in cell death.

Effect of high cortisol levels in seal serum

To test the possibility that naturally high circulating cortisol levels are responsible for the anti-inflammatory effects of seal serum, we exposed isolated seal monocytes to FBS medium supplemented with hydrocortisone prior to LPS exposure (constant 100 ng/mL for each hydrocortisone treatment). There was no effect of hydrocortisone (10-10,000 ng/mL) on LPS-induced *Il6* expression in seal monocytes (Figure S2), suggesting that cortisol did not mediate or impact the effect of WSS.

Effect of high lipid levels in seal serum

Next, we considered the potential for lipid levels to influence LPS exposure, either as an organismal protective strategy or a technical artifact of LPS sequestration by native lipoproteins. We matched lipid contents in WSS and FBS, then repeated the LPS exposure in mouse monocytes. WSS consistently reduced *Il6* and *Il1β* expression in RAW cells stimulated with LPS. Delipidation did not impair the anti-inflammatory capacity of WSS (Fig. 6). Further, lipid supplementation did not enhance the anti-inflammatory ability of FBS (Fig. 6). Finally, we investigated an alternative *in vivo* hyperlipidemic model, by assaying LPS-induced IL6 protein production in whole blood of control versus obese LDLR knock out mice (20-weeks on a high fat diet). In contrast to WSS and FBS experiments where lipid supplementation (or depletion) had no effect, mice fed a high fat diet had a pro-inflammatory response, with increased IL6 production compared to wild type mice ($F_{2,20}$=14.45, p=0.0001; Fig. 7).

DISCUSSION

In this study, we demonstrated that seals have a lower *ex vivo* inflammatory response to LPS compared to humans, and that this difference may, in part, be explained by a serum derived factor. This investigation takes a unique, multi-species approach by combining *ex vivo* responses to inflammatory challenge with controlled, *in vitro* experiments in live cells to tease apart components of the response.

Relevance of LPS stimulation and observed responses

LPS is an agonist of toll-like receptor 4 (TLR4). TLRs are a type of pattern recognition receptor (PRR) that help recognize molecules broadly shared by pathogens but different from host molecules. These receptors are an important part of the innate immune system that has evolved to provide rapid recognition of and protection from both pathogens and endogenous pro-inflammatory molecules released by cell damage (Okin and Medzhitov, 2012). Upon binding of LPS and TLR4 activation, a series of downstream cell signaling events occur, which includes the production of cytokines, notably IL6, which promotes fever and synthesis of acute phase proteins. We chose to focus on IL6 as it is a rapid, sensitive marker of acute inflammation, and has been used in large human clinical trials as a surrogate for the inflammatory response to critical illness (Brower et al., 2000), but has also been shown to play a critical role in lung inflammation/injury in mice upon exposure to environmental air pollutants (Yu et al., 2002). LPS is also known to promote the secretion of other pro-inflammatory cytokines, including IL1β, TNFα from human PBMCs (Eggesbø et al., 1994) and TNFα, IL8, KC-like from canine and pinniped PBMCs (Levin et al., 2014). TNFα is potent chemoattractant for neutrophils and also stimulates the acute phase response. IL8 is also a neutrophil chemotactic factor. IL1β has fever-producing effects and contributes to the pain associated with inflammation. Importantly, TNFα and IL1β help regulate the development of lung ischemia-reperfusion injury (Krishnadasan et al., 2003). In addition, LPS has also been shown to promote the secretion of the anti-inflammatory cytokine IL10 (Chanteux et al., 2007), which helps to downregulate the expression of cytokines produced by T helper 1 cells and may help protect against lung injury, in part by inhibiting TNFα and IL1β (Shanley et al., 2000). A variety of interleukins (including IL6) have been explored to evaluate the health and disease status of stranded marine mammals, as well as resolution of the immune response during rehabilitation (reviewed by Levin, 2018).

