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The Gulf War-associated compounds DEET, pyridostigmine bromide, and permethrin, in combination with desert dust metals have subtle adverse effects on blood brain barrier function in an *in-vitro* model

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ABSTRACT

In an effort to better understand factors that may play a role in the development of Gulf War Illness, we examine the effects of 3 organic compounds and 9 metals of interest to the military population that served during the Gulf War on the function and stability of a model of the human blood brain barrier (BBB). Using a BBB model consisting of human brain endothelial cell and astrocyte cell co-cultures in a transwell membrane system, we exposed transwells to organic compounds and metals either individually or as a combination of an organic followed by a metal and assessed changes in BBB function. Assays included trans-epithelial electrical resistance (TEER), translocation of two different sized fluorescence markers or metals of interest from the luminal side to the abluminal side of the BBB, and changes in expression of tight-junction proteins and cytokines. Several co-exposures resulted in reduced TEER values, although minimal or no effects were seen on transfer of the fluorescent markers and metals across the membrane. Expression of tight junction proteins was also unaffected, but some cytokines increased synergistically with several co-exposures. Of all combinations, pyridostigmine bromide, permethrin, and depleted uranium had the most consistent effects. The cause of Gulf War Illness is not well understood, and interactions of pesticides, prophylactics, and metals may factor into symptom development more than any single exposure. Understanding these interactions may also shed light on risk factors for other populations potentially exposed to similar contaminants in their environment.

KEYWORDS: Gulf War Illness, pyridostigmine bromide, permethrin, DEET, desert dust, metal, toxicity, blood brain barrier.

INTRODUCTION

Gulf War Illness (GWI) refers to the chronic multi-symptom illness characterized by cognitive problems, fatigue, and muscle pain affecting close to 700,000 US military personnel who served in the Persian Gulf War in 1990-1991. A variety of exposures to common in-theater chemicals have been suggested for causing GWI, but the large number of personnel affected and the multi-symptom nature of GWI does not fit any single known exposure scenario, suggesting instead a multi-exposure condition [1].

Three organic compounds in particular have surfaced as potential candidates: Pyridostigmine bromide (PB), an investigational nerve gas prophylactic self-administered to military personnel; N,N-Diethyl-*m*-toluamide (DEET), an insecticide/ insect repellent spray widely used by the military;

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and permethrin (PM), a synthetic pesticide spray also commonly used during the Gulf War [2-11]. Alone and if recommended use is followed these chemicals have been shown to be safe, but at higher levels they can be toxic [12, 13] and there is no way to know the actual dose exposure to personnel as this wasn't carefully tracked. PB in particular, alone or in combination with DEET, PM, or stress, could be linked with illnesses in Gulf War veterans, particularly with disruption of the blood brain barrier (BBB) and neurological deficits [14-30].

Another potentially important component comes from respiratory exposure to local fine-grained sand particles, deemed "desert dust," which contained high levels of a variety of metals as well as microbial contaminants [31-35] which were deemed to be exceeding the Military Exposure Guidelines (MEG) over 97% of the time for personnel during Operation Iraqi Freedom and Operation Enduring Freedom [36]. Many studies have shown that internalized metals are capable of crossing the BBB, especially in the presence of other pollutants that increase BBB permeability, and can induce neuronal injury, behavioral changes, and neurological diseases [37-61]. Local populations do not experience GWI symptoms, further supporting a hypothesis of a multi-exposure scenario.

We hypothesize that PB, PM, or DEET adversely affects BBB permeability, allowing inhaled metals to enter the brain and result in neuronal injury. To test this, as shown previously in Hoffman & Kalinich [62] we determined sub-toxic doses of 3 organic compounds (10 μM) and 9 metals of interest (1 μM) for human BMEC and astrocyte cell cultures, assessing viability and markers of oxidative stress in response to these compounds alone or in combined exposures. Here, we expand upon this foundation and assess the effects on BBB function and inflammatory response using an *in vitro* transwell BMEC and astrocyte co-culture model.

MATERIALS AND METHODS

Cells and cell culture

Human astrocytes: Normal human astrocytes (male) were purchased from ScienCell Research Labs (Carlsbad, CA, USA). Cells were maintained in Dulbecco's Minimal Essential Medium (D-MEM) with Glutamax (Gibco, Grand Island, NY, USA) supplemented with N2 Supplement CTS (Gibco) and Fetal Bovine Serum One-Shot (10%, Gibco) and were not used past cell passage 8 for all experiments.

Human brain endothelial cells: Human brain microvascular endothelial Cells (BMECs, male) were obtained from Cell Systems (Kirkland, WA, USA) and cultured in Vasculife VEGF Basal Medium supplemented with the associated VEGF LifeFactors Kit (Lifeline Cell Technology, Frederick, MD, USA) with the following modifications. The LifeFactors Kit fetal bovine serum was not used and only 10 ml of the LifeFactors-provided L-glutamine was used as a supplement. In addition, iCell Endothelial Cell Medium Supplement (Cellular Dynamics International, Madison WI, USA) was added to a final concentration of 10%. Cells were not used past cell passage 8 for all experiments.

Culture set-up: Stock cell cultures were maintained in tissue culture flasks (75 cm^2 area) at 37 ºC in a humidified atmosphere of 5% CO_2. For endothelial cells, the flasks were treated with 6 ml of Quick-Coat (Angio-Proteomie, Boston, MA, USA) to provide a suitable substrate for attachment. For both cell lines, medium was refreshed every other day and cells were subcultured when 70-80% confluent based upon direct microscopic observation. Briefly, medium was aspirated from the flasks and the flasks were washed once with phosphate-buffered saline (PBS, Gibco). After aspirating the PBS, 3 ml of StemPro Accutase Cell Dissociation Reagent (Gibco) was added and the flask left at room temperature for up to 8 min to allow the cells to dissociate from the flask. Once detached, fresh medium was added, and the cell suspension pipetted to break up any clumps. The cell suspension was centrifuged at 240 x g for 10 min after which the cell pellet was resuspended in a suitable amount of the appropriate medium and transferred to new flasks. Cell numbers and viability were determined using trypan blue (Gibco) exclusion with the Bio-Rad Model TC-20 Cell Counter (Hercules, CA, USA).

Organics and metals for treatment

Organic compounds of interest: The following compounds were tested: pyridostigmine bromide (PB), permethrin (PM), and N,N-diethyl-*m*-toluamide (DEET) (all Sigma Chemical, St. Louis, MO, USA). PB, PM, and DEET were only slightly soluble, if at all, in water. Stock solutions were prepared in dimethylsulfoxide (DMSO, Invitrogen, Carlsbad, CA, USA) before dilution to appropriate working concentrations in cell culture medium (final concentration of DMSO: 0.01%). Media controls include the same final concentration of DMSO.

Metals of interest: Metals to be tested included aluminum (Al as aluminum chloride hydrate), iron (Fe as iron chloride hexahydrate), depleted uranium (DU as uranyl nitrate hexahydrate), nickel (Ni as nickel chloride hexahydrate), cobalt (Co as cobalt chloride hexahydrate), copper (Cu as copper chloride dihydrate), strontium (Sr as strontium chloride hexahydrate), manganese (Mn as manganese chloride tetrahydrate), and zinc (Zn as zinc chloride). All metals were obtained from Sigma Chemical except for uranyl nitrate hexahydrate which was from Fluka Chemical (Ronkonkoma, NY, USA). Metals were dissolved in the appropriate cell culture medium and diluted to provide proper working concentrations.

In vitro Blood-Brain Barrier (BBB) model system

An *in vitro* system using transwell inserts was used to assess BBB functionality after organic and metal exposure. Briefly, 24-well plates with Corning (Corning, NY, USA) Costar 6.5 mm inserts with a polyester membrane (0.4 μm pore size) were used to co-culture astrocytes on the abluminal (brain) side and endothelial cells on the luminal (blood) side of the transwell. On the basolateral (abluminal) side of the inverted transwell, 80 μl of a 3.125 x 10^5 cells/ml suspension was added and the transwells incubated for 4 h at 37 ºC and 5% CO_2. After that, the plate was flipped and 100 μl of 1 x 10^6 cells/ml was added to the apical (luminal) side of the transwell. The plates were incubated for 24 h as described above. After that, as a measure of the integrity of tight junctions and thus the overall health of the cells, Trans-Epithelial Electrical Resistance (TEER) was determined. Measurements were taken prior to addition of the organic compounds, 24 h after organic compound addition, and 24 h after metal addition. Resistance (R, in ohms, Ω) measurements were taken with the EVOM2 Epithelial Volt-Ohm Meter (World Precision Instruments, Inc., Sarasota, FL, USA) using the STX2 chopsticks electrode to determine the trans-epithelial electrical resistance (TEER) value, which is calculated as $(R_{target} - R_{blank})$ x (M_{area}), where M_{area} is the membrane area of the transwell (0.33 cm^2); TEER units are expressed as $\Omega \bullet cm^2$.

A second assessment of BBB functionality used a modification of previously published procedures [63, 64] to determine perturbations of tight junctions by using fluorescently labeled dextran molecules of different molecular weights. In this procedure, transwell plates were prepared as above. At 24 h after metal exposure (and 48 h after organic exposure), 75 μl of medium from the apical side of the transwell was removed for determination of inflammatory cytokines and 200 μl removed from the basolateral side for metal translocation analysis and replaced with 200 μl of fresh astrocyte medium. The remaining medium on the apical side of the transwell was aspirated, the transwell washed with 100 μl of PBS, and then 100 μl of endothelial medium containing 100 μg/ml of fluorescein (FITC)-labeled dextran (4 kDa, Sigma) and rhodamine (TRITC)-labeled dextran (70 kDa, Sigma) was added and the plates incubated for 2 h. After that 2 x 100 μl aliquots were removed and placed in wells of a black 96-well plate for fluorescence measurements. Using the BioTek Synergy Model H1M Multimodal Plate Reader with GEN5 Software, BioTek Instruments (Winooski, VT, USA), excitation/ emission values for fluorescein (485 nm/544 nm) and rhodamine (520 nm/590 nm), standard curves, and the amount of the 4 kDa and 70 kDa dextran that crossed the *in vitro* blood-brain barrier could be determined.

