In Vitro - In Vivo Comparison of the Toxicity of Diesel Emission Primary Particles Derived from Biodiesel Blends of Different Feedstock Origin

Subramanian Karthikeyan[1], Dalibor Breznan[1], Prem Kumarathasan[2], Errol Thomson[1], Debbie Rosenblatt[3], Greg Rideout[3], Renaud Vincent[1]
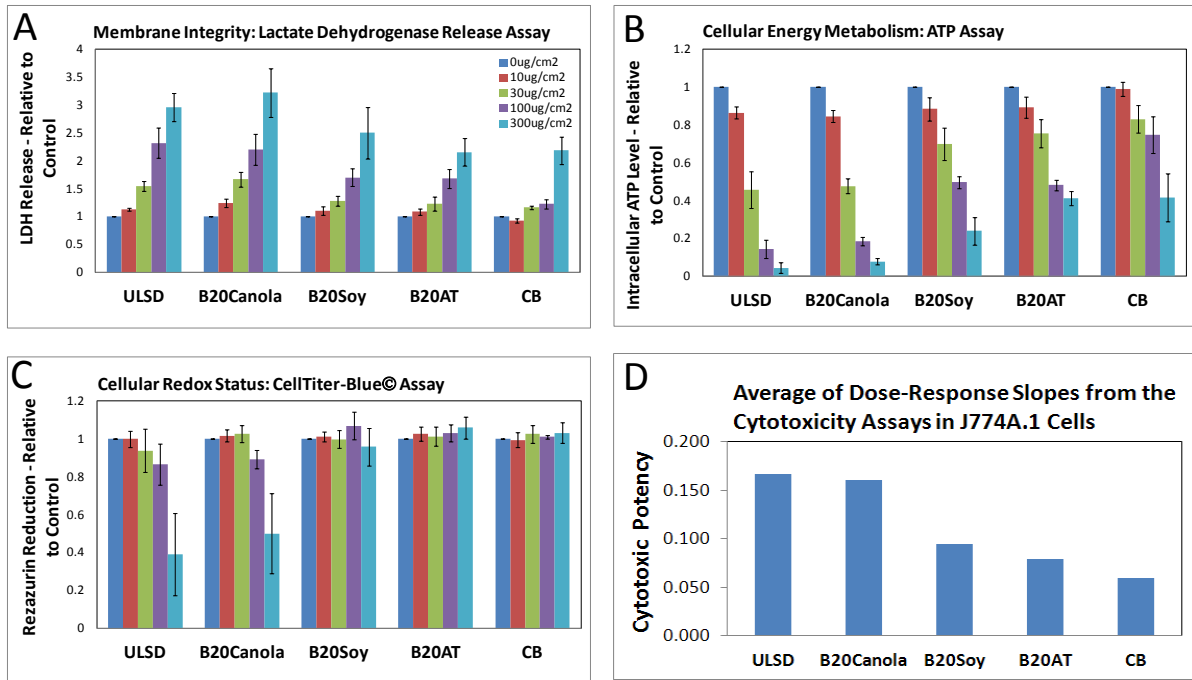
[1]Inhalation Toxicology Laboratory, Hazard Identification Division, Environmental Radiation and Health Sciences Directorate, Health Canada; [2]Proteomics Laboratory, Mechanistic Studies Division, Environmental Radiation and Health Sciences Directorate, Health Canada; [3]Emissions Research and Measurement Section, Air Quality Research Division, Science and Technology Branch, Environment Canada