The diving seal model system

There are many physiological features of diving seals that likely contribute to a pro-inflammatory milieu. The unique ecological niche exploited by deep-divers exposes them to stressors that would adversely affect terrestrial mammals. In addition to repeated episodes of lung collapse and re-expansion due to high hydrostatic pressure, the tissues of diving seals experience profound hypoxia and IR events resulting from breath-hold exercise, which could produce significant injury in a non-adapted system. Seals also have consistently high blood cholesterol levels, and transiently high triglycerides associated with phases of nursing, weaning, and adult foraging (Sakamoto et al., 2009; Schumacher et al., 1992). Degree of adiposity has been positively linked to circulating cytokine levels in elephant seals (Peck et al., 2016). Yet, there is no evidence that seals suffer from atherosclerosis or vascular disease, despite being hypercholestrolemic by human standards. Indeed, seals have been proposed as models for investigating naturally occurring protection against issues stemming from IR and diet-induced obesity, namely oxidative stress (Zenteno-Savín et al., 2002) and metabolic syndrome (Houser et al., 2013). Teleologically, therefore, it would not be surprising that diving mammals have evolved multiple strategies to mitigate organ damage from the above stresses. While changes in surfactant function and the structure of distal airways are likely adaptations for diving in marine mammals (Foot et al., 2006), our data suggest that a modified innate immune response is another.

The innate immune response is initiated rapidly by resident populations of white blood cells, producing non-targeted effects such as acute inflammation mediated by cytokines. This arm of the immune system is expected to be the most responsive under hypoxic conditions that mimic the low oxygen tension environments of wounds. While hypoxia limits adaptive immune cell functionality, it directly promotes innate immune cell recruitment and activation (Sica et al., 2011). Consequently, anti-inflammatory effects that target this immune arm may be most relevant in protection against dive-induced tissue injury.

While this experiment applied LPS/endotoxin *ex vivo* and *in vitro* to stimulate and study immune responses under controlled conditions, LPS exposure may also be biologically relevant for Antarctic seals. Proteobacteria, the dominant phylum of gram-negative bacteria where LPS occurs, represent the major component of skin microbiome (Unpublished Observations), and gram-negative rods have been cultured from skin swabs in this species (Mellish et al., 2010), indicating their presence even in polar ecosystems. Proteobacteria have also previously been identified in the fecal microbiome (12.9% relative abundance) of Weddell seals (Banks et al., 2014), suggesting that gram negative bacteria are an important part of their gut microbial community. A healthy gut barrier prevents the translocation of live bacteria into the tissues and circulation, a process that if defective would expose animals to acute infectious and inflammatory challenge, and could produce sepsis (Balzan et al., 2007; Schuijt et al., 2013). The integrity of the gut barrier faces constant challenge from hypoxia-reoxygenation across foraging bouts. Diving seals demonstrate regional differences in organ perfusion (Zapol et al., 1979), with visceral tissues of Weddell seals thought to remain consistently vasoconstricted during submergence (Davis et al., 1983; Guppy et al., 1986), and experimental evidence in another pinniped predicts that digestion is at least partially deferred to the surface period (Rosen et al., 2015). In addition to their increased risk of bacterial translocation and exposure to bacterial endotoxin, endogenous ligands of TLR4 (*e.g.* HSP70, HMGB1 proteins) may also be released in response to IR (Kaczorowski et al., 2009). Consequently, studying the response to a TLR4 ligand in seals is likely to have pathophysiologic relevance.