Metal analysis by inductively coupled plasma mass spectroscopy (ICP-MS)

All compounds used in this study were of the highest grade available. Plastic ware and other disposables were also obtained from Thermo Fisher Scientific. Samples were dissolved in

ultrapure nitric acid (Fisher Scientific, Newark, DE, USA) and metal content determined using an inductively coupled plasma-mass spectrometer (XSeries 2 ICP-MS System, ThermoFisher, Madison, WI, USA) equipped with a Cetac ASX520 Autosampler (Cetac Technologies, Omaha, NE, USA). High-pressure liquid argon, 99.997%, was used for the plasma gas. The instrument was calibrated with external standards of the appropriate metal standard (SPEX CertiPrep, Metuchen, NJ, USA) in 2% HNO_3. The sample probe was washed with a constant flow of 2% nitric acid between measurements to prevent carryover. Quantitative analysis was obtained by reference to the slope of the calibration curve (counts per second/ng per liter) as well as an internal standard. Limit of Detection (LoD)/Limit of Quantitation (LoQ), in ppb, are as follows: Al - 0.38/0.44; Fe - 1.08/1.85; Ni - 0.17/0.21; Sr - 0.01/0.05; Co - 0.03/0.06; Cu - 0.24/0.54; Mn - 0.45/0.6; Zn - 2.80/3.01; U - 0.02/0.07.

BBB protein marker and inflammatory cytokine expression

The following procedure was used to provide samples to assess changes in protein expression relevant to BBB function and inflammatory cytokine response of endothelial cells treated with metals after the presence or absence of Gulf War-associated organic compounds. The basic experimental design was to plate the cells on Day 1, replace the medium on Day 2, add organic compounds (if required) on Day 3, add metals (if required) on Day 4, and harvest on Day 5. Briefly, brain microvascular endothelial cells were harvested as above and re-plated on 6-well tissue culture plates at a concentration of 2 x 10^5 cells/well and the plates returned to the incubator. The next day the medium was aspirated and replaced with fresh medium (2 ml/well). If required, on the next day the appropriate organic compound was added to a non-toxic dose (as determined above) and the plates returned to the incubator. The next day, if required, appropriate metals were added, again at a non-toxic dose as previously determined and the plates returned to the incubator. The next day, 1 ml of medium was removed from each well, placed in a 1.5 ml tube and centrifuged at 18,400 x g for 10 min to pellet any detached cells. The supernatant (0.8 ml) was then removed, placed in a fresh 1.5 ml tube and stored at -80 ºC until needed for the cytokine assays. The remaining medium in the wells was aspirated and the wells washed with 2 ml of PBS (Gibco). After aspirating the PBS, 1 ml of Accutase was added to each well and the plates incubated at room temperature for 8 min to detach the cells. The cell suspension was placed in a 1.5 ml tube and centrifuged at 18,400 x g for 10 min to pellet the cells. After removing the supernatant, the cell pellets were stored at -80 ºC until required for protein expression analysis.

Protein expression using the ProteinSimple Wes: Cell pellets were re-suspended in RIPA buffer (Thermo, Waltham, MA, USA) plus Halt Protease and Phosphatase Inhibitor Cocktail (Thermo) and homogenized in a Bullet Blender (Next Advance, Troy, NY, USA) with 0.5 mm glass beads (NextAdvance) (settings speed 6, 5 min x 2 runs) and then centrifuged at 1340 x g for 10 min. Total protein from each sample supernatant was measured by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA), in triplicate, against a BSA standard curve, on a spectrophotometer (BioTek Synergy Model H1M Multimodal Plate Reader with GEN5 Software), and read at 595 nm. Proteins of interest were quantified using an automated capillary-based size sorting chemiluminescent system 'WES' from ProteinSimple (San Jose, CA, USA). All procedures were performed using manufacturer's reagents (ProteinSimple, 12-235 kDa kit) according to the user manual with some adjustments: samples are aliquoted to 1 µg/µl before mixing 4 µl with 1 µl fluorescent master mix, then denatured at 94 ^oF (34.4^oC) for 4 min, given a quick spin, and loaded on the plate at 4 µl per well. In run settings, stacking time was changed to 18 sec, separation time to 31 min, and antibody diluent time to 30 min. Antibodies against the proteins occludin, ZO-1, and claudin-5 were obtained from Thermo Fisher, while those against beta-actin were purchased from Cell Signaling (Danvers, MA, USA). Peak values were determined using Compass Software (ProteinSimple). Target proteins are presented as a ratio of the target protein expression normalized to within-sample β-actin expression in arbitrary units. The ProteinSimple Wes system uses capillary size

separation and chemiluminescent detection, and an example of the raw data and corresponding traditional Western blot image derived from it using the same cell cultures mentioned in this publication can be found in our previous publication (Hoffman & Kalinich) [62].

Cytokine determination using Meso Scale Discovery (MSD): The ability of the various treatments to induce various cytokines was assessed using the U-PLEX Human Biomarker Group 1 kit (Meso Scale Discovery, Rockville, MD, USA). The following biomarkers were assessed in the collected endothelial cell supernatants: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), and Interferon gamma (IF-γ). The Meso Scale U-PLEX uses proprietary linkers to couple to biotinylated capture antibodies. The complexes were self-assembled onto distinct spots on the U-PLEX assay plate. After sample (25 µl of cell supernatant) incubation and washing, an electrochemiluminescent detection antibody was added and the plate read in a Meso Scale Discovery QuickPlex Reader. Concentrations of the various cytokines were determined by comparison to a standard curve.

Statistical analyses

Analyses were performed using GraphPad Prism Software (version 8.4.2, La Jolla, CA, USA). There were treated wells per group (n = 4) for all experiments, and experiments were repeated independently 3 times each. One-way Analysis of Variance (ANOVAs) with Sidak's multiple comparisons post-hoc tests were performed, with specific group comparisons or any other statistical analyses noted. For metal transfer experiments, metal-only transfer values were compared to each corresponding organic+metal group within an organic exposure set using multiple t-tests and a false discovery rate (FDR) approach using the two-stage set-up method of Benjamini, Krieger, and Yekutieli with Q = 1%. Specific post-hoc *P* values less than 0.05 were considered significant.

RESULTS

We hypothesize that combined exposures of sub-toxic levels of DEET, PB, or PM adversely affect the viability of cells involved in the BBB, altering its permeability and function, potentially allowing metals solubilized from inhaled desert dust particles to enter the brain. To test this, a transwell BBB model using co-cultures of human BMEC and astrocyte cells were exposed to DEET, PB, or PM, followed by nickel, cobalt, strontium, zinc, manganese, copper, iron, aluminum, or depleted uranium and assessed for any changes in function and cytokine response.

Trans-epithelial electrical resistance (TEER) after exposure to organic and metal compounds

One of the major functions of the blood brain barrier is to prevent passive transport of compounds from the blood to the interstitial space of the brain. One way to measure this function is by measuring the trans-epithelial electrical resistance (TEER), which is representative of how tight of a barrier the BBB has formed between cells. The higher the measured resistance, the less passive transport is expected to occur. Using an *in-vitro* model of the BBB, the co-culturing of BMEC and astrocytes in a transwell plate, cells were grown over the course of several days and treated concurrently to account for changes over time in tight junction formation. On the first two days all cells received media alone to allow for optimal tight junction growth. The next day the media-only group received only media, and all other groups received media with the indicated organic. On the third day the media-only group received only media, the organic-only group received media with the indicated organic, and the organic plus metal group received media with the indicated organic and metal co-exposure. Although TEER readings were completed each day before media change, data presented here is from the final reading, with values normalized to a set of wells with no cell culture to control for resistance values of the membrane alone. Analysis is presented as a one-way ANOVA with Sidak multiple comparisons tests specifically for the media-only control vs the organic alone (i.e. effect of organic), and then the organic alone vs each organic plus metal group (i.e. does the metal have any effect beyond exposure to the organic compound) (Figure 1: A) DEET plus metals, ($F_{(10,153)} = 4.913$, $p < 0.0001$); B) PB plus metals, ($F_{(10,153)} = 5.50$, $p < 0.0001$); DEET plus metals, ($F_{(10,153)} = 9.825$, $p < 0.0001$).

