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1 **Methyl mercury (MeHg) *in vitro* exposure alters mitogen-induced lymphocyte**
2 **proliferation and cytokine expression in Steller sea lion (*Eumetopias jubatus*) pups**

3

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24

25 **Abstract**

26 Steller sea lions (*Eumetopias jubatus*, SSLs) are managed as two distinct population
27 segments within U.S. waters: the endangered western distinct population segment and the
28 recently delisted eastern distinct population segment. Recent studies reported concentrations
29 of mercury in several tissues collected from young SSLs in the Aleutian Islands that were at or
30 above concentrations found to negatively impact health in other fish-eating mammals.
31 However, there are limited studies which have investigated the range of mercury
32 concentrations that may negatively influence the SSL immune system. This study assessed
33 relationships between methyl mercury (MeHg+) concentrations and two immune functions,
34 lymphocyte proliferation and cytokine expression. Peripheral blood mononuclear cells
35 (PBMCs) were isolated and cryopreserved from pups on three rookeries within the western
36 distinct population segment: Chiswell Island, Ulak, and Agattu Islands. Lymphocyte
37 proliferation and cytokine expression were assessed *in vitro* using thawed PBMCs with
38 exposure to MeHg+ (unexposed control, 0.001, 0.01, and 0.1 µg/ml). Lymphocyte proliferation
39 was measured without and with stimulation with a T cell mitogen (ConA) and B cell mitogen
40 (LPS) and the concentration of cytokines was measured in the cell culture supernatant (with
41 and without ConA or LPS). Spontaneous lymphocyte proliferation was significantly increased
42 at 0.01 and 0.1 µg/ml. T lymphocyte proliferation was significantly increased at 0.001 µg/ml
43 and 0.1 µg/ml, while B lymphocyte proliferation was decreased at 0.1 µg/ml. Cytokine
44 concentrations for INFγ, IL-10, IL-6, and TNFα were reduced at 0.1 µg/ml upon either T or B
45 cell mitogen stimulation, with the exception for IL-10, where 0.1 µg/ml reduced IL-10
46 concentration compared to unstimulated cells. These data suggest immune functions were
47 affected by MeHg+ exposure requiring *in vivo* follow up investigations. The observed

48 modulation of immune functions is of concern as any toxicant-induced modulation may
49 adversely affect the health of individuals, particularly younger animals undergoing periods of
50 critical development.

51

52 Keywords: Steller sea lion; Methyl mercury; Immune; Lymphocyte proliferation; Cytokine

53 **1. Introduction**

54 Within U.S. waters, Steller sea lions (*Eumetopias jubatus*, SSLs) are managed as two
55 distinct population segments, with the western distinct population segment listed as
56 endangered and the eastern distinct population segment listed as threatened, until its delisting
57 in 2013 (NMFS, 2013; W. Pitcher et al., 2007). Within the western distinct population segment,
58 variable population trajectories have been found across the geographic range with some
59 rookeries continuing to decline (Fritz et al., 2014). The declining or depleted portions of the
60 western distinct population segment may be facing numerous biotic or abiotic stressors
61 including toxicant exposure, altered prey availability, fisheries competition, killer whale
62 predation, and disease. The population declines and slow recoveries of the western distinct
63 population segment of SSLs have been the focus of continuing research and debate, with
64 environmental toxicants including organochlorine compounds (OCs) and mercury being
65 hypothesized as contributing factors (Atkinson et al., 2008; Barron et al., 2003; Beckmen et al.,
66 2002; Holmes et al., 2008). Recent studies have reported relatively high concentrations of
67 mercury in tissues (hair and blood) sampled from SSL pups, lending support to the hypothesis
68 that mercury may be impacting the recovery of SSLs in some areas within the western distinct
69 population segment (Castellini et al., 2012; Kennedy et al., 2019; Rea et al., 2013).

70 Mercury is a widespread element in the environment and has both natural (e.g. volcanic
71 activity, geological deposits) and anthropogenic (e.g. incineration of coal, gold mining) sources
72 (Pacyna et al., 2006). Like other contaminants, mercury biomagnifies and bioaccumulates in
73 upper trophic animals and has been shown to have deleterious effects on the nervous,
74 immune, and endocrine systems (Basu et al., 2006; Tan et al., 2009; Wolfe et al., 1998).
75 Across the range of SSLs, total mercury concentrations in hair and pups less than three
76 months of age had high concentrations suggesting exposure and accumulation of mercury *in*
77 *utero*. Further, total mercury levels in hair grown *in utero* (a proxy for maternal exposure) were

78 significantly elevated in pups whose mothers fed on higher trophic prey, as determined by
79 analysis of stable nitrogen, δ15N (Rea et al., 2013), suggesting that diet is a significant source
80 of mercury in SSLs. These findings suggest that adult females and their pups are potentially at
81 risk for deleterious effects of mercury that may include disruption of the immune system.

82 Mercury exposure for very young pinnipeds is through transplacental and
83 transmammary transfer (Jones et al., 1976; Wagemann et al., 1988). In the western distinct
84 population segment, several SSL pups had hair and blood mercury concentrations
85 approaching or exceeding concentrations found to negatively impact fish-eating mammals
86 (Basu et al., 2007; Castellini et al., 2012; Rea et al., 2013; Riget et al., 2011; Wren et al.,
87 1988). Among SSL pups from the western distinct population segment, whole blood total
88 mercury concentrations ≥0.11 mg/kg were correlated with lower concentrations of haptoglobin,
89 an acute phase protein (Kennedy et al., 2019). In marine mammals, a high percentage of
90 mercury in the blood is in the methylated form (Das et al., 2008), which may interact with blood
91 immune cells and modulate their function. Monomethyl mercury ($MeHg^+$) was shown to
92 modulate two immune functions, lymphocyte proliferation and cytokine expression, in free-
93 ranging and captive harbor seals (Das et al., 2008; Kakuschke et al., 2008). However, it is
94 unclear what mercury concentrations affect the health of SSLs and only a few studies to date
95 have undertaken the task of assessing the relationships between mercury exposure and
96 immune functions in SSLs (Kennedy et al., 2019).