Evidence for a serum-derived protective factor

Our data suggest that a seal serum-based factor mediates the cytokine response to LPS stimulation, although we cannot completely rule out a species-specific difference in immune cell responses as well. The importance of seal serum in blunting the inflammatory response of seal monocytes was clear. *Il6* expression and induction of cytokines from isolated cells was attenuated in the presence of WSS compared to FBS. Although isolated seal monocytes responded to LPS with a clear increase in *Il6* mRNA production (~300× under standard culture conditions), it is noteworthy that the magnitude of this response was muted in comparison to similar studies in isolated human monocytes by our group (Hoeft et al., 2017). A previous study examining LPS-induced cytokine production in isolated PBMCs also suggests that harbor seals (*Phoca vitulina*), gray seals (*Halichoerus grypus*) and harp seals (*Pagophilus groenlandicus*) have slightly reduced cell level inflammatory responses compared to dogs (*Canis familiaris*) under similar culture conditions, although between-species comparisons were not explicitly made (Levin et al., 2014). Despite some methodological differences between studies prohibiting quantitative comparisons of LPS responses, it generally appears that magnitude of change in pro-inflammatory cyto/chemokines are lowest in deep-diving Weddell seals among the five carnivores. It is possible that diving mammals, including Weddell seals, additionally have immune cell-level adaptations that reduce the effects of dive-related challenges. For example, both elephant seal platelets and beluga (*Delphinapteurus leucas*) immune cells respond differently from human cells to increased hydrostatic pressure (Field and Tablin, 2012; Thompson and Romano, 2015; Thompson and Romano, 2016), and their responses to inflammation may also differ. However, in our hands the human monocyte response to acute LPS-stimulation is on average ~10× stronger than the seal response (Hoeft et al., 2017), whereas the response in whole blood is markedly increased (>100× higher in human versus seal). A more pronounced difference between species response *in vitro* versus *ex vivo* supports the idea that seal serum, and not a difference in properties of immune cells themselves, is the primary anti-inflammatory factor.

We identified and tested two elements of seal serum that are expected to differ between species, and which may provide an anti-inflammatory benefit. Weddell seals demonstrate extremely rapid cortisol turnover rates and high total cortisol levels (Barrell and Montgomery, 1989; Bartsh et al., 1992; Constable et al., 2006; Shero et al., 2015), a trait suggested to relate to protection from high-pressure nervous syndrome (Liggins et al., 1979; Liggins et al., 1993). Steroids have been shown to have an anti-inflammatory effect by multiple mechanisms, including inhibition of NF-kB (Ray and Prefontaine, 1994; Van Der Burg et al., 1997) and inhibition of cytokine gene expression (Berghe et al., 2000). High dose steroids are commonly used as anti-inflammatory agents in human (Hench et al., 1950) and veterinary medicine (Pedersen et al., 1976). It is therefore possible that high circulating cortisol levels may have an anti-inflammatory effect in seal serum. However, we found no evidence that hydrocortisone pre-treatment (at biologically relevant levels for Weddell seals) was immunosuppressive in monocytes. This concurs with observations from fasting elephant seals (females during molting and breeding) that report no link between circulating cortisol and IL6 levels (Peck et al., 2016). We also considered that the known hyperlipidemic baseline in seals may interfere with inflammatory responses. For instance, both northern elephant seals (Tift et al., 2011) and Weddell seals (Schumacher et al., 1992) have high levels of high density lipoprotein (HDL) and HDL-cholesterol relative to humans. There is evidence that HDL binds to and sequesters LPS, preventing effective experimental stimulation of immune cells (De Nardo et al., 2014). However, the fact that delipidating seal serum did not change the anti-inflammatory effect of WSS on RAW cells, that LPS responses in WSS-treated RAW cells approaches the levels in FBS-treated cells at a moderately high dose of LPS (100 ng/ml), and that both Weddell seals and elephant seals have a measurable *ex vivo* response to low dose LPS (1 ng/ml) argue against an effect mediated purely by LPS sequestration.

Our findings raise several intriguing questions that will require further exploration. LPS requires the presence of other cofactors, including CD14, MD2 and lipopolysaccharide binding protein (LBP) for optimal function (Lee et al., 2012). It is possible that one or more of these protein cofactors is reduced in seals. While we have primarily focused on the TLR4 ligand LPS, it would be informative to examine the effect of other TLR ligands to determine whether there is a general attenuation of the inflammatory response across multiple ligands. The response to endogenous TLR ligands such as HMGB-1 (an endogenous TLR4 ligand) would be especially interesting to study because TLR4 is implicated in the pathogenesis of IR injury in many organs (Yang et al., 2017; Zhao et al., 2014).

CONCLUSIONS

The results presented here reveal a significantly attenuated inflammatory response to the TLR4 ligand LPS in seal blood compared to humans and support our hypothesis that deep-diving seals respond to acute inflammatory stimuli differently from humans. The data suggest the presence of a serum borne factor that blunts the inflammatory response to LPS in these deep divers. While high cortisol and lipid levels do not appear to account for this relative anti-inflammatory effect, serum proteins may be an attractive target for further investigation. There is evidence that modulation of inflammation by serum proteins may be responsible for the markedly different response to inflammation in mice compared to humans (Lin et al., 2015). Uncovering the identity of the factor(s) may help us understand more about seal biology and potentially have translational implications for reducing the sequelae of IR, particularly in the context of acute lung injury and solid organ transplants.