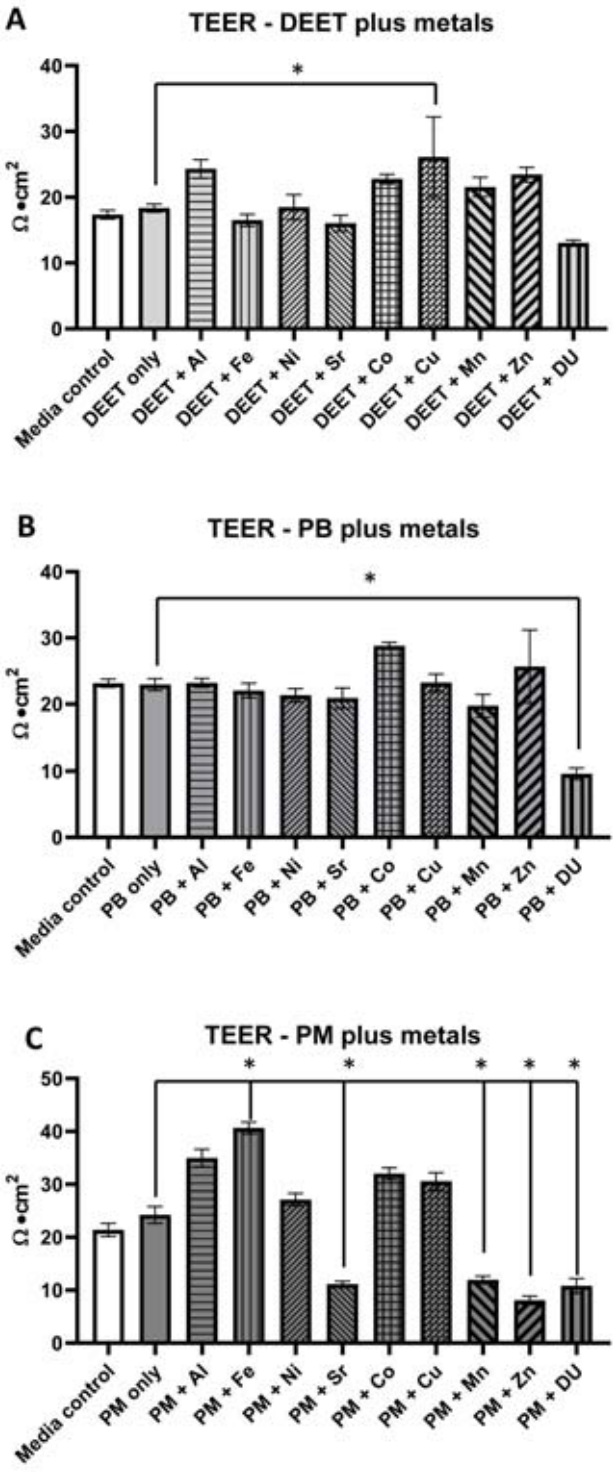

Figure 1. Trans-epithelial electrical resistance (TEER) of a BBB transwell model after exposure to organic and metal compounds. TEER values of resistance across a membrane with co-cultures of BMEC and astrocytes are given in $\Omega \bullet cm^2$. White bar represents media-only control. Solid bars indicate organic treatment, pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) DEET plus metals, B) PB plus metals, C) PM plus metals. * indicates significant p-value for comparison between groups indicated with connected lines.

DEET, PB, and PM alone had no significant effect on the TEER reading compared to the media-only control. DEET + Cu exposure significantly increased the TEER value over DEET alone (*p = 0.0039), suggesting a possible protective effect. PB + DU exposure significantly decreased the TEER value over PB alone (*p < 0.0001), suggesting DU may work synergistically with PB to make the BBB more leaky. Multiple metals had significant effects on TEER when combined with PM compared to PM alone. Fe increased TEER value (*p = 0.0004), while Sr, Mn, Zn, and DU all decreased TEER value (*p = 0.0092, *p = 0.0179, *p = 0.0005, and *p = 0.0012, respectively).

Fluorescence transfer after exposure to organic and metal compounds

Tracking movement of different-sized fluorescent molecules from the endothelial side of a BBB transwell model to the astrocyte side of the transwell can indicate if the integrity of the BBB is intact or compromised and leaky. BMEC and astrocytes were co-cultured in a transwell setup and exposed to a media-only control, each metal alone, an organic (DEET, PM, or PB) alone, or the organic plus each individual metal. A no-cell well condition was also included as a baseline for how much fluorescence transfer is obstructed by the transwell membrane alone. FITC dextran (4 kDa) or TRITC dextran (70 kDa) was then introduced to the endothelial side, and after incubation, the amount of fluorescence signal was measured in the astrocyte side. Analysis is presented as a one-way ANOVA with Sidak multiple comparisons tests specifically for the no-cell control vs media-only control (i.e. the baseline of how much fluorescence is prevented from crossing with the BMEC and astrocyte co-culture in place), the media-only control vs the metal alone or organic alone (i.e. effect of metal or organic), and then the organic alone vs each organic plus metal group (i.e. does the metal have any effect beyond exposure to the organic compound) (Figure 2: A) 4 kDa FITC, metals alone, $F_{(10,33)} = 28.65$, $p < 0.0001$; B) 70 kDa TRITC, metals alone, $F_{(10,33)} = 20.69$, $p < 0.0001$; C) 4 kDa FITC, DEET plus metals, $F_{(11,36)} = 74.77$, $p < 0.0001$; D) 70 kDa TRITC, DEET plus metals, $F_{(11,36)} = 79.83$, $p < 0.0001$; E) 4 kDa FITC, PB plus metals, $F_{(11,36)} = 34.60$, $p < 0.0001$; F) 70 kDa TRITC, PB plus metals, $F_{(11,36)} = 31.01$, $p < 0.0001$; G) 4 kDa FITC, PM plus metals, $F_{(11,36)} = 128.5$, $p < 0.0001$; H) 70 kDa TRITC, PM plus metals, $F_{(11,36)} = 86.67$, $p < 0.0001$).

For both sizes of fluorescent compound in all treatment experiments, the media-only control prevented fluorescent signal from passing across the membrane compared to the no-cell control (*p < 0.0001 for all eight sets). None of the metals and none of the organics (DEET, PB, and PM) alone had any significant effect on fluorescence transfer compared to the media-only control. Additionally, there were no combinations of an organic compound plus metal treatment compared to the corresponding organic compound alone with a significant effect on fluorescence control, suggesting no effect of organic compounds or organic plus metal exposure on the integrity of the BBB. We also note that only around half the 4 kDa FITC dextran was prevented from crossing the membrane by the media-only cell control compared to the no-cell control, whereas all of the larger 70 kDa TRITC dextran was prevented from crossing the barrier.

Metal transfer after exposure to organic and metal compounds

Similar to the fluorescence transfer, we wanted to assess how much of each metal was able to cross the BBB in conjunction with exposure to the organic compounds of interest. Transwells were set up with co-cultures of BMECs and astrocytes and exposed to 1 µM of a known amount of metal on the BMEC side of the transwell following exposure to each organic of interest or media alone to determine if exposure to the organic compound facilitated metal translocation. Metal concentration was measured on the astrocyte side of the transwell, and presented as a percentage of the total metal exposure (% translocated metal), presented in Figure 3. Of the metal-only exposures, all metals crossed the membrane to varying degrees with the exception of Fe, which did not transfer at all (one-way t-test comparing % translocated metal to a value of 0; Fe: no detectable metal crossed, Zn: *p = 0.0131, Al *p = 0.004, DU *p = 0.0003, all other metals: *p < 0.0001). The metal-only exposure groups served

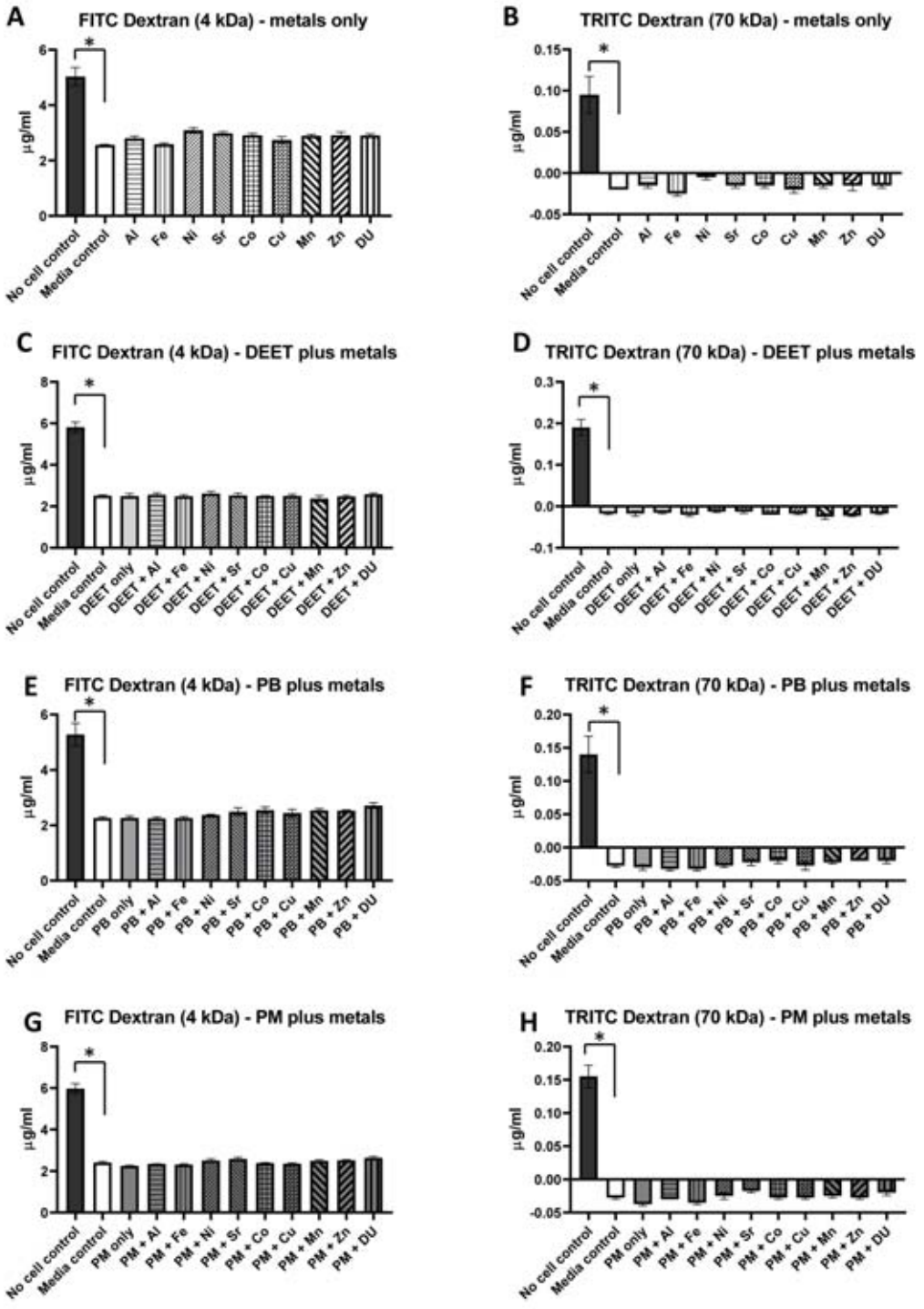

Figure 2. Transfer of fluorescent markers (4 kDa and 70 kDa) across a BBB transwell model after exposure to organic and metal compounds. Data presented as µg/ml of FITC dextran (4 kDa) or TRITC dextran (70 kDa) that crossed over to the astrocyte side of the BBB model transwell after a known concentration presented to the endothelial side after exposure to organic and metal compounds. Dark gray bar indicates no-cell control wells. White bar represents media-only control. Light gray bars indicate organic treatment, pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) DEET plus metals, B) PB plus metals, C) PM plus metals. * indicates significant p-value for comparison between groups indicated with connected lines.