97 Lymphocytes are responsible for the acquired or adaptive immune response in
98 vertebrates. T lymphocytes are primarily responsible for mounting a cell-mediated immune
99 response, while B lymphocytes are primarily responsible for mounting a humoral immune
100 response, both which require the expansion of naïve or memory cells through the process of
101 lymphocyte proliferation (Owen et al., 2013). Lymphocyte proliferation is the first step in a
102 proper immune response to create effector lymphocytes, which are necessary to eliminate a

103 current antigen, or memory lymphocytes, which help eliminate the same antigen the host may
104 encounter in the future, responding with a more rapid and enhanced response compared to the
105 first encounter. SSL pups with a developing immune system may be susceptible to the effects
106 of toxicants, such as MeHg+, on critical T and B lymphocyte immune functions. SSL pups
107 respond to *in vitro* stimulation, with T cells responding to an antigenic challenge as early as
108 five days following birth (Keogh et al., 2010). However, SSL pups exhibited a decrease in T
109 cell proliferation with age during the early postnatal period (Keogh et al., 2010). Toxicant-
110 induced immunomodulation may increase an individual's susceptibility to infectious pathogens,
111 leading to increased morbidity and mortality (Ross et al., 1996; Van Loveren et al., 2000).

112 Cytokines are small cell-signaling proteins, which are produced and released by cells of
113 the immune system in response to a stimulus (pathogen or tissue damage). Pro-inflammatory
114 cytokines, secreted in the beginning of an inflammatory response, include interleukin (IL)-1, IL-
115 6, IL-8 and tumor necrosis factor alpha (TNFα). Anti-inflammatory cytokines, secreted to
116 dampen an inflammatory response, include IL-4, IL-10, and IL-13. Cytokines are also used to
117 define the direction of an immune response orchestrated by T helper (Th) cells. Th1 cells
118 secrete interferon gamma (IFNγ), IL-2, IL-12 and TNFα, which promote cell-mediated immunity
119 to help combat intracellular pathogens, whereas Th2 cells secrete IL-4, IL-10, and IL-5, which
120 promote humoral immune responses to help combat extracellular pathogens (Owen et al.,
121 2013).

122 Several papers suggest that the toxicant-induced immunomodulation is likely to result in
123 adverse health outcomes, such as disease susceptibility in marine mammals. For example, in
124 a series of papers assessing the relationship between toxicant exposure and changes in
125 immune functions and host disease resistance, a good correlation was found between
126 changes in the immune tests and altered host resistance in that there were no instances where

127 host resistance was altered without affecting an immune test(s) (Luster et al., 1994; Luster et
128 al., 1993; Luster et al., 1992). Using a semi-field study in which captive harbor seals were fed
129 herring from the contaminated Baltic sea, or from the less contaminated Atlantic Ocean, those
130 seals fed the contaminated (with polychlorinated biphenyls) herring had impairments to several
131 immune functions (e.g. natural killer cell activity, T lymphocyte proliferation, delayed typed
132 hypersensitivity), suggesting that the contaminated herring were immunotoxic (Ross et al.,
133 1996). The authors suggested that the impaired immune functions could results in diminished
134 host resistance and an increased incidence and severity of infectious disease. In a recent
135 review of the immunotoxic effects of contaminants and toxicants, including mercury, in marine
136 mammals, the authors concluded that exposure to immunotoxic contaminants may have
137 significant population level consequences as a contributing factor to increasing anthropogenic
138 stress in marine wildlife and infectious disease outbreaks (Desforges et al., 2016).

139 The goal of this study was to assess the effects of *in vitro* exposure to increasing
140 concentrations of $MeHg^{+}$ on lymphocyte proliferation and cytokine expression. These data will
141 contribute to understanding the role of toxicants in SSL recovery within the western distinct
142 population segment population.

143

144 **2. Methods and Materials**

145 *2.1. Blood samples*

146 We sampled 28 SSL pups (15 female, 13 male) at three rookeries within the western
147 distinct population segment. Sampling occurred on June 30, 2016 on the northern shore of
148 Chiswell Island (59°35'13" N, 149°34'50" W) in the northern Gulf of Alaska, June 27, 2017 at
149 Gillon Point on Agattu Island (54°24'26" N, 173°21'46" W), and on June 30, 2017 at Hasgox
150 Point, Ulak Island (51°18'39" N, 178°59'12" W). While under isoflurane anesthesia (Heath et

151 al., 1997; Merrick et al., 1996), blood samples were collected using standard aseptic
152 techniques via the caudal gluteal plexus (20 G 1.5 inch needle) directly into sodium heparin
153 blood tubes. Blood samples were centrifuged and buffy coats were cryogenically preserved
154 (fetal calf serum with 10% DMSO) and stored at -150°C or colder until further analysis, as
155 previously described (Keogh et al., 2010).
156

157 *2.2. Peripheral blood mononuclear cell (PBMC) preparation*

158 Cryopreserved buffy coats were quickly thawed in a 37◦C water bath and added to a
159 conical tube containing 10 times the volume of warm Hank's Balanced Salt Solution (HBSS;
160 Life Technologies, Grand Island, NY) and centrifuged at 300g for 10 min. The pellet was re-
161 suspended in 20 ml HBSS and centrifuged at 300g for 10 min. The pellet was re-suspended in
162 10 ml HBSS and passed through sterile nylon wool to remove any clumps or cellular debris.
163 The cell solution was centrifuged at 300g for 10 min and re-suspended into Dulbecco's
164 modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY) supplemented with
165 1mM sodium pyruvate, 100 mM non-essential amino acids, 25mM HEPES, 2mM L-glutamine,
166 100 U/mL penicillin, 100 mg/mL streptomycin and 0.25 mg/mL Fungizone (all obtained from
167 Life Technologies, Grand Island, NY, USA), along with 10% fetal bovine serum (Hyclone,
168 Logan, UT, USA), hereafter referred to as complete DMEM. The PBMCs were counted with a
169 hemocytometer using the trypan blue exclusion dye to measure cell viability. Cell viability was
170 typically >90%.