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Competing interests

The authors declare no competing interests.

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FIGURES

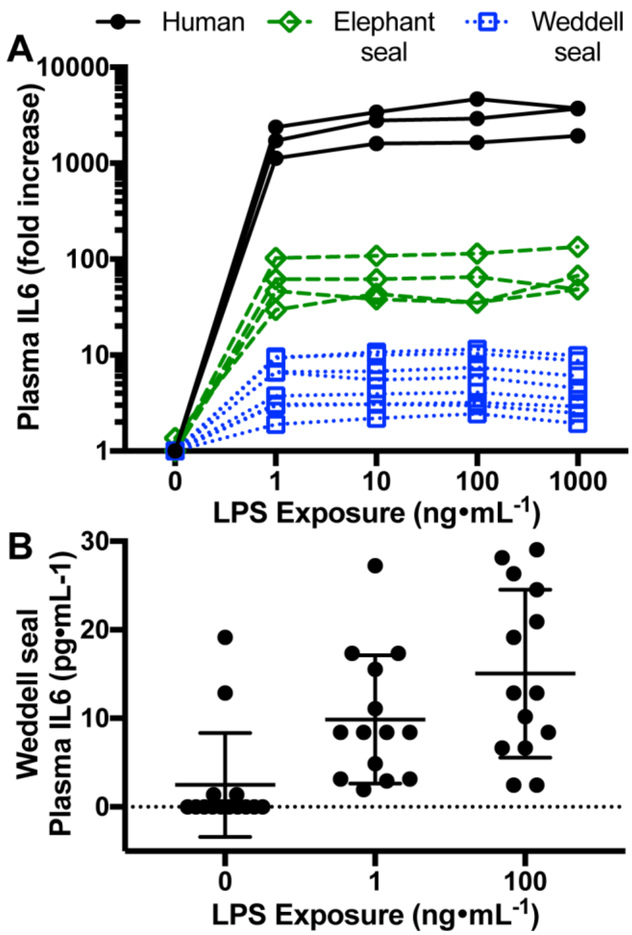

Figure 1. Interleukin-6 (IL6) production is reduced in the blood of deep-diving seals. (A) IL6 protein production following a 4-h LPS *ex vivo* exposure in whole blood in three species (n=3 humans, n=4 elephant seals, n=8 Weddell seals). Plasma IL6, measured by IL6 Quantikine ELISAs, was lower in the plasma of the two seals compared to humans (two-tailed Sidak posthoc $p<0.0001$ for both seals). Weddell seals had the smallest relative increase in plasma IL6 of all species tested. (B) The low, ~6-fold response of plasma IL6 to 100ng/mL LPS exposure in Weddell seals was confirmed using a bead-based multiplex cytokine panel (n=14).

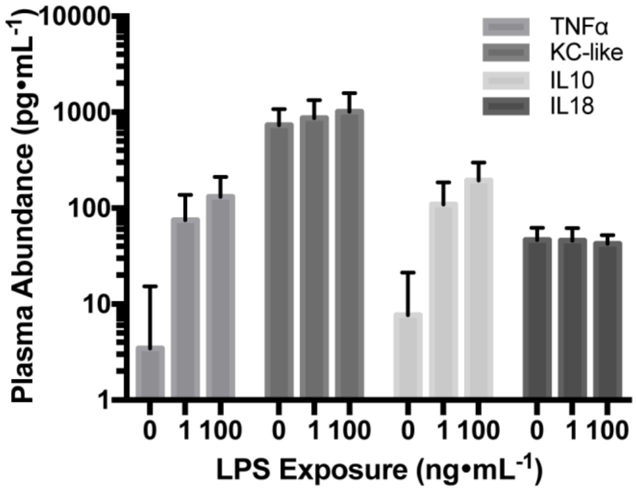

Figure 2. LPS-responsive cytokines are detected in Weddell seal plasma. Four additional cyto- and chemokines were detected in Weddell seal plasma at baseline, and following a 4-h ex vivo exposure to LPS (1 and 100 ng/mL, n=14 for each condition). Only tumor necrosis factor-alpha (TNFα) and interleukin-10 (IL10) increased with LPS. Data are summarized mean ± stdev.