as the control for each of the organic + metal comparisons.

Surprisingly, for all three organic + metal experimental sets, transfer of Cu, Co, and DU were decreased after each organic exposure compared to the metal exposure alone (DEET Figure 3A: Cu t-ratio = 5.079, *p = 0.0023; Co t-ratio = 9.667, *p < 0.0001; DU t-ratio = 11.32, *p < 0.0001; PB Figure 3B: Cu t-ratio = 4.527, *p = 0.004; Co t-ratio = 6.724, *p = 0.0005; DU t-ratio = 12.27, *p < 0.0001; PM Figure 3C: Cu t-ratio = 9.053, *p = 0.0001; Co t-ratio = 7.668, *p = 0.0003; DU t-ratio = 12.12, *p < 0.0001). Additionally, Mn transfer across the membrane was also decreased

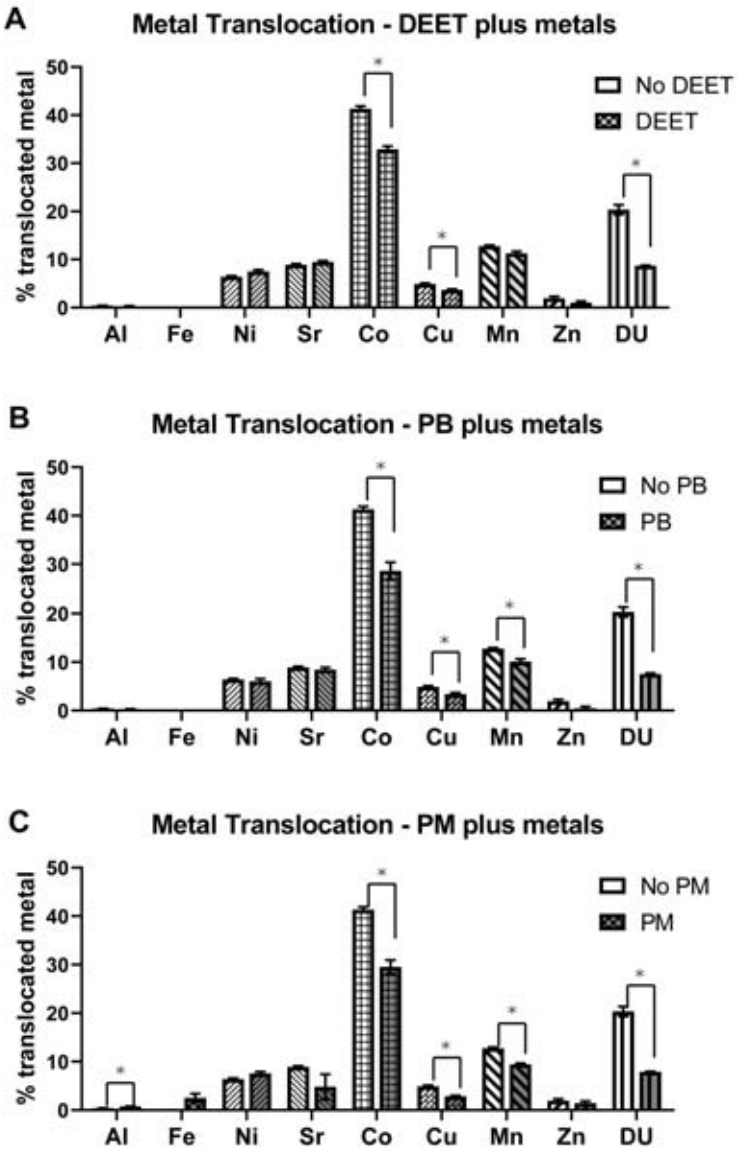

Figure 3. Transfer of metals across a BBB transwell model after exposure to organic and metal compounds. Data presented as percent of metal translocated to the astrocyte side of the BBB model transwell from a known concentration presented to the endothelial side after exposure to media or organic compounds. White bar represents media only control. Gray bars indicate organic treatment, pattern indicates subsequent metal treatment. A) DEET plus metals, B) PB plus metals, C) PM plus metals. * indicates significant p-value for comparison between groups indicated with connected lines.

when in combination with both PB (Figure 3B: t-ratio = 4.468, *p = 0.0043) and PM (Figure 3C: t-ratio = 9.602, *p = 0.0001) compared to Mn transfer alone. Al transfer was increased after exposure to PM (Figure 3C: t-ratio 5.839, *p = 0.0009). It is also of interest to note that although it did not reach statistical significance, Fe transfer across the membrane also increased from 0% to 2.53% after exposure to PM.

BBB tight junction protein expression after exposure to organic and metal compounds

We examined the effects of organic and metal compound exposure to BMEC cell cultures on the expression of BBB-related proteins, occludin, ZO-1, and claudin 5 because of their function in BBB tight junction formation and thus, permeability [65]. In each experimental set, BMECs were cultured alone and exposed to a media control, an organic compound alone, or the organic compound in combination with one of the metals. Statistical analysis is presented as a one-way ANOVA of the entire set, with post-hoc Sidak's multiple comparisons test comparing media to the organic compound alone, to determine the effect of the organic compound, followed by individual comparisons of the organic compound versus the organic plus each individual metal, to determine any further effects of metal exposure. Graphs are presented as arbitrary units, where the chemiluminescence expression level of the target protein is normalized to the expression level of β-actin within each sample.

For the DEET + metals experimental set, there were no significant differences in expression of any protein after any treatment, with the exception of DEET + Fe compared to DEET alone on occludin (Figure 4, ANOVA - $F_{(10,32)} = 1.658$, p = 0.1347, Sidak comparison – p = 0.0348). Occludin expression would be expected to decrease if the BBB were disrupted, but DEET + Fe actually resulted in a higher expression of occludin than DEET alone, suggesting a possible protective effect, or possibly overexpression to compensate for a loss of function. All protein results are shown in Figure 4 (B) ZO-1, $F_{(10,33)} = 2.126$, p = 0.0507; C) claudin 5, $F_{(10,33)} = 0.4763$, p = 0.8933). For the PB + metals' experimental set, there were no significant differences in the

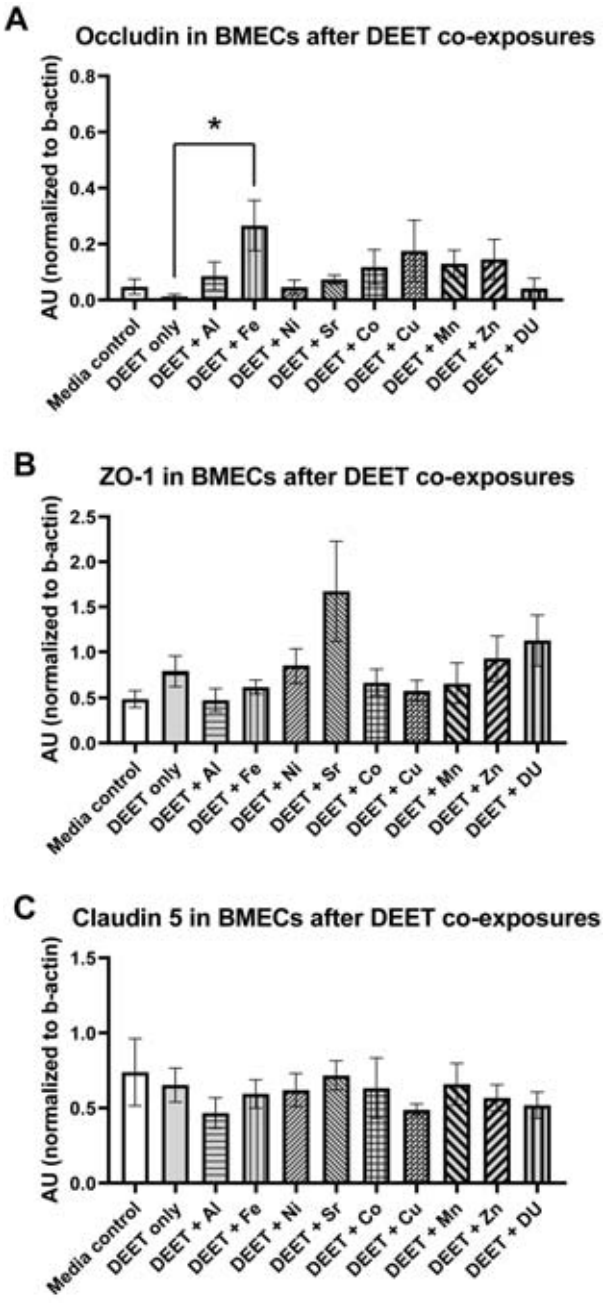

Figure 4. BBB protein expression in BMEC cultures after exposure to DEET and metal compounds. Data presented as arbitrary units of ratio of chemiluminescence value of target protein over within-subject value of β-actin. White bar represents media-only control. Light gray bars indicate DEET treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) occludin expression, B) ZO-1 expression, C) claudin 5 expression. * indicates significant p-value for comparison between groups indicated with connected lines.