171 PBMC isolation was confirmed using BD FACScan flow cytometry (Becton Dickinson,
172 Rutherford, NJ). CellQuest software (Becton Dickinson Immunocytometry System, San Jose,
173 CA) was used to identify PBMCs (lymphocytes and monocytes) based on their cell morphology
174 using cell size (forward scatter) and granularity (side scatter) (Figure 1), as performed with
175 other pinnipeds (Bogomolni et al., 2016a; Bogomolni et al., 2016b).

176

177 *2.3. Mitogen-induced lymphocyte proliferation*

178 Preliminary data showed that *in vitro* exposure of PBMCs to 0.5 and 1 µg/ml $MeHg^+$
179 caused direct cell mortality as measured using propidium iodide and flow cytometry (data not
180 shown). Therefore, 0.1 µg/ml was chosen as the highest concentration tested, which did not
181 cause direct cell toxicity. MeHgCl (Sigma-Aldrich, St Louis, MO) concentrations were prepared
182 in complete DMEM at 0 (unexposed control), 0.001, 0.01, and 0.1 µg/ml. Importantly, this
183 range included whole blood MeHg+ concentrations within the ranges measured in Chiswell
184 SSL pup whole blood (range: 0.028 to 0.066 µg/ml; Rea, Castellini, Keogh et al., unpublished
185 results), Pacific harbor seal pups (Van Hoomissen et al., 2015), bottlenose dolphins (Reif et
186 al., 2015), and humans (Jung et al., 2013).

187 T and B cell mitogen-induced proliferation was evaluated as described previously (Levin
188 et al., 2005; Levin et al., 2009; Mori et al., 2008; Mori et al., 2006). Briefly, 2 x 10^5 PBMCs/well
189 in complete DMEM were plated, in triplicate, in 96-well flat-bottom tissue-culture plates
190 (Falcon, Becton Dickinson, NJ). Cells were incubated at 37°C with 5% CO_2 for a total of 66
191 hours with concanavalin A (ConA 1 µg/ml; Millipore Sigma, St. Louis, MO), lipopolysaccharide
192 (LPS O111:B4, 5.0 µg/ml; Millipore Sigma, St. Louis, MO) or complete DMEM (no mitogen), as
193 well as $MeHg^+$ as selected above. Both ConA and LPS mitogens have been shown to
194 stimulate marine mammal lymphocytes, including in SSLs (de Swart et al., 1993; Keogh et al.,
195 2010; Mori et al., 2008; Mori et al., 2006). Lymphocyte proliferation, as measured by the
196 incorporation of 5-bromo-2′-deoxyuridine (BrdU), a thymidine analogue, during the last 18
197 hours of incubation and was detected by enzyme-linked immunosorbent assay (ELISA; Cell
198 Proliferation ELISA BrdU, Roche Diagnostics, USA) using a plate reader (Multiskan EX v.1.0)
199 at 450 nm with a reference wavelength of 690 nm.

200

201 *2.4. Cytokine concentrations*

202 PBMCs were cultured for the collection of supernatants to measure secreted cytokines,
203 as previously described (Levin et al., 2014). Briefly, 2 x 10^5 PBMCs/well (from n=5 individuals)
204 in complete DMEM were plated, in duplicate, in 96-well flat-bottom tissue-culture plates
205 (Falcon, Becton Dickinson, NJ). Cells were incubated at 37°C with 5% CO_2 for a total of 48
206 hours with ConA (1 µg/ml), LPS (5.0 µg/ml) or complete DMEM (no mitogen), as well as the
207 selected concentrations of $MeHg^+$ listed above. After 48 hours, plates were centrifuged for 10
208 min at 220g and the cell culture supernatant was collected and pooled from duplicate well.
209 Samples were immediately frozen and stored at -80°C until analysis.

210 Cell culture supernatant cytokines were quantified (pg/ml) using the commercially
211 available Millipore Canine Cytokine/Chemokine Magnetic Bead Panel (Millipore Sigma, St.
212 Louis, MO and the Bio-Plex® 100/200™ System at the University of Connecticut. The cytokine
213 panel was previously validated to measure cytokines in several pinniped species (Levin et al.
214 2014). Samples were prepared and analyzed according to the manufacturer's instruction with
215 quality control measures. All quality control values were within the manufacturer's specified
216 concentration ranges for each run.