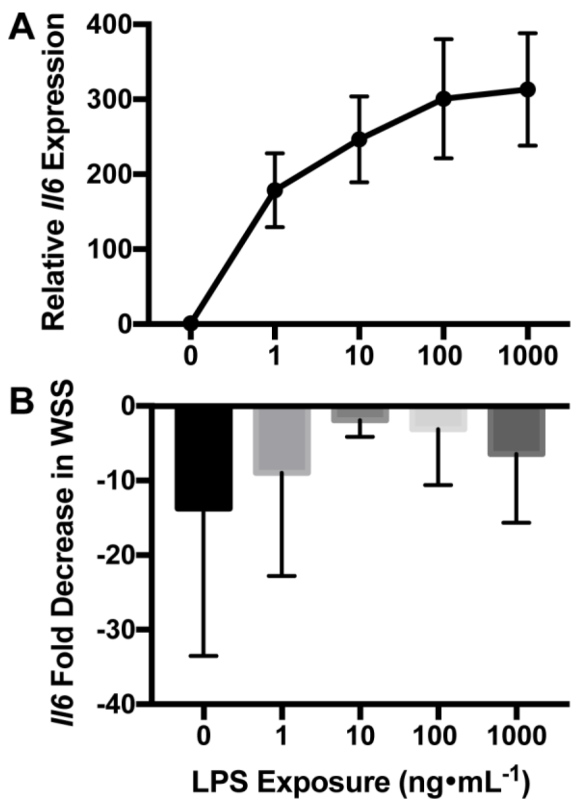

Figure 3. Weddell seal serum (WSS) reduced *Il6* expression in isolated seal monocytes. (A) LPS stimulation (1-1000 ng/mL concentrations) robustly induced *Il6* expression in isolated Weddell seal monocytes (n=12 individuals, 3 replicate wells plated per individual) under standard cell culture conditions (DMEM supplemented with 10% FBS). (B) In response to the same LPS stimulation (in n=8 additional animals, 3 replicate wells per individual per serum treatment) *Il6* expression was relatively reduced in monocytes cultured with their own serum (WSS), versus commercially available FBS (overall serum effect, two-tailed 2-way ANOVA, $F_{1,6}$=8.476; p=0.027, no significant interaction effect between serum type and LPS dose). Data are summarized mean ± stdev.