expression of any protein after any treatment. All protein results are shown in Figure 5 ((A) occludin, $F_{(10,32)} = 1.692$, $p = 0.1258$; B) ZO-1, $F_{(10,33)} = 0.3921$, $p = 0.9409$; C) claudin 5, $F_{(10,33)} = 0.2610$, $p = 0.9856$). For the PM + metals' experimental set, there were also no significant differences in the expression of any protein after any treatment. All protein results are shown in Figure 6 ((A) occludin, $F_{(10,33)} = 0.6181$, $p = 0.7874$; B) ZO-1, $F_{(10,32)} = 0.3662$, $p = 0.9524$; C) claudin 5, $F_{(10,32)} = 0.1738$, $p = 0.9971$).

Cytokine expression after exposure to organic and metal compounds

Similar to the protein expression experiments, we also examined the effects of organic and metal compound exposure on changes in 9 cytokines in BMEC cultures (IL-2, IL-8, IL-12p70, IL-1β, GM-CSF, IFN-γ, IL-6, IL-10, and TNF-α). In each experimental set, BMECs were cultured alone and exposed to a media control, a metal alone, an organic compound alone, or the organic compound in combination with one of the metals. Statistical analysis is presented as a one-way ANOVA of the entire set, with post-hoc Sidak's multiple comparisons test comparing media to the organic compound alone and individual comparisons of the organic compound versus the organic plus each individual metal.

For the metal-alone experimental set all cytokine results are shown in Figure 7 ((A) IL-2, $F_{(9,20)} = 3.784$, $p = 0.0063$; B) IL-8, $F_{(9,20)} = 4.399$, $p = 0.0028$; C) IL-12p70, $F_{(9,20)} = 21.79$, $p < 0.0001$; D) IL-1β, $F_{(9,20)} = 1.688$, $p = 0.1577$; E) GM-CSF, $F_{(9,20)} = 1.325$, $p = 0.2854$; F) IFN-γ, $F_{(9,20)} = 2.494$, $p = 0.0426$; G) IL-6, $F_{(9,20)} = 729.9$, $p < 0.0001$; H) IL-10, $F_{(9,20)} = 2.463$, $p = 0.0447$; I) TNF-α, $F_{(9,20)} = 1.316$, $p = 0.2896$). Compared to media alone, the only metal alone with no effect on IL-2 was Sr. All other metals alone statistically decreased IL-2 levels: Al: *p = 0.0020, Fe: *p = 0.0046, Ni: *p = 0.0021, Co: *p = 0.0008, Cu: *p = 0.0234, Mn: *p = 0.0186, Zn: *p = 0.0294, and DU: *p = 0.0461. For IL-8, Zn and DU had no effect compared to media alone, while the rest of the metals decreased IL-8; Al: *p = 0.0047, Fe: *p = 0.0064, Ni: *p = 0.0029, Sr: *p = 0.0481, Co: *p = 0.0414, Cu: *p = 0.0103, and Mn: *p = 0.0481. IFN-γ was increased only by DU exposure

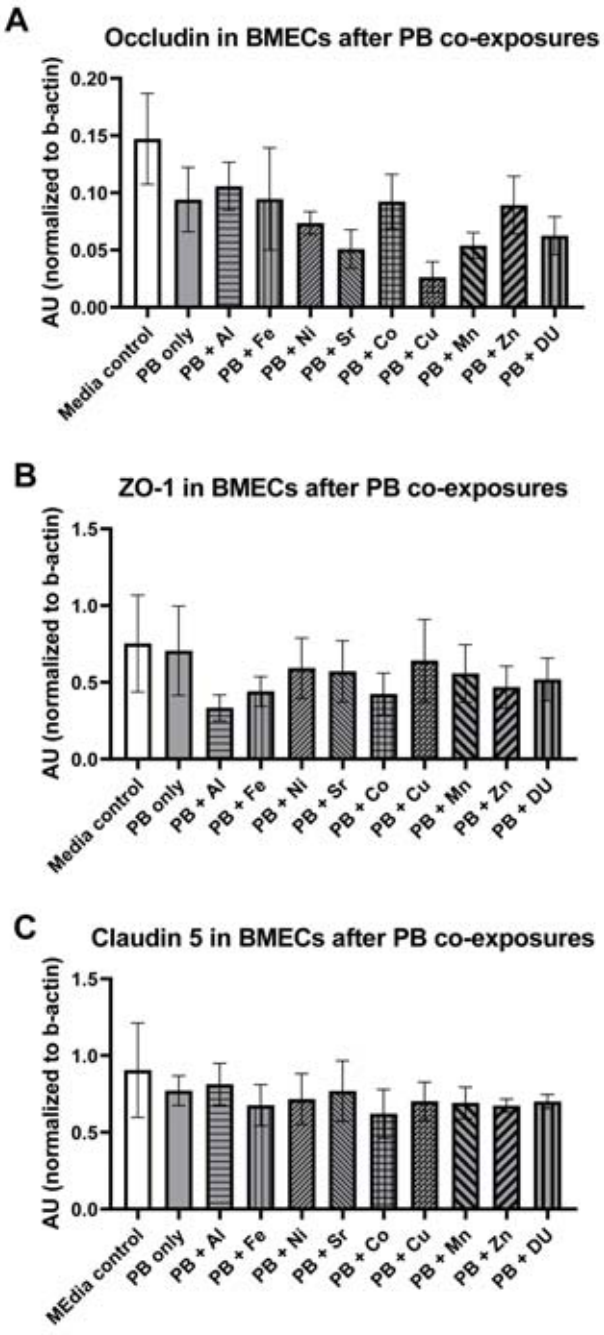

Figure 5. BBB protein expression in BMEC cultures after exposure to PB and metal compounds. Data presented as arbitrary units of ratio of chemiluminescence value of target protein over within-subject value of β-actin. White bar represents media-only control. Light gray bars indicate PB treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) occludin expression, B) ZO-1 expression, C) claudin 5 expression. No significant differences.

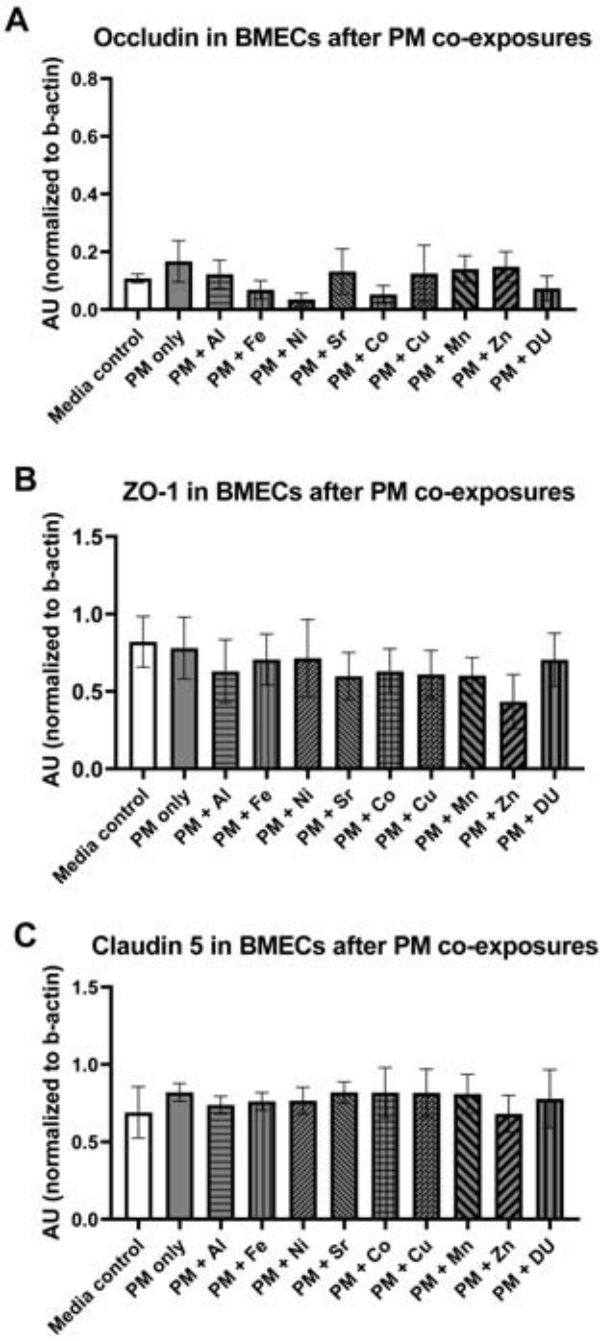

Figure 6. BBB protein expression in BMEC cultures after exposure to PM and metal compounds. Data presented as arbitrary units of ratio of chemiluminescence value of target protein over within-subject value of β-actin. White bar represents media-only control. Light gray bars indicate PM treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) occludin expression, B) ZO-1 expression, C) claudin 5 expression. No significant differences.

compared to media only control (*p = 0.0060). All metals decreased the expression of IL-12p70 and IL-6 compared to media (*p < 0.0001 for all). IL-1β, GM-CSF, IL-10, and TNF-α expression were not significantly affected by any metal. When taken together these results would suggest a subtle decrease in cytokine response regardless of specific metal.

