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Thermal and Cytokine Responses to Endotoxin Challenge
During Early Life

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Running Head: Lipopolysaccharide and Neonatal Cytokine Response

ABSTRACT:

Sudden infant death syndrome (SIDS) remains the leading cause of infant mortality beyond the neonatal period. An increase in body temperature as a result of high environmental temperature, over-wrapping of infants and/or infection are associated with SIDS. Endotoxins such as lipopolysaccharide (LPS) and heat stress may perturb cardiorespiratory function and thermoregulation. Although LPS-mediated body temperature and cytokine responses are well-documented in older animals, the capacity of LPS to induce fever and cytokine response in young rats remains unclear. Therefore, we sought to investigate the acute effects of LPS on body temperature and cytokine concentrations in rat pups. Postnatal day 7 rat pups were divided into three groups. Group 1 was administered LPS intraperitoneally (200µg/kg). Group 2 received saline at volume equal to LPS group. Group 3 received no treatment. Pups were placed in custom-made chambers maintained at ambient temperature of 33°C. Body surface temperature was continuously monitored for four hours. Thereafter, the rats were euthanized and serum collected for cytokine analysis. We demonstrate that LPS treatment increased MIP-1 α , IL-10, MCP-1, IP-10, fractalkine and TNF- α with no concurrent rise in body surface temperature. Although neonatal rats produced an array of cytokines in response to LPS, there was no evidence of fever.

Key Words: Inflammation, Fever, Sudden Infant Death Syndrome (SIDS), Neonatal, Cytokines

38 INTRODUCTION

39 Sudden infant death syndrome (SIDS), defined as the sudden death of an infant that remains
40 unexplained after a thorough case investigation, including performance of a complete autopsy,
41 examination of the death scene and review of the clinical history (Moon et al. 2007). An increase
42 in body temperature as a result of high environmental temperature, over-wrapping of infants
43 and/or infection are associated with SIDS. Similarly, prone sleeping position that may interfere
44 with heat dissipation is the single most important extrinsic risk factor for SIDS. Since the
45 successful advocacy of “back to sleep” position, the rates of SIDS have markedly decreased, yet
46 SIDS remains the leading cause of infant death beyond the neonatal period (Moon et al. 2007).

47 Antigens such as lipopolysaccharide (LPS), once recognized by antigen presenting cells,
48 stimulate an inflammatory response in the host through the actions of multiple cytokines via
49 prostanoid dependent and independent pathways (Blatteis et al. 2000; Fabricio et al. 2006; Roth
50 and De Souza 2001; Zampronio et al. 2015). Inflammatory responses may cause fever, i.e. an
51 increase in body temperature in response to change in thermoregulatory set point (Zampronio et
52 al. 2015). However, body temperature may also be unnaturally increased by high ambient
53 temperature due to overheating the dwelling and/or overwrapping of infants resulting in
54 hyperthermia. Although a number of well-designed studies have investigated the role and
55 mechanisms of fever and cytokine responses in the context of LPS administration in adult
56 rodents including rats (Koenig et al. 2014; Long et al. 1990; Luheshi and Rothwell 1996;
57 Machado et al. 2007; Roth and De Souza 2001; Rummel et al. 2011a), to our knowledge, limited
58 data are available during early life (Fraifeld and Kaplanski 1998). Thus, it is critical to elucidate
59 if LPS administration leads to increased body temperature and/or increased circulating pro-

inflammatory cytokines in neonatal rats and whether it is possible to delineate the effects of infection per se versus increased body temperature caused by fever. Recent studies from our laboratory have investigated inflammatory responses to LPS administration in young rat pups, however, the animals were also exposed to hypoxia and restraint stress (McDonald et al. 2016a; McDonald et al. 2016b); both factors may impact the inflammatory responses (Doherty and Blatteis 1980; Fournier et al. 2015; Ricciuti and Fewell 1992). Elucidating the effects of LPS on body surface temperature and cytokine responses is the first step in quantifying the interaction of LPS-mediated cytokine and thermal responses. Therefore, the specific aims of this study were: 1) to elucidate if LPS administration affects body temperature over time within a controlled thermal environment and 2) to investigate the LPS-induced cytokine response in one week old rat pups. We hypothesized that LPS administration would result in inflammatory cytokine response with concurrent rise in body temperature over time.