217

218 *2.5. Statistics*

219 The effect of *in vitro* exposure on each response variable (e.g. proliferation or cytokine
220 concentration) was assessed using linear mixed effects models (LME). Exposure
221 concentration was set as the fixed effect and sea lion ID was set as the random-effect in order
222 to account for the natural variation in individual responses. The unexposed control (complete
223 DMEM alone) was set as the reference concentration and was used to compare all other
224 treatments. Concentration−response relationships were described with log−logistic models. All
225 statistical and graphical analyses were carried out using R version 3.5.3 (R Development Core

226 Team, 2019) with the *nlme* and *DRC* packages and the graphical package *ggplot2* (Pinheiro et
227 al., 2017; Ritz et al., 2015; Wickham, 2016). The null hypothesis was rejected at $\alpha = 0.05$.
228

229 3. Results

230 *3.1. Lymphocyte flow cytometric profile*

231 A representative scatterplot and density plot of the flow cytometric profile of SSL
232 PBMCs is shown in Figure 1 and confirmed that thawed buffy coats contained viable PBMCs.
233 PBMCs were easily distinguished based on relative cell size (forward scatter) and granularity
234 (side scatter), as previously observed for pinnipeds (Bogomolni et al., 2016a; Bogomolni et al.,
235 2016b).
236

237 *3.2. Mitogen-induced lymphocyte proliferation*

238 The effects of MeHg+ on lymphocyte proliferation are illustrated in Figure 2. Without
239 mitogen, spontaneous lymphocyte proliferation was significantly increased at 0.01 µg/ml
240 (112% of control; p=0.0004) and 0.1 µg/ml (112% of control; p=0.0004) $MeHg^+$. ConA-induced
241 lymphocyte proliferation was significantly increased at 0.001 µg/ml (107% of control; p=0.0119)
242 and 0.01 µg/ml (111% of control; p<0.0001) $MeHg^+$. LPS induced lymphocyte proliferation was
243 decreased at 0.1 µg/ml (76% of control; p<0.0018) MeHg+.
244

245 *3.3. Cell culture supernatant cytokine concentrations*

246 Cytokine expression in the presence of MeHg+ is illustrated in Figure 3. Of the 13
247 cytokines measured using the canine cytokine kit, concentrations were above the minimum
248 detection limits (Table 1) for six cytokines, including IFNγ, IL-6, IL-10, TNFα, IP-10, and KC-
249 like. All other cytokines were below the minimum detection limit and were not further analyzed.

250 For IFNγ, 0.1 µg/ml MeHg+ reduced ConA-induced expression (69% of control; p=0.0021). For
251 IL-10, 0.1 µg/ml MeHg+, reduced both unstimulated expression (60% of control; p=0.039) and
252 LPS-induced expression (58% of control; p=0.0024). For IL-6, 0.1 µg/ml MeHg+ reduced both
253 ConA-induced expression (56% of control; p=0.0015) and LPS-induced expression (78% of
254 control; p=0.0009). For TNFα, 0.01 µg/ml MeHg+ reduced LPS-induced expression (92% of
255 control; p=0.0136), while 0.1 µg/ml MeHg+ reduced both ConA-induced expression (50% of
256 control; p=0.0001) and LPS-induced expression (84% of control; p=0.0001). No significant
257 changes in cytokine expression were detected for IP-10 and KC-like (data not shown).
258

259 **4. Discussion**

260 This study describes the effects of *in vitro* MeHg+ exposure on lymphocyte proliferation
261 and cytokine expression for SSL pups in Alaska (United States). For both measures of immune
262 function, the highest MeHg+ concentration tested, 0.1 µg/ml, significantly reduced either
263 mitogen-induced lymphocyte proliferation or cytokine expression. However, only modest
264 changes were detected for lymphocyte proliferation (no more than +/- 15% of control), while
265 changes in cytokine expression were more marked, with reductions of up to 50% in some
266 cases.
267

268 *4.1 Lymphocyte proliferation*

269 In previous studies using harbor seal and human PBMCs (Das et al., 2008), the
270 reduction in mitogen-induced lymphocyte proliferation occurred at ~1.1 µg/ml MeHg+, which
271 was approximately ten times higher than the highest concentration used in the current study,
272 0.1 µg/ml, resulting in a greater than 75% reduction in proliferation. However, in the same
273 previous study, the concentrations that caused a decrease in lymphocyte proliferation also

274 induced a significant increase in cell mortality, which could suggest that direct cytotoxicity
275 contributed to the decrease in proliferation the authors observed in that study. Preliminary data
276 from this study showed that 1 µg/ml MeHg+ resulted in a marked decrease in PBMC viability,
277 therefore, 0.1 µg/ml was chosen as the highest concentration tested in this current study. In a
278 different study using captive harbor seals, *in vitro* exposure of blood lymphocytes to MeHg+
279 between 0.125 to 0.5 µg/ml (0.6 and 2.3 µM) resulted mostly in a decrease in lymphocyte
280 proliferation, although with a different T cell mitogen, pokeweed mitogen (PWM; 2 µg/ml)
281 (Kakuschke et al., 2008).

282 The MeHg+ concentration that resulted in a 50% decrease in ConA-induced lymphocyte
283 proliferation (inhibitory concentration, IC_{50}) was reported as 0.17 and 0.12 µg/ml for harbor and
284 greys seals, respectively (Desforges et al., 2016), much higher than IC_{50} values reported for
285 beluga whales and bottlenose dolphins, both at 0.039 µg/ml (Desforges et al., 2016). In the
286 present study, no concentration reduced proliferation by more than 50%, hence, the IC_{50} is
287 presumably above 0.1 µg/ml MeHg+. These data suggest that the threshold for
288 immunosuppressive effects may be higher in SSL pups, compared to some species of
289 cetaceans and pinnipeds, at least for effects on lymphocyte proliferation. Mechanisms involved
290 in the detoxifying of mercury in these species, including the role of selenium, may account for
291 observed differences in IC_{50} values and should be further explored. Although statistically
292 significant changes were observed in lymphocyte proliferation, the magnitude of change was
293 no more than 15% above or below unexposed control cells. It is unclear at this time if these
294 changes would translate into biological significant health consequences in exposed animals.