For the DEET + metals' experimental set all cytokine results are shown in Figure 8 ((A) IL-2, $F_{(10,22)}$ = 0.4218, p = 0.9207; B) IL-8, $F_{(10,21)}$ = 2.747, p = 0.0245; C) IL-12p70, $F_{(10,22)}$ = 1.232, p = 0.3250; D) IL-1β, $F_{(10,22)}$ = 0.5463, p = 0.8386; E) GM-CSF, $F_{(10,22)}$ = 0.6357, p = 0.7681; F) IFN-γ, $F_{(10,21)}$ = 0.9052, p = 0.5454; G) IL-6, $F_{(10,22)}$ = 3.264, p = 0.0099; H) IL-10, $F_{(10,21)}$ = 0.7160, p = 0.7008; I) TNF-α, $F_{(10,22)}$ = 1.481, p = 0.2119). DEET alone had no effect on the expression of any of the cytokines. In conjunction with DEET, compared to DEET alone, there were no significant changes in cytokine expression from DEET + metal exposures. We would expect to see differences due to effects seen from exposure to metals alone, which may indicate a dampening effect of DEET exposure prior to metal exposure, and thus a possible synergistic increase in cytokine response regardless of metal.

For the PB + metals' experimental set all cytokine results are shown in Figure 9 ((A) IL-2, $F_{(10,22)}$ = 2.011, p = 0.0828; B) IL-8, $F_{(10,22)}$ = 3.286, p = 0.0096; C) IL-12p70, $F_{(10,22)}$ = 5.796, p = 0.0003; D) IL-1β, $F_{(10,21)}$ = 1.250, p = 0.3180; E) GM-CSF, $F_{(10,22)}$ = 0.8838, p = 0.5618; F) IFN-γ, $F_{(10,22)}$ = 6.457, p = 0.0001; G) IL-6, $F_{(10,22)}$ = 4.301, p = 0.0021; H) IL-10, $F_{(10,22)}$ = 2.717, p = 0.0243; I) TNF-α, $F_{(10,22)}$ = 4.834, p = 0.0010). PB alone had no effects on any cytokines. In conjunction with PB, compared to PB alone, Ni increased TNF-α (*p = 0.0388). Cu decreased IFN-γ and IL-6 (*p = 0.0018 and *p = 0.0283, respectively). Mn increased TNF-α (*p = 0.0438). Zn decreased IL-8, IL-12p70, IFN-γ, and IL-6 (*p = 0.0086, *p = 0.0273, *p = 0.0003, and *p = 0.0078, respectively). There were no other significant changes in cytokine expression from PB + metal exposures. When considering these effects against the metal-only exposures, DU exposure alone had increased IFN-γ, but after exposure to PB the effect of DU was no longer

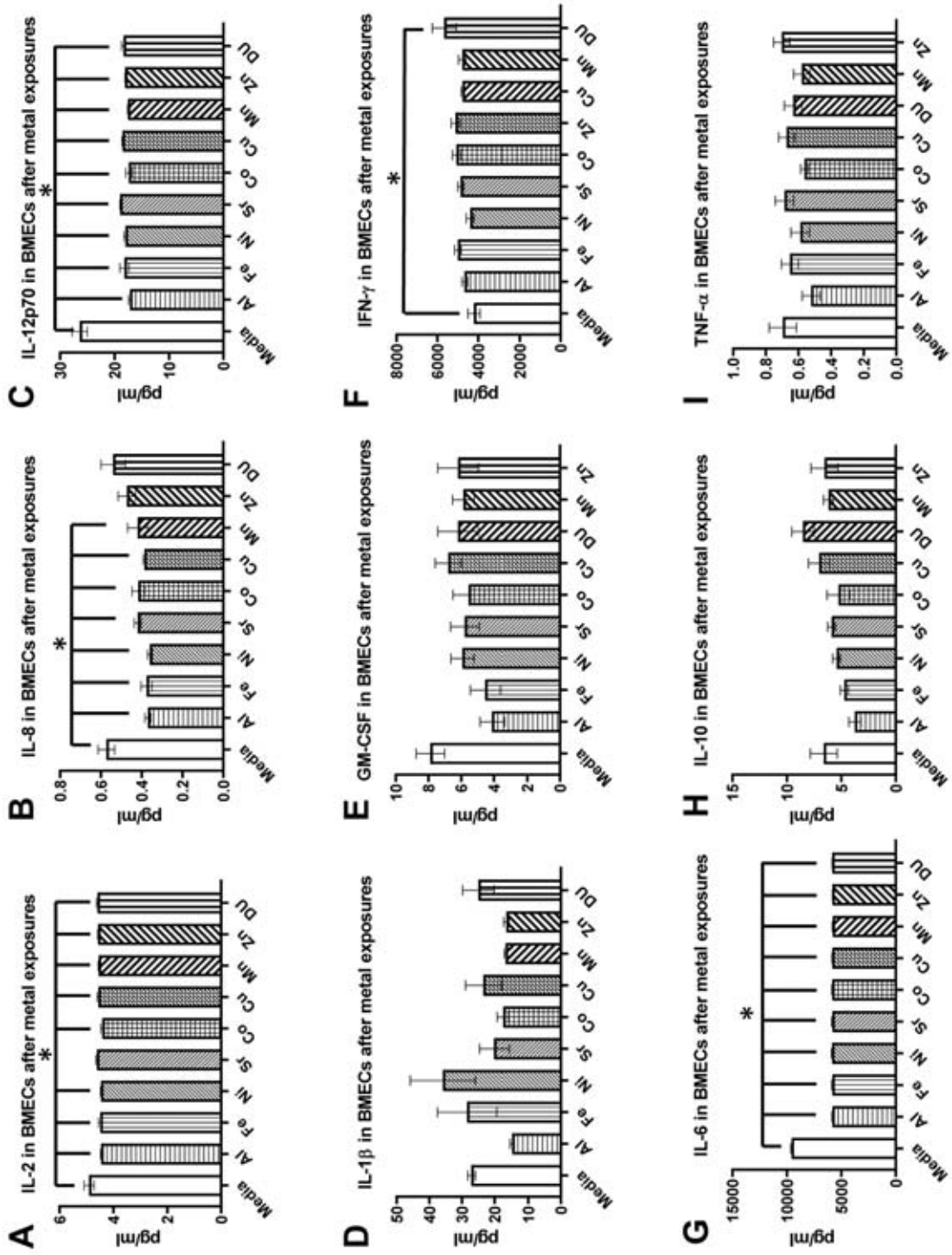

Figure 7. Cytokines in BMEC cultures after exposure to metals alone. Data presented as pg/ml of target cytokine. White bars indicate media-only treatments with or without metals. Pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) IL-2, B) IL-8, C) IL-12p70, D) IL-1β, E) GM-CSF, F) IFN-γ, G) IL-6, H) IL-10, I) TNF-α. * indicates significant p-value for comparison between groups indicated with connected lines.