MATERIALS and METHODS

Experiments were performed on 24 Sprague-Dawley rat pups from 8 litters on postnatal day 7. There were three experimental groups; Control, Saline and LPS. Each group comprised of pups from 8 litters. LPS was administered intraperitoneally (IP) at 200µg/kg. Saline was administered also IP at a volume equivalent to LPS injection. Control group received no treatment. No pup was studied on more than one occasion and pups for various experimental groups were randomly selected from the entire cohort of pregnant rats. A thermocouple was affixed to a cohesive bandage (Co-Flex, Andover, MA) as described previously (McDonald et al. 2016a; McDonald et al. 2016b). The pup was then placed in a custom-made double-walled glass chamber through which water circulated from a water bath, to maintain an ambient temperature of 33°C,

considered within thermoneutral range for a rat pup (Mortola 2001). The heated water circulated through the double-wall lumen without being in contact with the animals. The chambers were open-ended but covered loosely with parafilm punched with multiple holes to allow rat pups to breathe room air. Three experiments were performed in parallel with three chambers connected in series. Temperature in each chamber was monitored using a thermocouple. Ambient chamber temperature (T_c) and body surface temperature (T_{BS}) were recorded continuously and noted every 15 min for four hours. The duration of the study was chosen as previous studies have demonstrated an increase in body temperature during this period (Luheshi 1998; Machado et al. 2007; Roth and De Souza 2001). At the end of the experiment, rats were euthanized and blood samples were obtained, spun down and serum stored at -80°C for cytokines analysis. The time-interval between placement of rat pups in thermal chambers and/or saline/LPS injection, and blood sampling for cytokines analysis was four hours.

Cytokine analysis was performed by Eve Technologies, located at the University of Calgary, using the Rat Cytokine Array/Chemokine Array 27-Plex. The multiplex assay was performed using the Bio-Plex™ 200 system (Bio-Rad Laboratories), and a Milliplex rat cytokine kit (Millipore) according to their protocol. The 27-plex consisted of EGF, Eotaxin, Fractalkine, G-CSF, GM-CSF, GRO/KC/CINC-1, $\text{IFN}\gamma$, IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12(p70), IL-13, IL-17A, IL-18, IP-10, Leptin, LIX, MCP-1, MIP-1 α , MIP-2, RANTES, TNF α and VEGF.

Temperature measurements and cytokine data are presented as mean $\pm$ SD. T_c and T_{BS} data were analyzed using two- way ANOVA (LPS treatment x time). Cytokine data were tested for outliers using Grubbs test, maximum of one outlier was removed per group $P < 0.05$. Cytokine data were

then analyzed using one-way ANOVA (LPS treatment). Tukey's post-hoc test was performed if ANOVA reached significance ($P < 0.05$).

RESULTS

Animals

Body Mass

The body mass of the rat pups was not different between the three groups before the experiment; (Control 16.2 ± 5.3 ; Saline injection 16.61 ± 2.3 and LPS injection 16.9 ± 2.5 g; $p = 0.84$).

Thermal chamber (T_C) and body surface temperature (T_{BS})

The T_C temperature remained constant throughout the experiment and each chamber was within 0.2°C (Figure 1). T_{BS} was plotted against time and while the temperature increased slightly over four hours in all groups (control, saline or LPS; 1°C , 1.13°C and 1.5°C respectively). The treatment was not a significant factor (treatment $p = 0.7$, time $p < 0.0001$ and interaction $p = 0.9$, Figure 1).

Cytokines

The serum concentrations of 27 cytokines/chemokines (pg/ml) and statistical analysis are presented in Table 1 and Figure 2. Fractalkine ($p < 0.0001$), IL-10 ($p = 0.003$), IP-10 ($p = 0.002$), MCP-1 ($p = 0.01$), MIP-1 α ($p = 0.01$) and TNF α ($p = 0.0003$) concentrations were significantly higher in the LPS treatment group compared with those observed in both saline and control groups (Figure 2). IL-17A ($p = 0.006$) had higher concentrations in both saline and LPS groups compared with the control group values, however, these values were similar between saline- and LPS-treated groups (Table 1). Overall, IL-6 and MIP-2 serum concentrations were higher in the

LPS-treated group but did not reach statistical significance ($p>0.05$; Table 1). Non-significant increases were observed in EGF and GRO/KC concentrations (Table 1). The remainder of the cytokine concentrations were equivalent between the groups.