295

296 *4.2 Cytokine expression*

297 To the authors' knowledge, this is the first study to measure cytokine expression, at the
298 protein level, from the cell culture supernatant of SSL PBMCs following *in vitro* exposure to

299 MeHg+. Notably, measurable increases in cytokine concentrations were detected when SSL
300 PBMCs were stimulated with the ConA and/or LPS mitogens, compared to unstimulated
301 PBMCs without *in vitro* MeHg+. exposure. These same cytokines were also shown to increase
302 their concentrations when PBMCs were stimulated with ConA and/or LPS in other pinniped
303 species, including harbor seals, grey seals, harp seals, and Weddell seals (Bagchi et al., 2018;
304 Levin et al., 2014), further demonstrating the likely cross-reactivity of the canine cytokine kit for
305 pinniped species.

306 IFNγ is an important T helper 1 (Th1) and pro-inflammatory cytokine, which activates
307 macrophages to help combat intracellular pathogens such as viruses, and 0.1 µg/ml MeHg+
308 reduced its concentration from ConA-induced PBMCs by about 30%. The decrease in IFNγ did
309 not correlate with a significant change in T lymphocyte proliferation at the same MeHg+
310 concentration. Interestingly, at the two lower concentrations tested, an increase in T
311 lymphocyte proliferation did not necessarily translate into an increase in this Th1 cytokine.

312 A second pro-inflammatory cytokine, IL-6, is an important mediator of fever and of the
313 acute phase response and is secreted by both T and B lymphocytes. MeHg+ at 0.1 µg/ml
314 reduced its concentration from both T and B stimulated lymphocytes, 56% and 78%,
315 respectively. The decrease in IL-6 did not correlate with a significant change in T lymphocyte
316 proliferation at the same concentration (and did not increase at concentrations that increased T
317 cell proliferation), but the decrease in IL-6 did follow a decrease in B lymphocyte proliferation
318 (0.1 µg/ml). Whether the latter is correlative or causal warrants further investigation.

319 A third pro-inflammatory cytokine, TNFα, is involved in systemic inflammation and the
320 acute phase response and can be secreted by CD4 lymphocytes, as well as macrophages.
321 MeHg+ at 0.1 µg/ml notably reduced its concentration from T stimulated lymphocytes by 50%,
322 while both 0.01 and 0.1 µg/ml MeHg+ reduce its expression in B stimulated lymphocytes, but

323 by only up to 15%. Again, as for IFNγ and IL-6, a decrease in TNFα did not correlate with a
324 significant change in T lymphocyte proliferation (and did not increase at concentrations that
325 increased T cell proliferation). Similar to IL-6, a decrease in TNFα did follow a decrease in B
326 lymphocyte proliferation at the same MeHg+ concentration (0.1 µg/ml).

327 IL-10 can help down-regulate a Th1 immune response as well as enhance B cell
328 survival, proliferation, and antibody production, and 0.1 µg/ml MeHg+ reduced its expression in
329 LPS-stimulated PBMCs, as well as unstimulated PBMCs. Notably, 0.1 µg/ml MeHg+ also
330 reduced B lymphocyte proliferation, and whether this relationship is correlative or causal
331 warrants further investigation. It is interesting to speculate whether a failure to down-regulate a
332 Th1 response was responsible for the increase in T lymphocyte proliferation, however, this is
333 difficult to confirm solely from *in vitro* experiments, but is worth further investigation.

334 Taken together, significant and distinct reductions in cytokine concentrations were
335 detected, especially those involved in pro-inflammatory cytokines, which may increase the risk
336 for pathogen infection and/or disease susceptibility in this species as previously documented
337 (Burek et al., 2005). In addition, the magnitude of change for cytokine secretion was more
338 pronounced in T stimulated lymphocytes than B stimulated lymphocytes, however, the
339 decrease in cytokines in B stimulated lymphocytes followed a decrease in B stimulated
340 lymphocyte proliferation and these relationships warrant further study. As with lymphocyte
341 proliferation, it is unclear at this time if these changes in cytokine concentrations would
342 translate into adverse health consequences in exposed animals.

343 In one other report that assessed the impact of MeHg+ on cytokine expression in
344 pinnipeds (at the mRNA level) the expression of cytokines IL-2, IL-4 and TGF-β was
345 investigated in harbor seal (*Phoca vitulina*) lymphocytes by real time quantitative PCR (n=5) at
346 concentrations of ~0.05 and 0.25 µg/ml (Das et al., 2008). IL-2 and TGF-β appeared highly

347 sensitive to *in vitro* MeHg exposure in regard to their dramatic decreases in gene expression at
348 0.2 and 1 µM, compared to control. However, IL-4 showed an increasing trend from control to
349 1 µM. From both the current and previous study, cytokines appear to be sensitive to the effects
350 of MeHg+ upon *in vitro* exposure, resulting in mostly a decrease in their expression at both the
351 molecular and protein levels.

352 It is important to note that the above *in vitro* experiments where conducted with PBMCs
353 isolated from whole blood, and *in vitro* studies assessing the impact of MeHg+ on lymphocyte
354 proliferation covered a range of Hg concentrations, including physiologically relevant
355 concentrations as reported in whole blood. However, Hg compartmentalization studies in
356 bottlenose dolphin (*Tursiops truncatus*) whole blood have reported 95% of total mercury
357 associated with the packed cells (primarily erythrocytes), while only 5% was in plasma (Correa
358 et al., 2013). Similarly, Aberg et al. (1969) reported 10 fold higher MeHg+ concentrations in
359 erythrocytes compared to serum after oral administration in humans. Thus,
360 compartmentalization of MeHg+ in whole blood may impact the exposure level of lymphocytes
361 *in vivo*, potentially reducing its immunotoxic effects. Furthermore, additional work should
362 investigate the partitioning of THg and MeHg+ among the white blood cells.