With the growing interest in the development and use of biodiesels as a substitute to petroleum diesel, potential modification of the toxicity of engine emissions resulting from the combustion of biodiesel or biodiesel-blended diesel fuels is a subject of concern (1). Physicochemical properties of both particle and gas phases of the diesel exhaust are a function of biodiesel feedstock (2), and hence feedstock may impact emission toxicity. We have previously shown that the toxic potency of particles in cellular bioassays (3) or in animals by inhalation exposures (4) is closely related to their chemical composition. In this work, the relative toxicity of diesel emission particles derived from the combustion of commercial ultra-low sulfur diesel (ULSD) or 20% (v/v) blends (B20) of biodiesels of different feedstock origin in ULSD was assessed using *in vitro* and *in vivo* platforms.
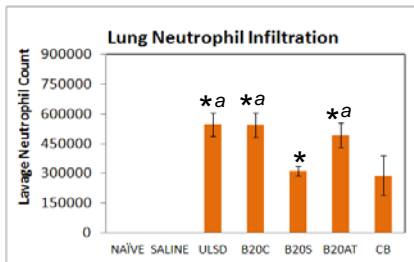
A heavy-duty diesel engine (Caterpillar C11, 2004 emission standard, OEM diesel oxidation catalyst) was operated at 25% and 50% loads using ULSD or B20 blends (20% v/v in ULSD) of canola (B20C), soy (B20S) or animal tallow (B20AT) biodiesels. Diesel emission primary particles (DEP) were collected on Teflon® filters, extracted with ethanol followed by water and freeze-dried. Particles were reconstituted in a particle suspension buffer, and the suspensions for the 25% and 50% loads were pooled at 2:1 ratio for use in cytotoxicity assays in J774A.1 cells *in vitro* and intratracheal instillation in mice. Carbon black (CB) was tested as a surrogate of the carbon structure of soot but lacking the bound polyaromatic hydrocarbon. *In vitro* toxicity of the particles was assessed using a murine macrophage (J774A.1) cell line. Briefly, J774A.1 cells were seeded in black-walled, clear bottom 96-well plates at a density of 40,000 cells/well in 100µl of Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum, and incubated for 24h at 37°C and 5% CO_2, with humidification. The cells were exposed to 0, 10, 30, 100 and 300 $\mu g/cm^2$ doses of ULSD, B20C, B20S, B20AT, and CB particles prepared in 100µl of serum-free cell culture media. The cells were incubated for an additional 24h period before assessment of cytotoxicity using an integrated bioassay that combined assays of cellular redox status (CellTiter Blue® Assay), energy metabolism (ATP assay) and membrane integrity (intracellular lactate dehydrogenase release assay). The cytotoxic potency (β) of the particles was determined from fold-effect = $(dose + 1)^\beta$ where β represents the slope of the dose response curve (3). *In vivo* toxicity was assessed in BALB/c mice (male, weight 26.4 ± 0.2g, mean ± standard error) by intratracheal instillation (under isofluorane anesthesia) of 250µg of DEP or CB (n=6). A group of animals were instilled with saline as the vehicle control (n=6). Finally, a group of naïve animals (no IT instillation, no anesthesia) was also used to confirm that saline instillation itself was benign (n=4). Necropsies were conducted 24h post instillation for the assessment of a number of toxicologically relevant endpoints.