DISCUSSION

We demonstrate that body temperature remains stable over time in a thermoneutral environment and while LPS increased serum concentrations of cytokines, it did not significantly increase body temperature. To our knowledge, this is the first study providing a large profile of cytokine concentrations in both naïve and LPS-treated rat pups. The data demonstrate that an LPS-induced change in cytokine concentrations can be independent of an increase in body surface temperature in one week old rat pups. We provide the much needed baseline data on thermal and cytokine responses to LPS that might form the basis of mechanistic studies on inflammation and temperature regulation during early life.

Evidence suggests that febrile response including the biphasic pattern is highly variable and dependent on species, sex, age, homeothermia, ambient temperature, dose of endotoxin and its route of administration, and prior antigenic exposure among others (Boissé et al. 2004; Fraifeld and Kaplanski 1998; Kluger et al. 1998; Rudaya et al. 2005; Zhao et al. 2008). Although fever is a hallmark of infection in adults, there are mixed reports on whether neonatal and some adult mammals develop fever in response to infection and whether newborns are capable of mounting a sufficient immune response (Gervassi and Horton 2014; Zhao et al. 2008). Fraifeld and Kaplanski (1998) summarized the pyrogenic responses at various postnatal ages and reported that 3 to 14 day old rats did not develop fever and the newborns in contrast to the weaning rats had prolonged hypothermic response. However, the studies were performed at 22°C ambient

temperature which is well below ($>10^{\circ}\text{C}$) the thermoneutral range. Given that the newborns of smaller species are unable to maintain their body temperature, the prolonged hypothermia likely reflected the ambient temperature rather than the effects of LPS treatment (Fraifeld and Kaplanski 1998).

Although the mechanisms of absence of LPS-associated fever in young animals remain unknown, several possibilities exist. Since cytokines interactions can be both synergistic and inhibitory, very high TNF- α concentrations observed in our study might have prevented the febrile response (Long et al. 1990). Furthermore, a dissociation between the rise of cytokines and initial phase of fever has been attributed to pyrogenic signals. It is, thus, possible that our naïve young animals lacked pre-formed pyrogenic factor and complement fragments (Roth and De Souza, 2001). Also, we did not observe an increase in leptin concentrations which has been postulated as one of the pyrogenic cytokines (Koenig et al. 2014). Though we have consistently observed increased levels of TNF- α , simultaneous IL-10 concentrations also increased following LPS challenge in our current and other studies (McDonald et al. 2016a; McDonald et al. 2016b). Endotoxin-induced fever in adult rats is associated with a significant rise of IL-10 (Tavares et al. 2005). IL-10 consistently increased in response to LPS in adult mice (Erickson and Banks 2011). In fact, IL-10 deficient mice exhibit exacerbated and prolonged fever in response to LPS (Kluger et al. 1998). Similar to findings in neonatal infants, serum cytokine samples show little TLR4-mediated IL-12p70 production IL-12(p70) but a high IL-10 response and therefore not strongly promoting a pro-inflammatory effect. It is likely, given the presence of cytokines in circulation in our study that neonatal animals can detect antigens/pyrogens, however there may be a self-limiting process to fever generation through anti-pyretic modulation or perhaps a blunted response at the level of the anterior hypothalamus. The most recent understanding of the innate

immune system is that it is preferentially polarized to an anti-inflammatory state, which in theory may leave young mammals susceptible to infection, as the body does not mount a sufficient inflammatory response to overcome the infection.

In adults, Toll-like receptor (TLR) activation initiates the process of cytokine production that triggers an innate immune response that predominately comprises of macrophages, monocytes and neutrophils. The acute phase response is the period in which cytokines, neutrophils and hormones are released into the plasma and lead to centrally coordinated sickness behavior in adults. Fever occurs alongside sickness behavior in adults and is a robust indicator of infection (Blatteis et al. 2000). Fever in adult rats occurs ultimately as the result of an upregulation of prostaglandin E (PGE) at the level of the ventromedial preoptic-anterior hypothalamus, however the mechanism that leads to this remains under debate and more than one route and mediator are possible. The puzzling fact about fever is that the rapid onset is unlikely to be mediated either by cytokines which need to be synthesized *de novo* or other humoral factors interfacing at the blood brain barrier which also need *de novo* (cyclooxygenase 2) COX2 and PGE signaling (Blatteis et al. 2000). Thus, it is likely that a neural mechanism leads to fever mediated via vagal afferents from the liver (complement, C5a) activation of Kupffer cells to release PGE2 causing norepinephrine release that activates constitutive COX2 and therefore rapid release of PGE2 as evidenced by studies on adult guinea-pig and rats but this too remains under debate (Fabricio et al. 2006; Li et al. 2006). Both pre- and full-term infants can increase their body temperature in response to PGE1 administration thus indicating that infants are responsive to PGs and absence of fever cannot be attributed to decreased sensitivity or lack of pyrogenic effects of PGs during early neonatal period (Heymann and Clyman 1982).