363

364 **5. Conclusions**

365 For the four cytokines above, a distinct decrease in cytokine expression corresponded
366 with a modest reduction in T and B lymphocyte proliferation at the highest MeHg+
367 concentration tested. At this point, it is unclear if there is a causal relationship between
368 decreases in lymphocyte proliferation and decreases in cytokine expression. It could be that
369 cytokine expression is more sensitive to the effects of $MeHg^{+}$ than is lymphocyte proliferation
370 and is a better indicator of the immunotoxic effects induced by MeHg+. Clearly, more data are
371 needed from *in vitro* and/or *in vivo* experiments, to translate what magnitude change (measure

in increase or decrease) in an immune function(s) would result in an adverse health outcome in exposed marine mammals. Further, compartmentalization of MeHg+ in whole blood may affect lymphocyte exposure levels *in vivo*. An additional study is underway to measure the same cytokines as above in SSL pup serum and assess their relationships with measured whole blood $MeHg^{+}$ concentrations. Together, these data will help direct efforts to accurately assess the impact of $MeHg^{+}$ on the immune health of SSL pups.

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524

525

526 **Figure Legend**

527

528 **Figure 1.** Representative scatterplot (left) and density plot (right) of SSL peripheral blood
529 leukocytes after cryopreservation. Leukocyte sub-populations were easily distinguished based
530 on forward scatter (relative size; x-axis) and side scatter (relative complexity; y-axis).
531 Neutrophils (R1) are large and complex; lymphocytes are smaller and less complex (R3), while
532 monocytes (R2) are slightly larger than lymphocytes and less complex than neutrophils.
533 Lymphocytes and monocytes make up the peripheral blood mononuclear cell (PBMC)
534 population.

535

536 **Figure 2.** Effects of increasing concentrations of monomethyl mercury (MeHg+) (unexposed
537 control, 0.001, 0.01, and 0.1 µg/ml) on mitogen-induced lymphocyte proliferation upon *in vitro*
538 exposure. Data are presented as a percent of the unexposed control and percent change
539 values are shown below the bar graph. *, $p<0.05$ compared to unexposed control for each
540 mitogen. Concanavalin A (ConA, 1 µg/ml) is a T cell mitogen and lipopolysaccharide (LPS, 5.0
541 µg/ml) is a B cell mitogen.

542

543 **Figure 3.** Effects of increasing concentrations of monomethyl mercury (MeHg+) (unexposed
544 control, 0.001, 0.01, and 0.1 µg/ml) on cytokine concentrations following *in vitro* exposure of
545 peripheral blood mononuclear cells (PBMC) to mitogens. No mitogen (NM), Concanavalin A
546 (ConA, 1 µg/ml), and lipopolysaccharide (LPS, 5.0 µg/ml). *, $p<0.05$ compared to unexposed
547 control for each mitogen.

Figure 1.