In vitro cytotoxicity analyses in the murine macrophage cell line J774A.1 showed that the potency of DEP was affected by the biodiesel feedstock in the B20 blends. Among the biodiesel blends, B20C generated the most potent particles, whereas tallow and soy biodiesel blending resulted in emission particles of reduced cytotoxicity by comparison to B20C (Figure 1 A-C). In general, particle toxicity contrasts derived from the three cytotoxicity assays were similar. Averaging the cytotoxic potencies of individual particles across the three different bioassays resulted in a consensus cytotoxicity ranking of ULSD > B20C > B20S > B20AT > CB (Figure 1D).
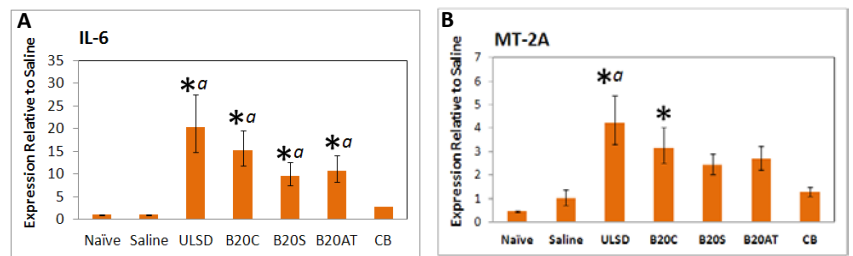
Analyses of bronchoalveolar lavage (BAL) collected from animals exposed to the particles showed that DEP exposure resulted in significantly (p<0.05) higher BAL neutrophil counts, compared to CB or saline exposures (Figure 2). ULSD and B20C, the particles with highest cytotoxic potency *in vitro* were also the
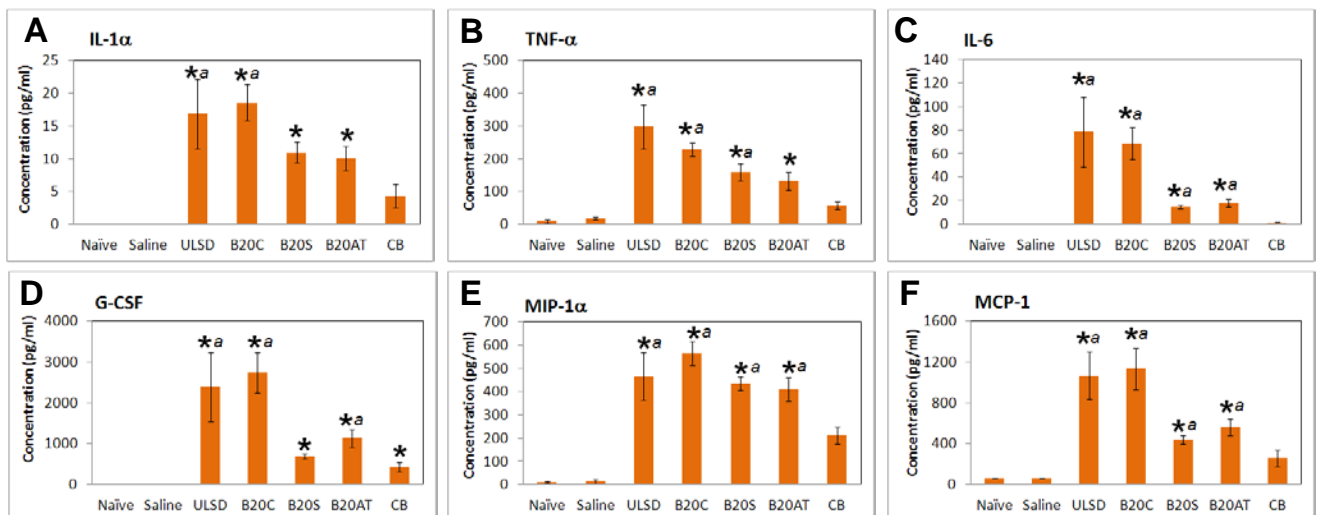
Figure 1: Cytotoxicity of different diesel emission particles and carbon black as measured by the panel of assays for lactate dehydrogenase release (A), cellular ATP (B) and cellular redox status (C). Toxic potencies of the different particles in each assay were calculated from the slopes of the dose-response curves using the equation fold effect = $(dose + 1)^{\beta}$. A consensus cytotoxic potency was then obtained across the three assays by averaging the assay-specific potencies (D).

Figure 2: Lung neutrophil infiltration assessed by bronchoalveolar lung lavage. Asterisks, significantly (p<0.05) different from the saline group. *a*, significantly different from CB. One-way ANOVA with particles as factor.

Figure 3: Effect of *in vivo* instillation of DEP and CB on the expression of interleukin-6 (IL-6) and metallothionein-2A (MT-2A) genes in the lung. Asterisks, significantly (p<0.05) different from the saline group. *a*, significantly different from CB. One-way ANOVA with particles as factor.

Figure 4: Changes in the levels of a panel of BAL cytokines in response to DEP or CB instillation. An * indicates significant (p<0.05) difference from the saline group; Asterisks, significantly (p<0.05) different from the saline group. *a*, significantly different from CB. One-way ANOVA with particles as factor.

Table 1: Correlation of *in vivo* endpoints of inflammation and oxidative stress to *in vitro* toxic potency

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most inflammogenic *in vivo* as measured by neutrophil influx in the lung. DEP instillation also increased the expression of a number of genes in the lung including interleukin-6 (IL-6) involved in inflammation and metallothionein-2A (MT-2A) involved in metal and oxidative stress responses (Figure 3). Consistent with neutrophilia and enhanced lung inflammatory gene expression, BAL levels of cytokines interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α) and IL-6 involved in inflammation and immune response, granulocyte colony-stimulating factor (G-CSF) and macrophage inflammatory protein-1α (MIP-1α) involved in neutrophil activation, and monocyte chemotactic protein-1(MCP-1) involved in macrophage chemoattraction were also significantly ($p<0.05$) elevated in response to DEP instillation (Figure 4). Across all of the *in vivo* endpoints, ULSD and B20C were the most potent. CB generally did not cause significant effects, while B20S and B20AT were of lower potency than ULSD and B20C. Note that saline control animals did not differ from naïve animals indicating that IT instillation of saline *per se* was innocuous. Magnitude of the lavage neutrophils, gene expression, and cytokine levels correlated significantly to the *in vitro* cytotoxic potencies of the particles (Table 1).