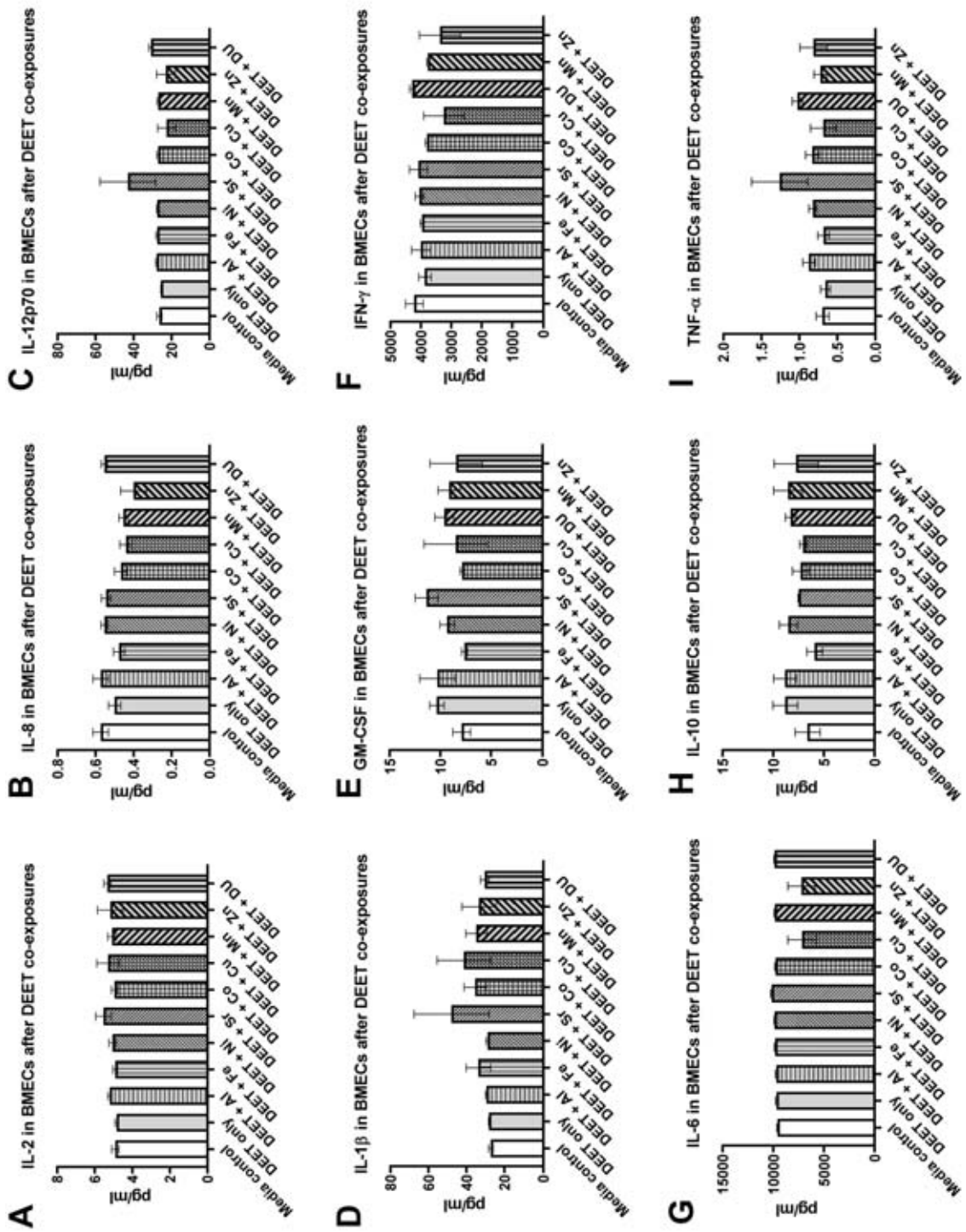

Figure 8. Cytokines in BMEC cultures after exposure to DEET and metal compounds. Data presented as pg/ml of target cytokine. White bar represents media-only control. Light gray bars indicate DEET treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) IL-2, B) IL-8, C) IL-12p70, D) IL-1β, E) GM-CSF, F) IFN-γ, G) IL-6, H) IL-10, I) TNF-α. No significant differences.

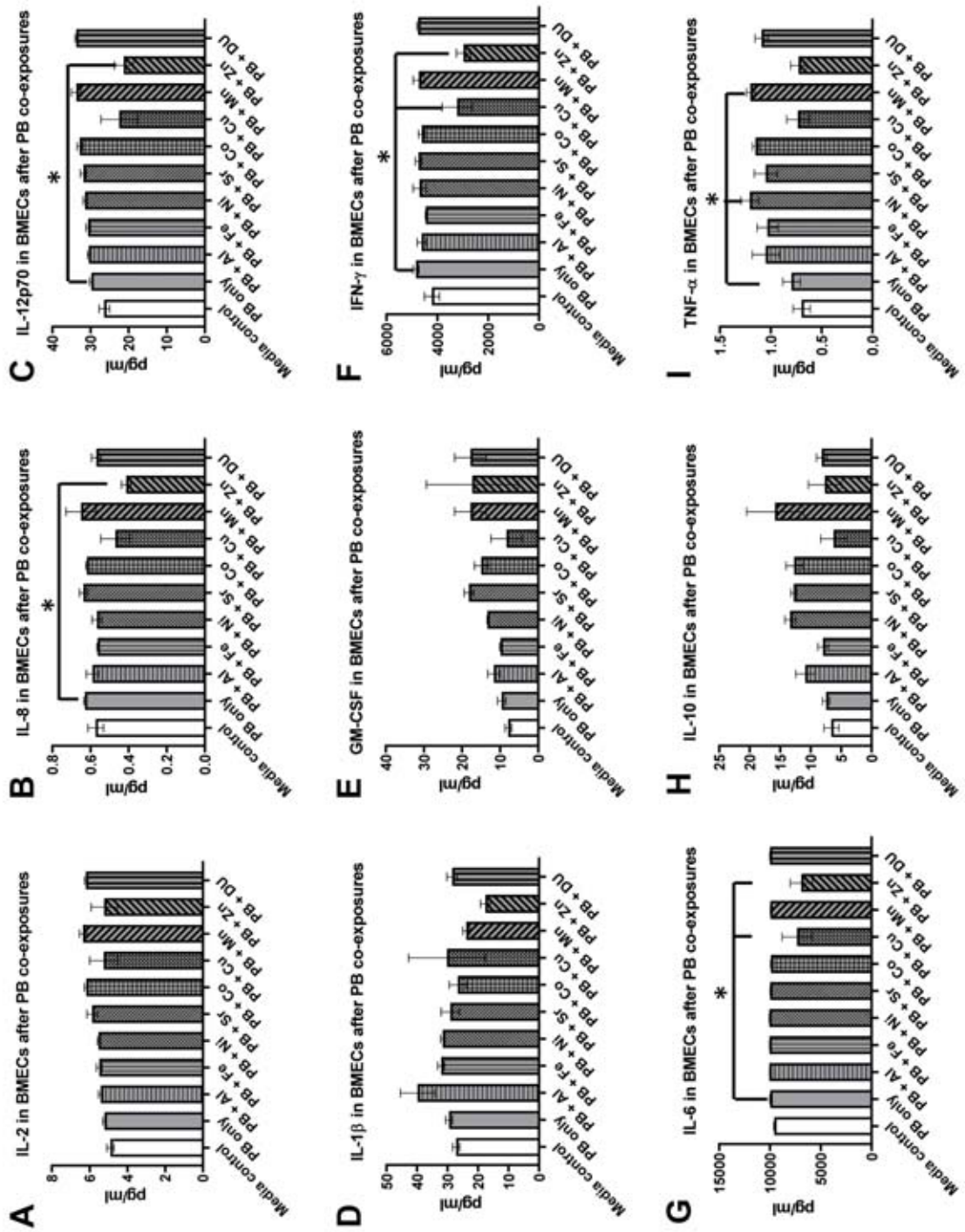

Figure 9. Cytokines in BMEC cultures after exposure to PB and metal compounds. Data presented as pg/ml of target cytokine. White bar represents media-only control. Light gray bars indicate PB treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) IL-2, B) IL-8, C) IL-12p70, D) IL-1β, E) GM-CSF, F) IFN-γ, G) IL-6, H) IL-10, I) TNF-α. * indicates significant p-value for comparison between groups indicated with connected lines.

significant, indicating a dampening effect of this co-exposure. However, PB resulted in a synergistic decrease in IFN-γ for exposure to Cu and Zn following exposure to PB while the metals alone had no effect. All metals alone had decreased IL-6 and IL-12p70 compared to media alone, but PB exposure had a dampening effect, where only Cu and Zn following PB exposure significantly reduced IL-6, and only Zn following PB reduced IL-12p70, but the effects in all three cases were reduced. Similarly, IL-2 and IL-8 were decreased by most metal exposures alone, but following exposure to PB this effect was prevented, with no significant changes in IL-2 or IL-8 in any metal after PB exposure that had been significant in metal only exposures. However, although Zn alone had no effect on IL-8 it decreased IL-8 following exposure to PB, indicating a synergistic effect of PB and Zn co-exposure. Metals alone had no effect on TNF-α, but the increase in TNF-α from both Ni and Mn after exposure to PB indicates a synergistic effect of this co-exposure as well. Overall, PB exposure prior to subsequent metal exposure prevented the levels of cytokines measured from decreasing, as well as increased TNF-α in two metals that had no effect alone, suggesting a synergistic effect of PB and metals on increasing TNF-α inflammatory response.

For the PM + metals' experimental set all cytokine results are shown in Figure 10 ((A) IL-2, $F_{(10,22)} = 7.077$, $p < 0.0001$; B) IL-8, $F_{(10,22)} = 16.28$, $p < 0.0001$; C) IL-12p70, $F_{(10,22)} = 14.35$, $p < 0.0001$; D) IL-1β, $F_{(10,21)} = 3.835$, $p = 0.0046$; E) GM-CSF, $F_{(10,21)} = 5.026$, $p = 0.0009$; F) IFN-γ, $F_{(10,22)} = 26.00$, $p < 0.0001$; G) IL-6, $F_{(10,22)} = 11.04$, $p < 0.0001$; H) IL-10, $F_{(10,22)} = 1.051$, $p = 0.4367$; I) TNF-α, $F_{(10,22)} = 2.510$, $p = 0.0346$). PM alone increased expression of IL-2 (*p = 0.0340), compared to media control, but had no effects on any other cytokines. In conjunction with PM, compared to PM alone, Cu decreased IL-12p70, GM-CSF, IFN-γ, and IL-6 (*p = 0.0002, *p = 0.0234, *p < 0.0001, and *p = 0.001, respectively). Zn reversed the effect of PM alone and decreased IL-2 (*p = 0.0014), and decreased IL-12p70, GM-CSF, IFN-γ, and IL-6 (*p < 0.0001, *p = 0.0179, *p = 0.0002, and *p < 0.0001, respectively). DU increased IL-8, IL-1β, and IFN-γ (*p < 0.0001, *p = 0.0013, and *p = 0.0002, respectively). There were no other significant changes in cytokine expression from PM + metal exposures. When considering these effects against the metal-only exposures, PM alone increased expression of IL-2 compared to the media-only control, but expression remained high for all metals except Zn, which returned to the level of Zn exposure alone. Since Al, Fe, Ni, Co, Cu, Mn, and DU had decreased IL-2 expression, PM therefore overrode the effects of these metals alone. PM exposure prior to subsequent metal exposures prevented the decrease in IL-8 seen in many metal-alone exposures. Similarly, PM also prevented the decrease in IL-12p70 and IL-6 seen after all metal exposures except for Cu and Zn. Exposure to Cu and Zn after exposure to PM resulted in a decrease in GM-CSF and IFN-γ that was not seen after exposure to the metals alone for either cytokine. DU alone had no effect on IL-1β or IL-8, but caused an increase in both cytokines following exposure to PM. PM exposure also resulted in a further increase in IFN-γ than DU alone. Overall many effects of metals alone were reversed, and several other cytokines were increased after co-exposure with PM, suggesting a synergistic effect of PM and metals on increasing cytokine response.