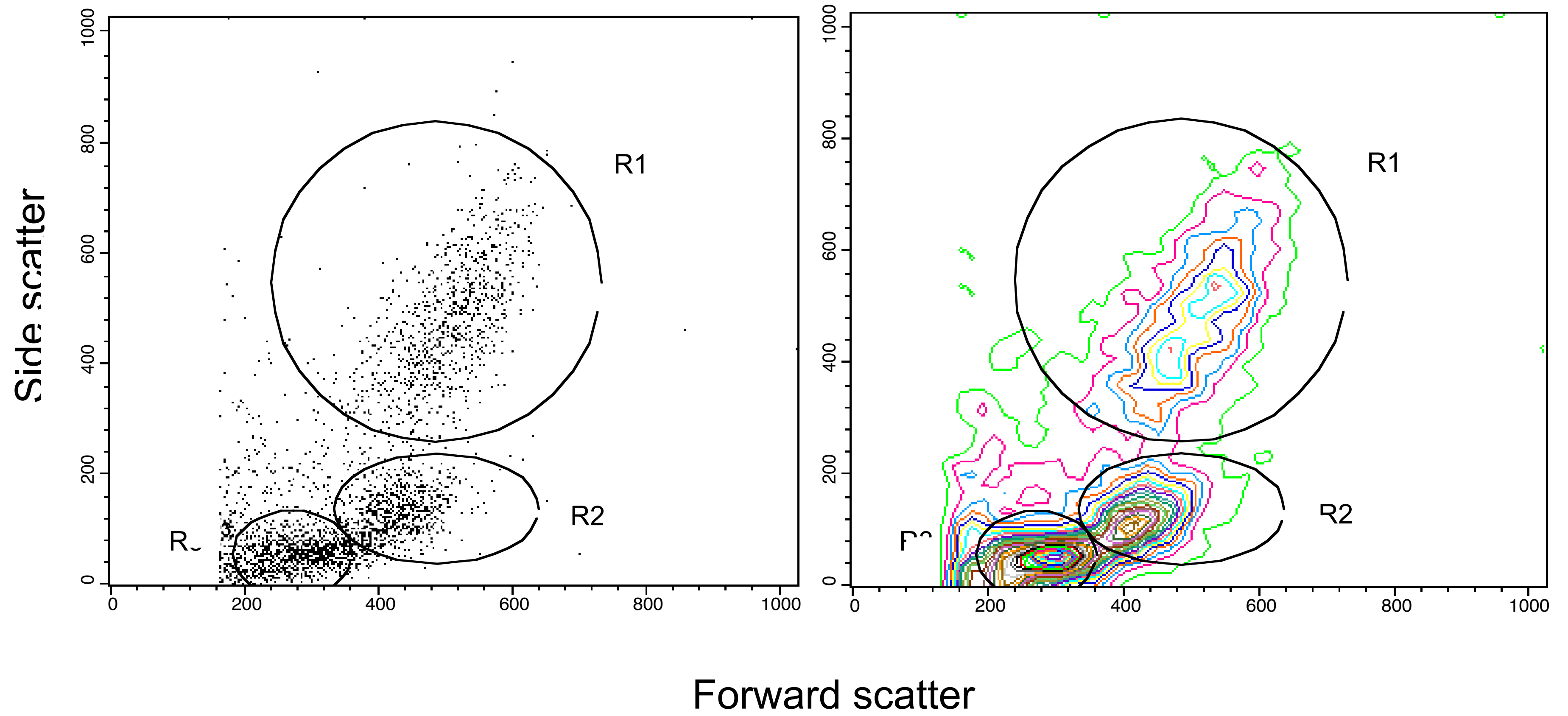
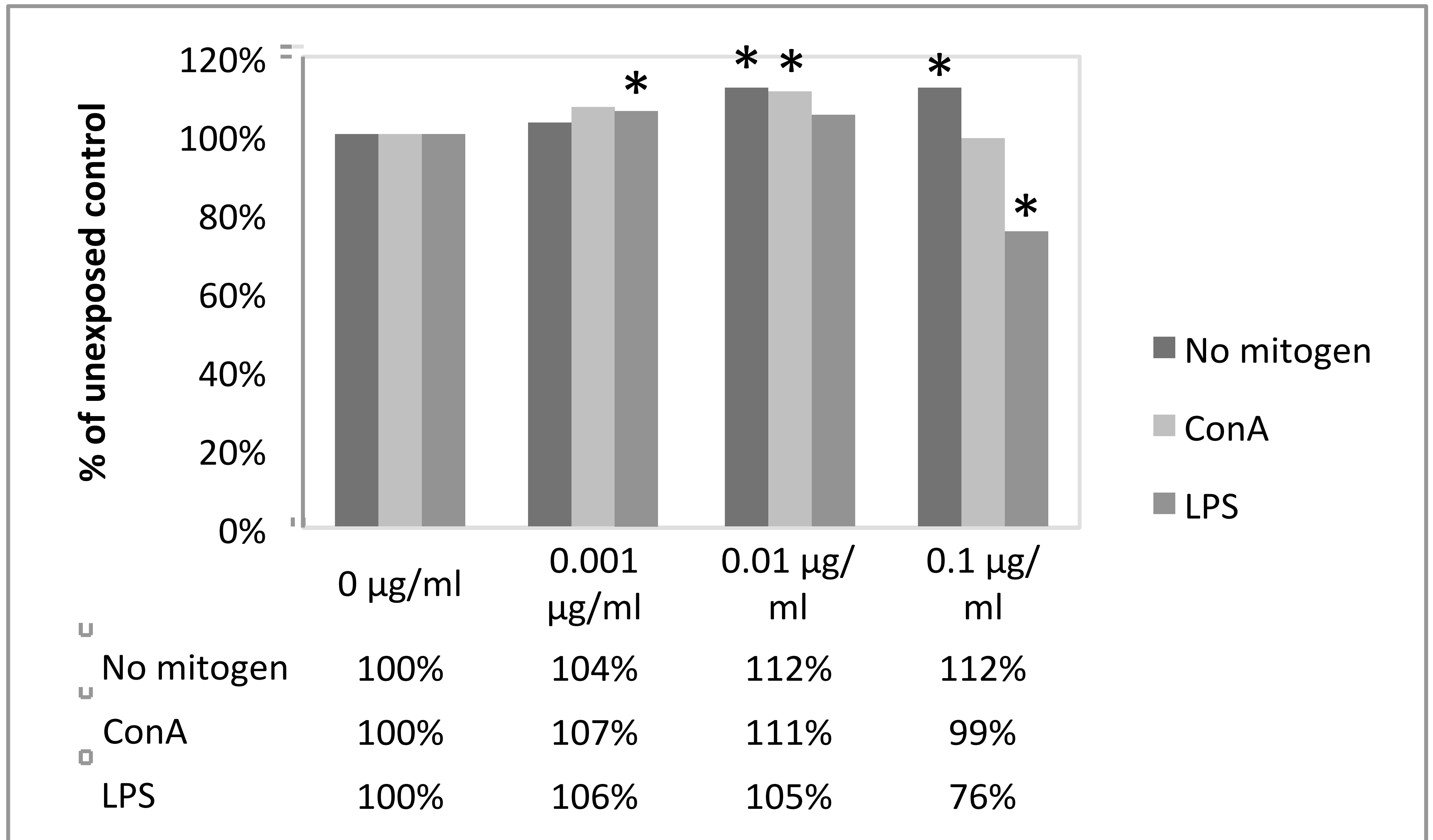
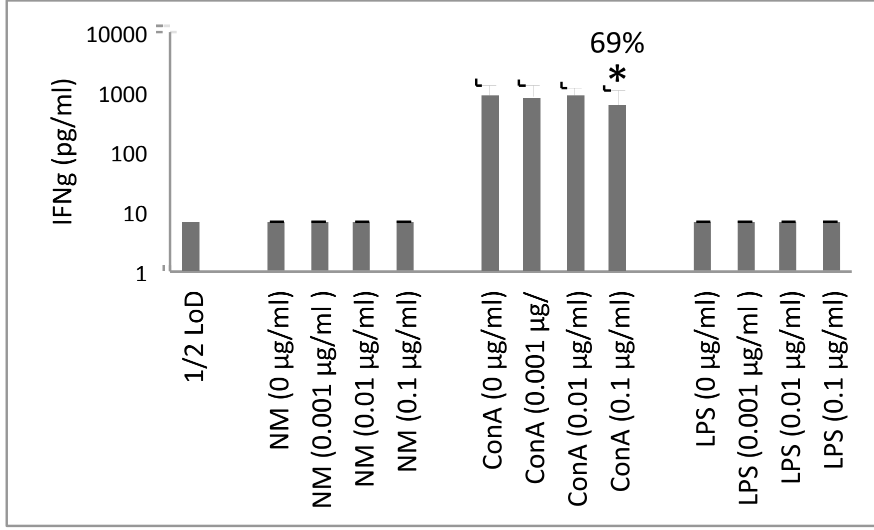