Although neural and humoral factors mediate the rapid onset of fever, there is a strong role for cytokine mediators in fever such as IL-6, TNF- α , IL-1 β , MIP1 α and MIP-1 β in adult rats (Fabricio et al. 2006; Zampronio et al. 2015). In adult rats, IL-6 was found to mediate fever response through mPEGS-1 in the brain and IL-6 deficient adult mice are resistant to fever and do not demonstrate an increase in PGE₂. TNF α is produced primarily by mast cells and functions to recruit monocytes, dendritic cells and polymorphonuclear cells. TNF- α is produced in response to endotoxin (Karck et al. 1988) and induces fever in adult rats (Steffenl et al. 1996). Our study shows an absence of fever to a moderate dose of LPS but a clear activation of pyrogenic cytokine production (eg. IL-6 and TNF- α). Nonetheless, IL-6 is known to have diverse pyrogenic capacities dependent on the concentration and species (Rummel et al. 2011b). In young rats, IL-6 played a strong role in fever genesis while TNF α modulated the initial pyrogenic phase but was not necessary for the later phase of LPS-mediated rise in body temperature (Harden et al. 2006). Thus, fever response may be dependent on expression of other immune mediators. Similar to the current study, IP-10, MIP-1 α , MCP-1 also increased in adult mice in response to LPS (Erickson and Banks 2011; Kopydlowski et al. 1999). Furthermore MCP-1 is also upregulated in adult rats following LPS administration (Uchiumi et al. 2004), though may not be directly pyrogenic (Zampronio et al. 2015). There was no observed LPS-induced increase in GRO (CXCL1) in our study, which is a mediator of prostaglandin dependent fever in adult rats (Soares et al. 2008). Moreover, IL-1 β was also not increased following LPS treatment, though in adult rats IL-1 β upregulation is small and more short-lived compared to other cytokines (Kakizaki et al. 1999).

In our study, a small but significant increase in IL-17A concentrations was observed in both LPS- and saline-treated animals. These changes might reflect the injection-related stress response

214 as mild thermal stress response has previously been reported (Rummel et al. 2011a). These
215 changes also highlight the significant impact of intraperitoneal injection on cytokine responses
216 regardless of the injected agent (vehicle and LPS in this case). It also brings up the point that in
217 studies where no naïve (control) group is included, the impact of various
218 manipulations/interventions on at least some of the cytokine responses might be at risk of under-
219 or over-estimation. An LPS-mediated increase in IL-17A has previously been documented by
220 Sun et al. (2015), however, the administered dose (500 µg/kg) was more than twice what we
221 used in our study.

222 Immunity comes at a high metabolic cost and may be an evolutionary adaptation to minimize
223 immune response over initial few days of growth and development. Fever is part of an acute,
224 centrally co-ordinated response which mediates a change in the thermoregulatory set point which
225 must be achieved by minimizing heat loss (vasoconstriction) or by heat generation that can be
226 accomplished by an increase in metabolic heat production (shivering or non-shivering
227 thermogenesis and decrease in heat loss) (Zampronio et al. 2015). Fraifeld and Kaplanski (1998)
228 suggest actual body temperature may not always reflect the functional state of the central
229 thermostat and increased PGE₂ production in hypothalamus would not directly lead to body
230 temperature elevation in rodents.

231 Our study has some limitations. Although we provide foundational data on a large number of
232 cytokines and demonstrate dissociation between fever and increased pro-inflammatory cytokines,
233 our study does not provide mechanisms underlying such observations. Furthermore, inclusion of
234 adult animals might have provided interesting comparative physiological data. Measurement of
235 rectal or intraabdominal temperature is a gold standard. In our study, we measured body surface

236 temperature as surrogate for rectal temperature. These measurements will not reflect body
237 temperature in older animals, however may be considered valid in very young rat pups as body
238 temperature at this age closely follows the ambient temperature which was kept within
239 thermoneutral range (Mortola 2001).