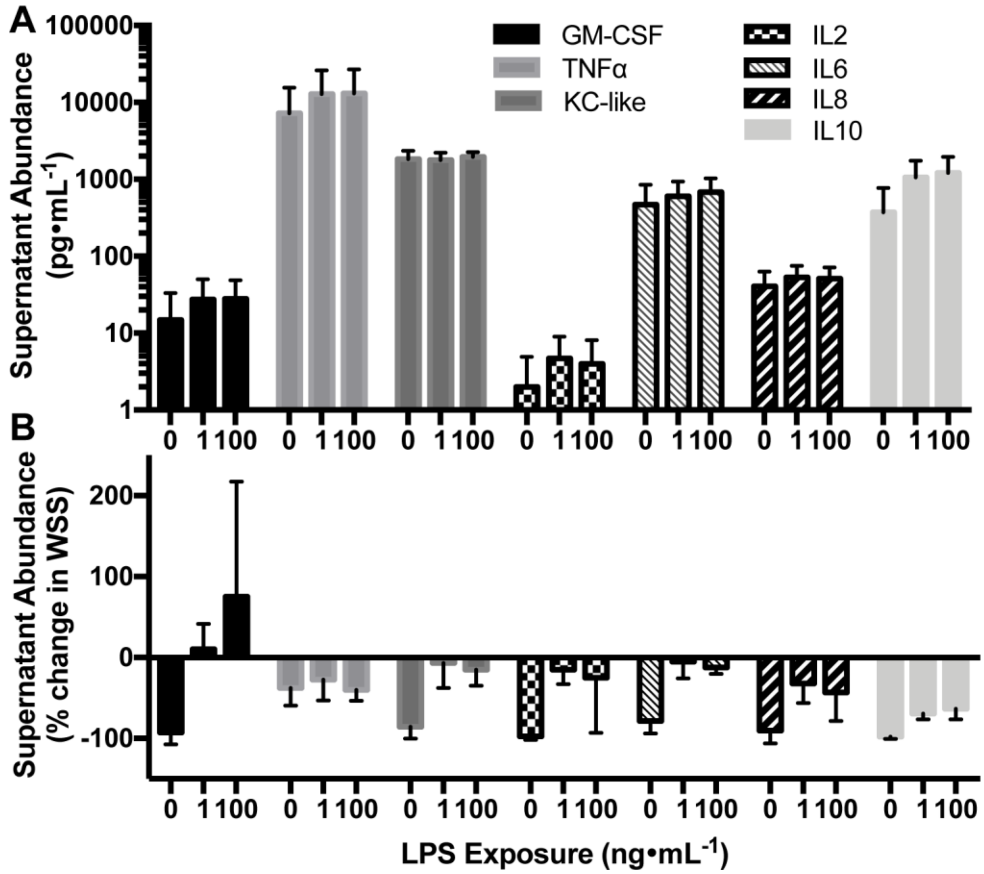

Figure 4. Weddell seal serum reduces overall inflammatory response of seal monocytes. (A) Seven cyto- and chemokines are detected in cell culture media from control and LPS-stimulated (1 or 100 ng/mL) Weddell seal monocytes (n=6 biological replicates, exposed to each dose and serum condition). (B) Production of IL6 (two-tailed 2-way ANOVA, $F_{1,5}$=13.77, p=0.014), IL8 ($F_{1,5}$=463.8, p<0.0001), IL10 ($F_{1,5}$=14.5, p=0.013), KC-like ($F_{1,5}$=18.18, p=0.008), and TNFα ($F_{1,5}$=7.369, p=0.042) by monocytes is significantly reduced during LPS exposure in cells cultured with WSS compared to FBS.