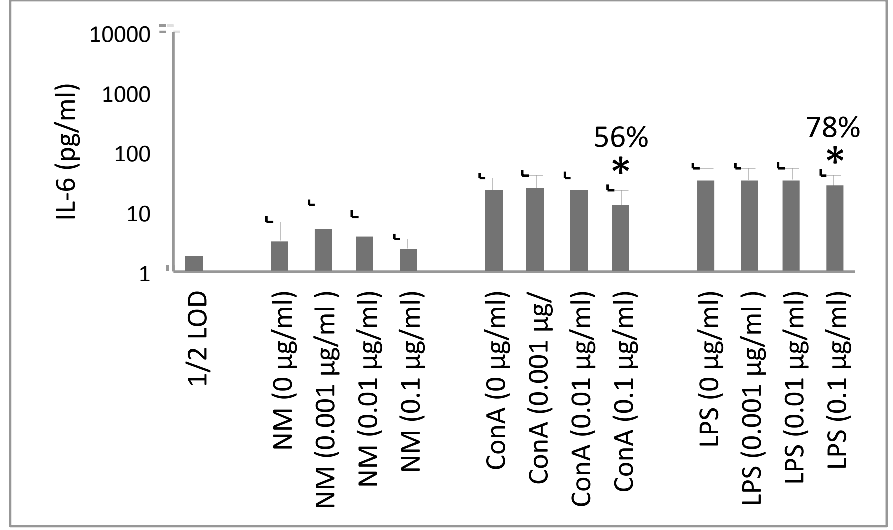
Figure 2.

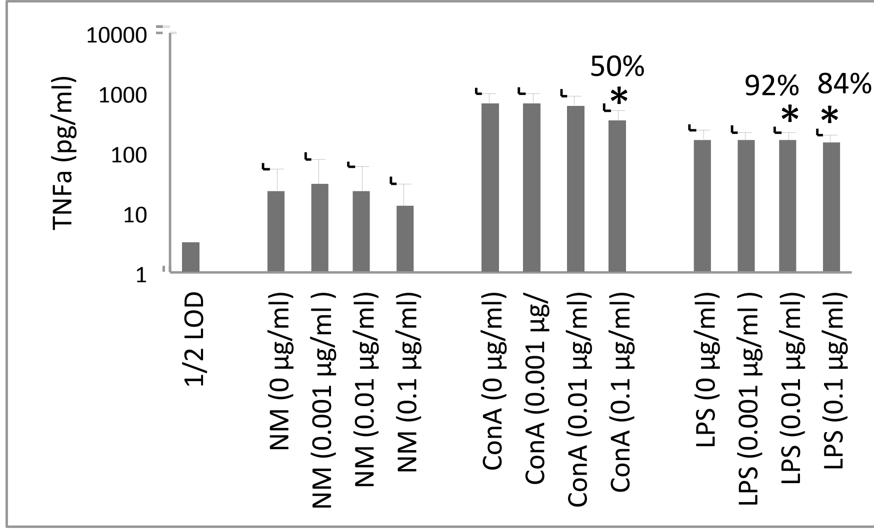


	0 μg/ml	0.001 μg/ml	0.01 μg/ml	0.1 μg/ml
No mitogen	100%	104%	112%	112%
ConA	100%	107%	111%	99%
LPS	100%	106%	105%	76%

Figure 3.







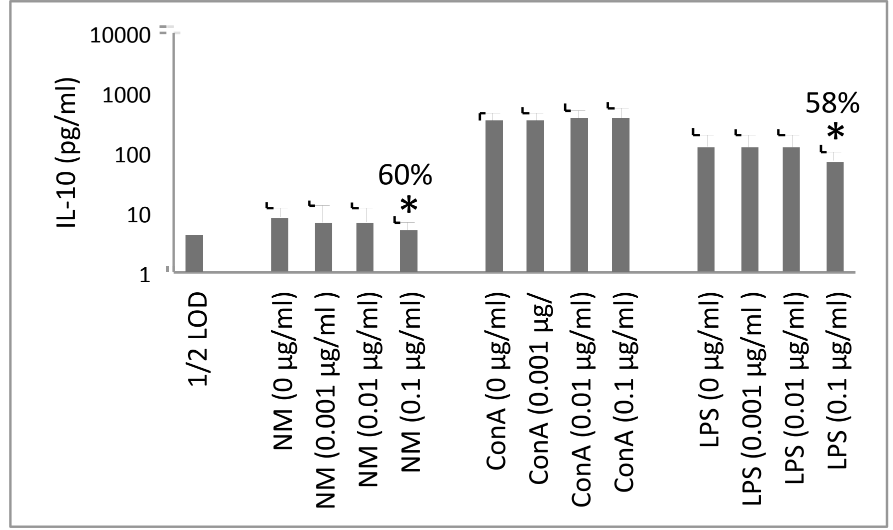


Table 1. Minimum detection concentrations for 13 cytokines

Cytokine	Minimum detection limit (pg/ml)
IFNγ	13.6
IL-10	8.5
IL-6	3.7
TNFα	6.1
IP-10	3.2
IL-2	3.5
IL-7	7.5
IL-8	21.7
IL-15	9.0
IL-18	5.8
KC-like	5.3
MCP-1	21.0
GM-CSF	9.2

Plasma
White blood cells
Red blood cells
MeHg+