240

Draft

241 In summary, we provide new observations on serum cytokine concentrations in naïve animals
242 and those exposed to a moderate LPS dose. Furthermore, we demonstrate that despite immune
243 activation by LPS, there was no corresponding rise in body temperature of one week old rat
244 pups. Thus, it is possible to delineate the effects of mild infection per se versus increased body
245 temperature caused by fever.

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246 **Ethics:** The University of Calgary Animal Care Committee approved the study protocols and all
247 experimental procedures followed the national guidelines set by the Canadian Council on Animal
248 Care.

249 **Authors' Contribution:** F.B.M, A.K, K.C and S.U.H designed the study. Experiments were
250 performed by A.K and K.C. F.B.M. A.K, A.I, M.E, S.U.H reviewed the data and performed
251 analysis. All authors participated in the manuscript preparation and final approval.

252 **Competing Interests:** None.

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369

371 **Legends**

372 *Figure 1*

373 *Body surface (circles) and ambient chamber temperature (squares) of control (open), saline*
374 *(grey) and LPS (solid) groups over the duration of the experiment.*

375 *Figure 2*

376 *Group data (mean $\pm$ SD) illustrating serum cytokine concentrations (pg/ml) of P7 neonatal rat*
377 *pups in control, saline and LPS groups. * $P < 0.05$ LPS vs. both control and saline group.*

378

Draft

379 *Table 1. Serum cytokine concentrations (pg/ml).*

	Control	Saline	LPS	One-way ANOVA
n	8	8	8	P
IL-1 α	83 $\pm$ 81	67 $\pm$ 65	157 $\pm$ 149	0.2
GCSF	32 $\pm$ 35	39 $\pm$ 43	32 $\pm$ 35	0.9
GMCSF	78 $\pm$ 12	85 $\pm$ 14	83 $\pm$ 16	0.59
Leptin	14763 $\pm$ 10585	10385 $\pm$ 2032	9000 $\pm$ 7408	0.32
IL-4	9 $\pm$ 5	8 $\pm$ 4	8 $\pm$ 3	0.76
IL-1 β	217 $\pm$ 186	509 $\pm$ 712	240 $\pm$ 231	0.38
IL-2	33 $\pm$ 24	19 $\pm$ 13	25 $\pm$ 18	0.41
IL-6	87 $\pm$ 89	92 $\pm$ 82	3286 $\pm$ 4709	0.04
EGF	0.014 $\pm$ 0.013	0.016 $\pm$ 0.014	7.1 $\pm$ 13	0.14
IL-13	0.2 $\pm$ 0.22	0.11 $\pm$ 0.1	0.10 $\pm$ 0.2	0.49
IL-12 (p70)	7.3 $\pm$ 2.7	9.4 $\pm$ 6.3	9.7 $\pm$ 6.4	0.67
IFN γ	72 $\pm$ 72	74 $\pm$ 72	185 $\pm$ 253	0.28
IL-5	23.5 $\pm$ 13.3	29.8 $\pm$ 14.0	31.8 $\pm$ 20.0	0.57
IL-17A	7.4 $\pm$ 5.8	17.2 $\pm$ 5.9*	15.4 $\pm$ 5.4*	0.006
IL-18	11750 $\pm$ 3608	12687 $\pm$ 6929	9101 $\pm$ 4031	0.37
GRO/KC	225 $\pm$ 227	224 $\pm$ 241	18281 $\pm$ 33288	0.14
VEGF	221 $\pm$ 20	224 $\pm$ 32	223 $\pm$ 66	0.99
LIX	4072 $\pm$ 849	3927 $\pm$ 698	4212 $\pm$ 1118	0.82
MIP2	124 $\pm$ 103	122 $\pm$ 103	1238 $\pm$ 1655	0.04
Rantes	7493 $\pm$ 1994	8376 $\pm$ 2720	7110 $\pm$ 2659	0.59
Eotaxin	10 $\pm$ 10	11 $\pm$ 10	10 $\pm$ 7	0.91

380 *Mean $\pm$ SD. *P<0.05 LPS and Saline groups vs. control group.*

Temperature over time

Figure 1

* $p<0.05$ Control and Saline vs. LPS

Figure 2