In conclusion, these data show that biodiesel feedstock can impact the toxicity of primary particulate emissions. Good correlation observed between *in vitro* toxic potency and *in vivo* neutrophil response and a number of other biological endpoints of health relevance validates the utility of our *in vitro* assay panel as a relevant and useful platform for assessing the pulmonary toxicity and other potential biological consequences of exposure to diesel emission particles. Further analyses should reveal feedstock-related as well as mechanistic drivers of toxicity for biodiesels and the data will contribute to the information used in selecting feedstock for biodiesel use.

References:

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METHODS

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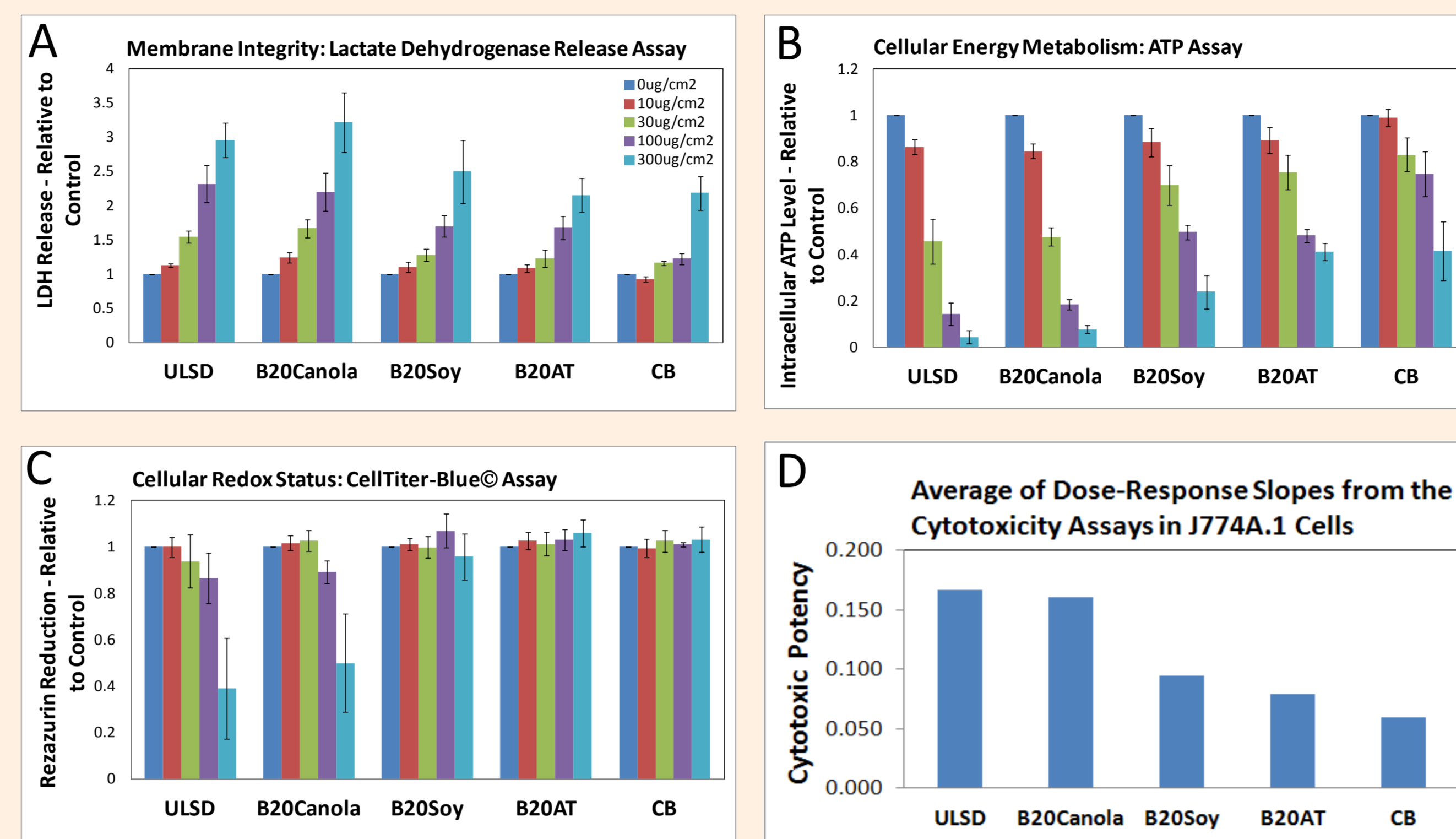
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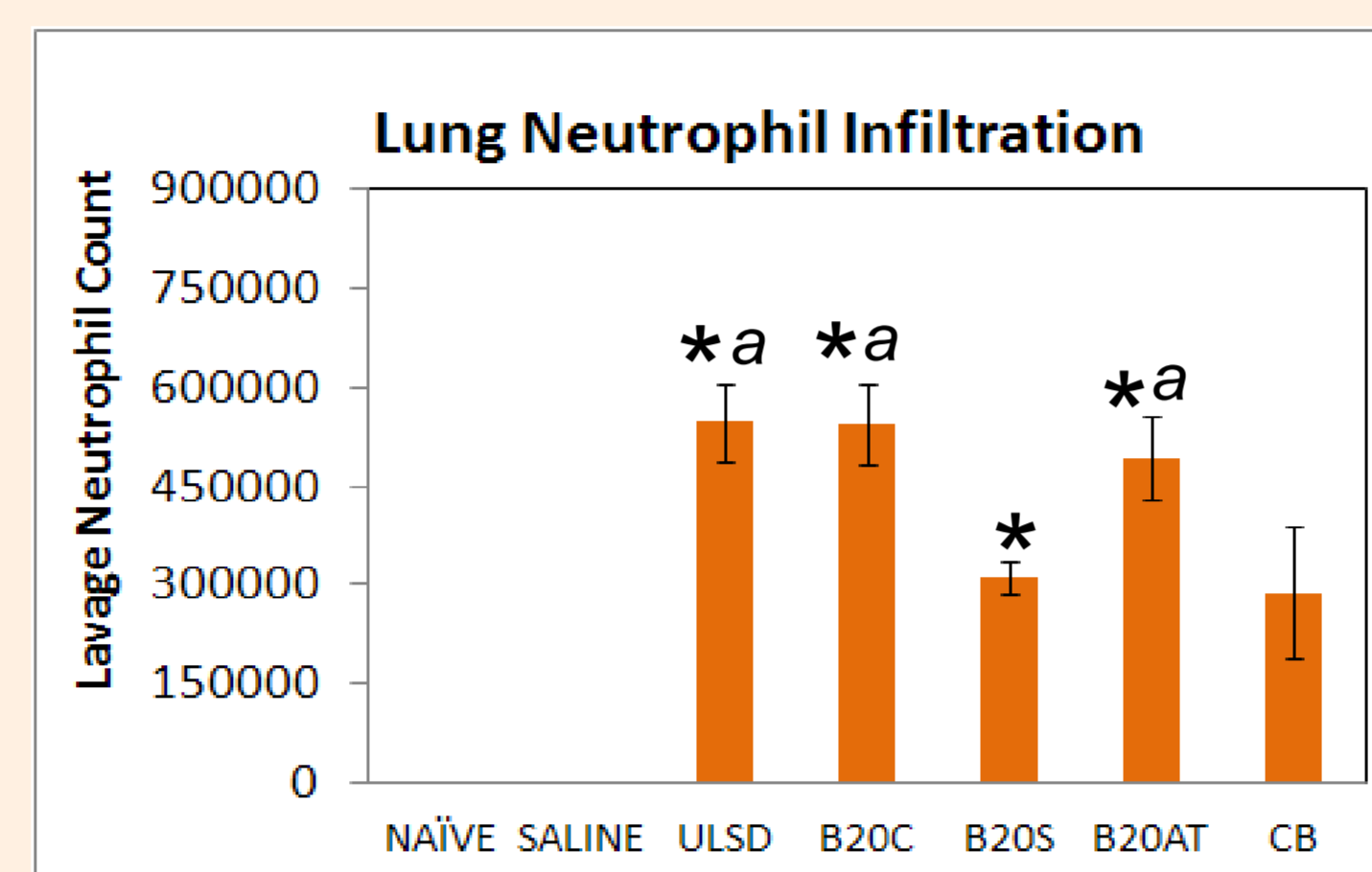
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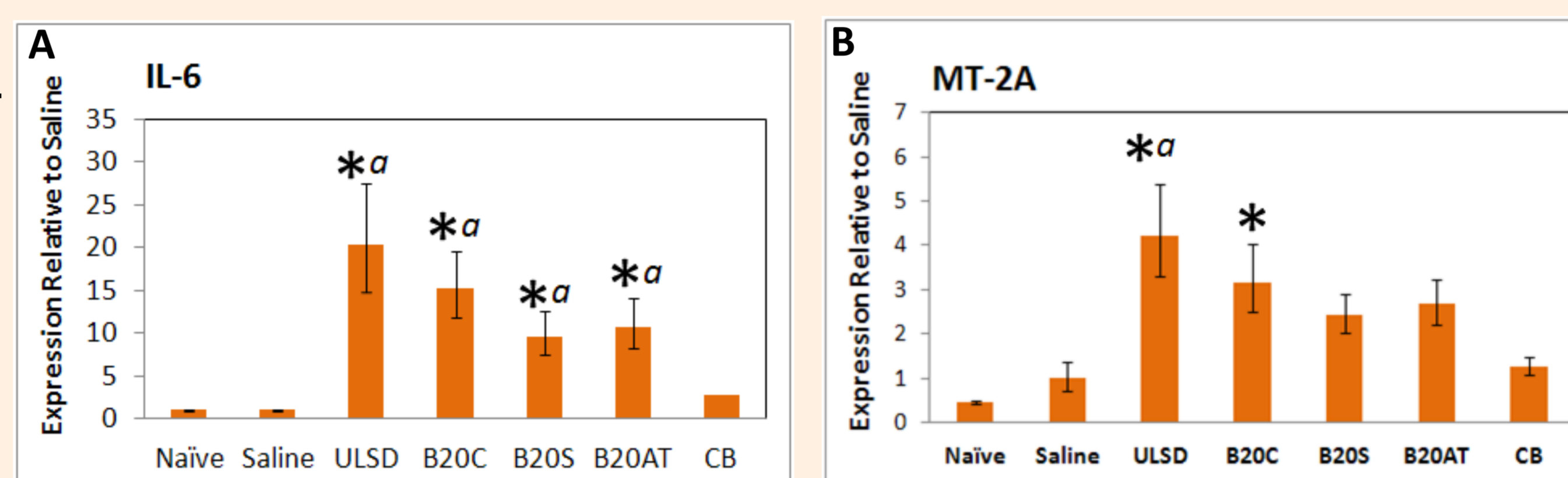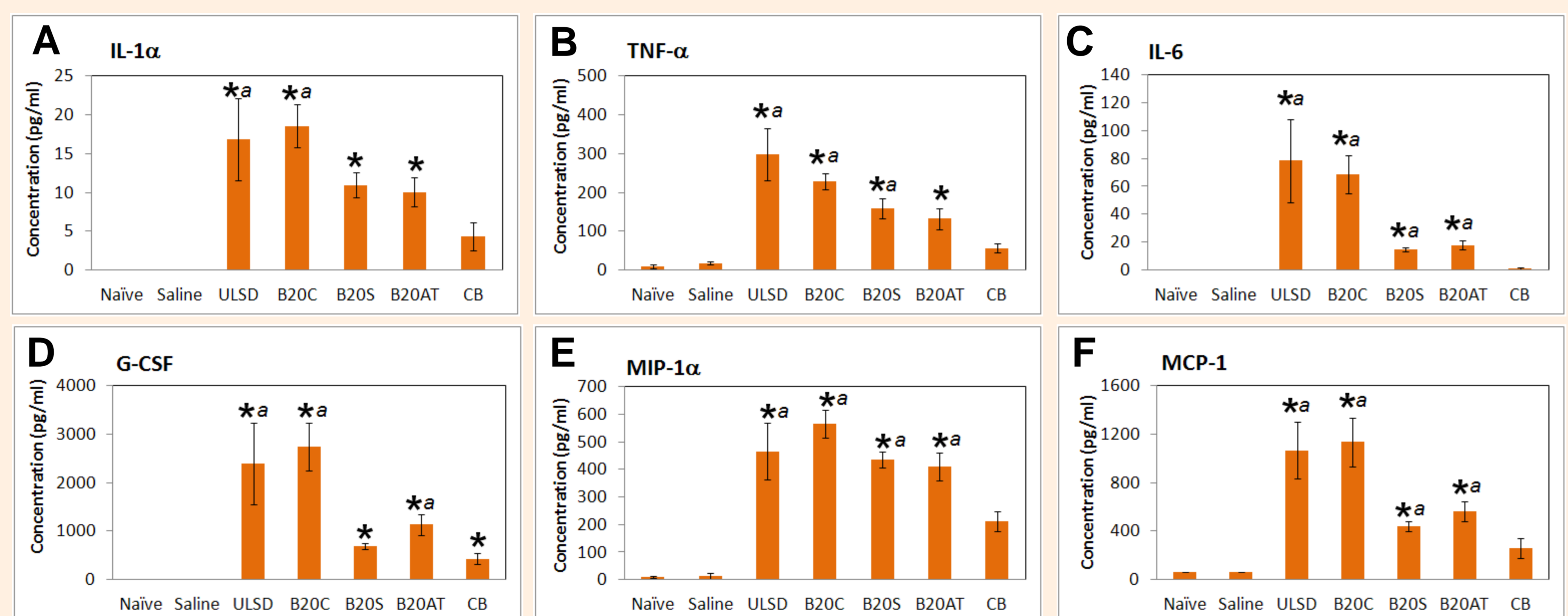
RESULTS AND DISCUSSION