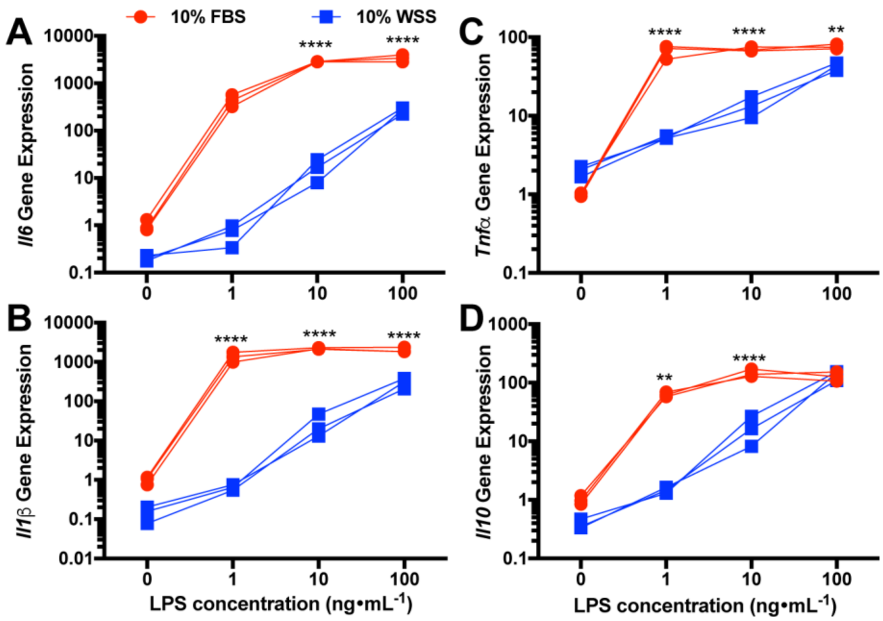

Figure 5. A mouse macrophage cell line (RAW 264.7) is protected from LPS-induced inflammatory responses by seal serum (WSS). mRNA expression of (A) *Il6*, (B) *Il-1b*, (C) *Tnfα*, and (D) *Il10* in mouse macrophages cultured in DMEM with 10% fetal bovine serum (FBS, black) or 10% Weddell seal serum (WSS, gray) was quantified after stimulation with LPS (1-100 ng/mL, n=3 wells per condition, error bars represent mean ± stdev). Target gene expression was normalized to *18s* as a reference gene, and expression levels are presented relative to the FBS condition in all panels. Asterisks denote significant differences between FBS and WSS at each LPS dose by two-tailed Sidak posthoc tests corrected for multiple comparisons, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

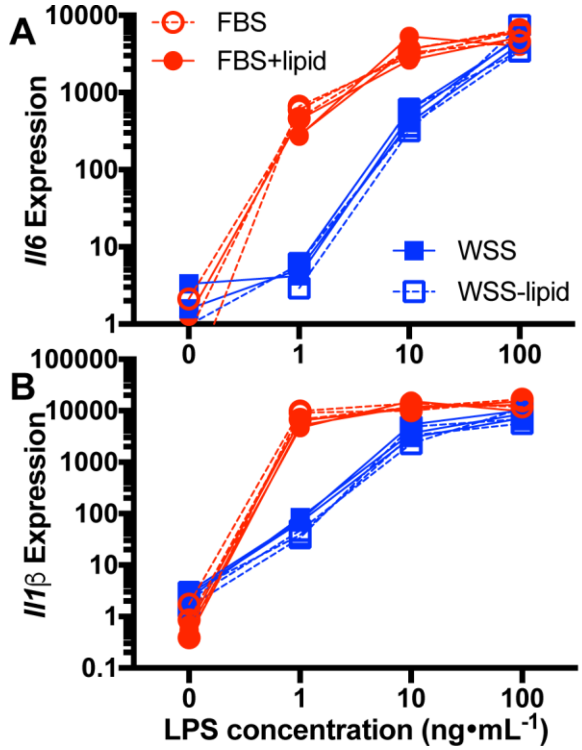

Figure 6. Lipid content of serum does not affect LPS-induced cytokine responses of mouse macrophages. Expression of (A) *Il6* and (B) *Il-1*b was measured in cells exposed to LPS (1-100 ng/mL, n=3 wells per condition, error bars represent mean ± stdev). Culture medium was provided in four conditions: either 10% FBS or 10% WSS, each ± lipid. mRNA expression of target genes is normalized to *18s* as a reference, and expression levels are presented relative to the FBS condition in both panels. Cytokine expression is significantly affected by LPS dose, serum condition, and the between-factors interaction, however no posthoc tests comparing FBS ± lipid or WSS ± lipid reached significance in any condition.

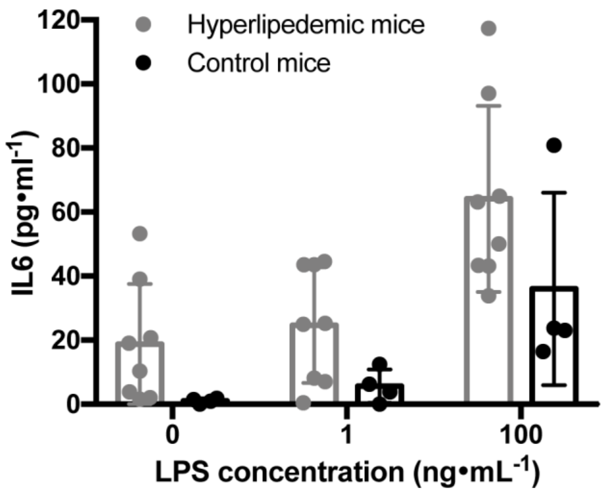

Figure 7. IL6 production is generally elevated in hyperlipidemic mice. Whole blood of control mice (n=4) had significantly reduced IL6 production across all conditions (2-way ANOVA, $F_{2,20}$=14.45, p=0.0001) when exposed to LPS (1 or 100 ng/mL), compared to mice fed a high-fat diet (n=8).