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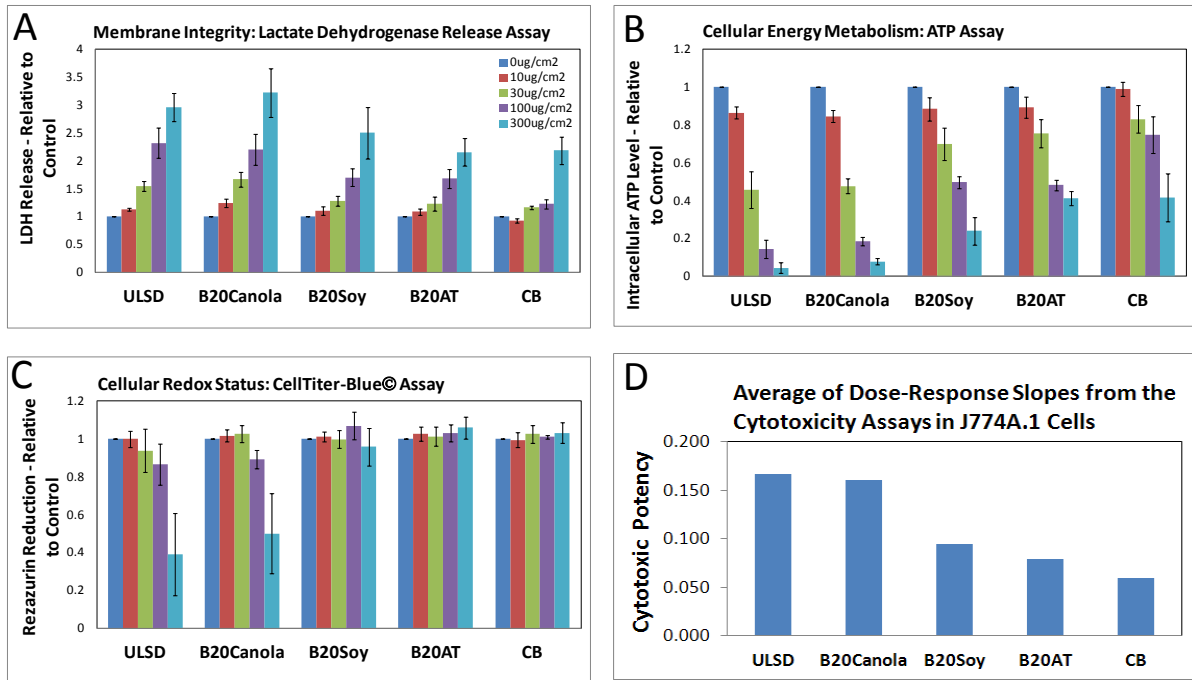

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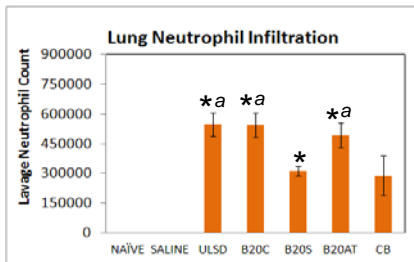
CONCLUSIONS

These data show that biodiesel feedstock impacts the toxicity of primary particulate emissions. Good correlation observed between *in vitro* toxic potency and *in vivo* neutrophil response and a number of other biological endpoints of health relevance validates the utility of our *in vitro* assay panel as a relevant and useful platform for assessing the pulmonary toxicity and other potential biological consequences of exposure to diesel emission particles. Further analyses should reveal feedstock-related as well as mechanistic drivers of toxicity for biodiesels and the data will contribute to the information used in selecting feedstock for biodiesel use.
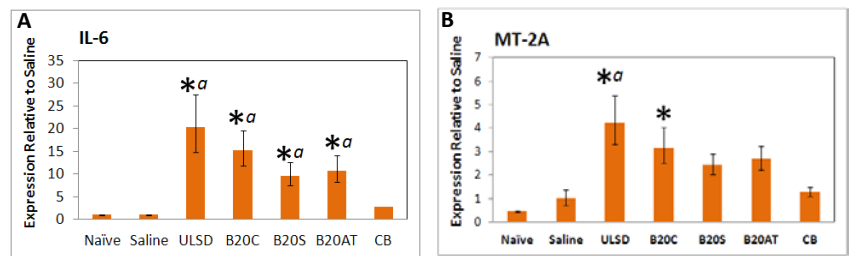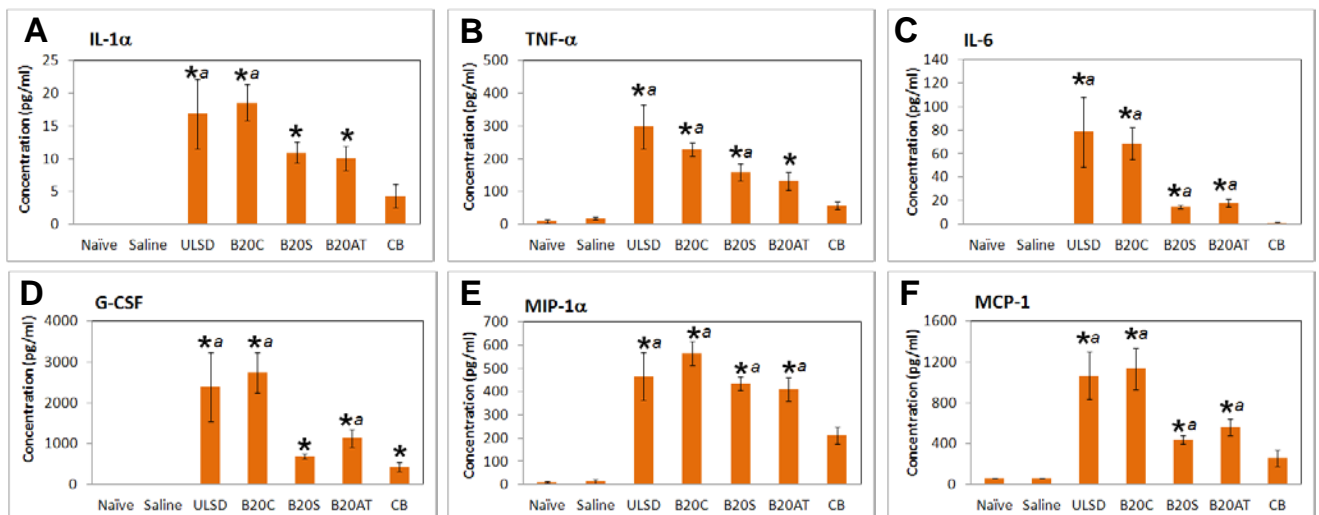
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