DISCUSSION

Currently, the multi-symptom syndrome described as Gulf War Illness, which applies to many US military personnel that served in Persian Gulf War in 1990-1991, has no known single-exposure scenario that applies to everyone diagnosed with GWI. Research instead suggests that multiple factors in combined exposure scenarios have created the complex GWI symptom profile. One proposed set of factors is that exposure to organic compounds in theatre at the time, such as DEET, pyridostigmine bromide, or permethrin, as well as metals and other microbes internalized through inhalation of contaminated "desert dust", could contribute to symptoms such as fatigue, anxiety, depression, and other cognitive problems seen in GWI as a result of their potential to disrupt the blood brain barrier. The BBB functions to heavily restrict movement of molecules from the circulatory system into the central nervous system

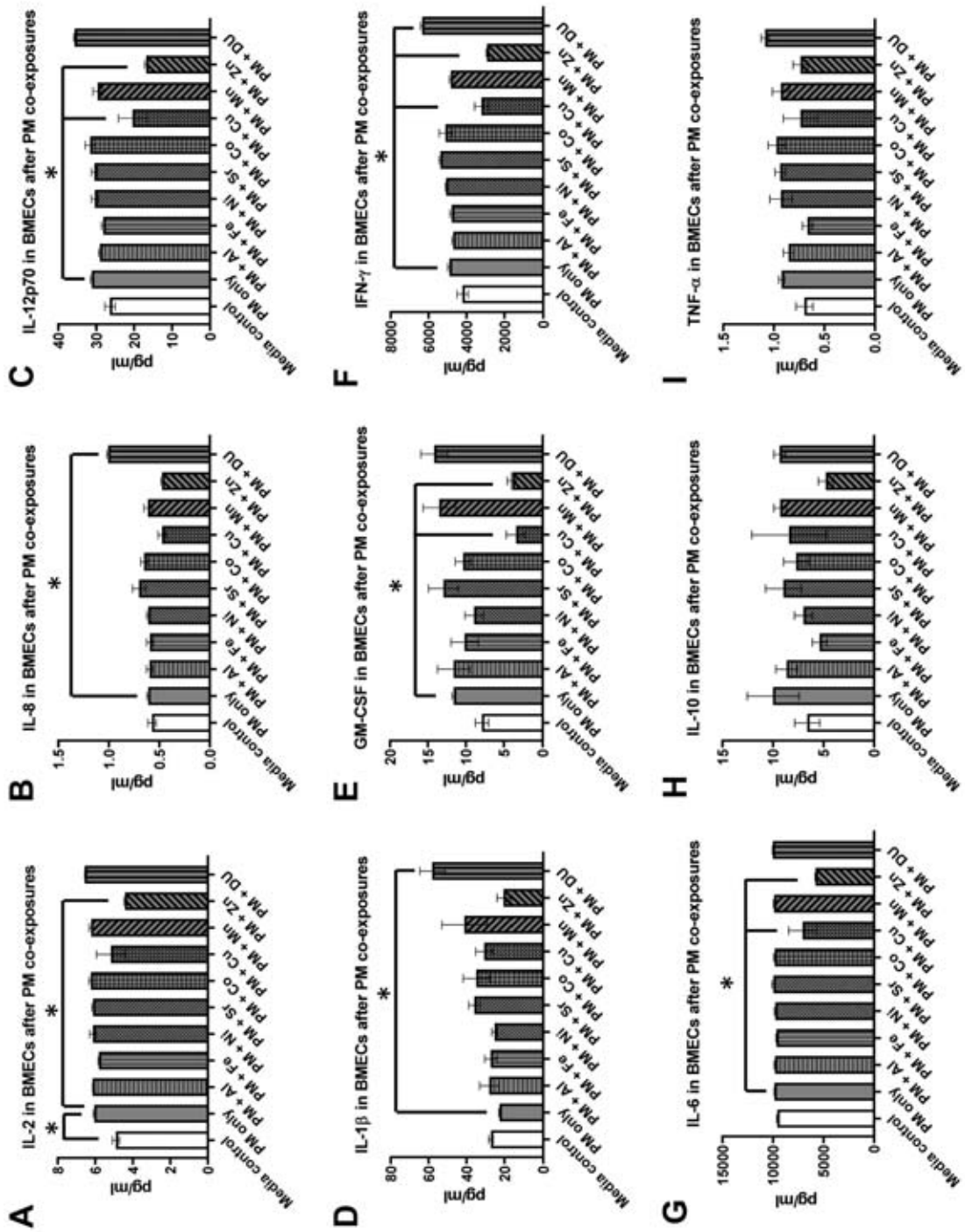

Figure 10. Cytokines in BMEC cultures after exposure to PM and metal compounds. Data presented as pg/ml of target cytokine. White bar represents media-only control. Light gray bars indicate PM treatment, and pattern indicates subsequent metal treatment with no pattern indicating media only instead of metal. A) IL-2, B) IL-8, C) IL-12p70, D) IL-1β, E) GM-CSF, F) IFN-γ, G) IL-6, H) IL-10, I) TNF-α. * indicates significant p-value for comparison between groups indicated with connected lines.

primarily through formations of tight junctions between endothelial cells and supported by astrocytes [65-67]. While there is no way to really know what doses personnel were exposed to, especially when it comes to metals, microbes, any other contaminants inhaled within desert dust, and actual versus recommended use of pesticides and prophylactics, we decided to work from the assumption of low dose exposures to several compounds of interest rather than overtly high acute exposures. Therefore we hypothesized that exposures of sub-toxic levels of DEET, PB, and PM, particularly in combination with concurrent exposure to sub-toxic levels of metals, would adversely affect the health and viability of cells involved in the BBB, which could lead to subtle changes in permeability and function.

Previously we examined a range of doses of the organic compounds and metals of interest to GWI and their effects on toxicity and viability on human BMEC and astrocyte cell cultures to establish a model of sub-toxic exposure for co-exposure conditions [62]. Not all compounds had the same toxicity profile across the entire dose range tested – for example, Sr did not appear toxic even at high levels. This may affect actual multi-exposure results. In mono-cultures of BMECs, we reported that Co and Mn in co-exposures with all three organics resulted in greater cell toxicity. DU increased nitric oxide (NO, oxidative stress marker) in BMEC cultures regardless of organic co-exposure, but Sr reduced NO levels in co-exposures with all three organics even though it had no effect alone, possibly a protective interaction between the organic compounds and Sr, especially considering Sr's lack of toxicity across investigated doses. We also reported no effects on other oxidative stress markers such as thiobarbituric acid reactive substances (TBARS), a byproduct of lipid peroxidation, protein carbonyl, or multiple protein markers (GPX4, catalase, SOD, MMP3, MMP9).

Here we wanted to investigate the effects of DEET, PB, PM, and nine metals of interest, in a multi-exposure scenario, on the functional aspects of the BBB, and did so by using human BMECs and human astrocytes co-cultured on a transwell membrane. In examining several markers of BBB function, we found that the transwell model did create an effective barrier to 4 kDa and 70 kDa fluorescence markers, regardless of organic, metal, or combined exposures. TEER, another marker of BBB functionality, was unaffected by DEET, PB, and PM alone at the experimental dose. DEET followed by Cu and PM followed by Fe increased TEER, which may reflect a protective effect, but multiple metals reduced TEER after exposure to PM. In particular, DU decreased TEER values, and thus decreased BBB functionality, after exposure to PM or PB. We were also interested in examining whether organic and metal combination exposures changed the ability of metals to cross over a BBB model. In the absence of organic compound exposure, all metals crossed the membrane to varying degrees with the exception of Fe, which did not transfer at all. Whether this is a result of Fe binding to the serum proteins used to supplement the BMEC growth medium is not known at this time. Surprisingly, metal translocation of several metals actually decreased after exposure to organic compounds, which is the opposite of what we predicted. Al was the only metal to significantly increase translocation across the BBB, but only after exposure to PM. Given that Fe did not cross the BBB on its own at all, it is interesting to note that it did showed a small amount of transfer after exposure to PM, although this did not reach statistical significance. Although the BBB tightly regulates which compounds can move from the circulatory system to the central nervous system, transport mechanisms exist to translocate essential metals across the BBB to in order to maintain metal homeostasis. In most cases these metal transporters are capable of translocating more than one metal across the BBB and more than one mechanism exists for the translocation of most essential metals. For example, divalent metal transporter-1 (DMT1) is capable of moving Fe across the BBB but can also transport Cu, Mn, and Al [68]. However, Fe can also be transported with the transferrin and melanotransferrin sytems, Cu with copper transporter-1 (CTR1), and both Zn and Mn with the ZIP transporter system [69]. Metallothionein has also been shown to transport both Zn and Cu across the BBB [70]. It is not currently known what transport mechanism would be involved in moving Ni, Sr, and Ni across the BBB since they are non-essential metals with

U and Ni in particular having no known biological function. While the ability of the 9 individual metals with and without pre-exposure to organic compounds to cross the BBB was assessed, it is not known whether metals were also transported into the BMECs and sequestered there, potentially affecting cell signalling and oxidative stress pathways.

No changes were observed in the expression levels of BBB tight junction proteins occludin, ZO-1, or claudin 5, with the exception of DEET + Fe, which increased occludin over DEET alone. This result again suggests a potential protective interactive effect involving Fe. It is important to note, however, that lack of change in expression level does not necessarily mean no change in function – we note a limitation of this work where we did not examine any changes in protein structure or location changes in the cell membrane versus cytoplasm, which was outside the scope of this project.

Cytokines represent a broad category of proteins involved in a variety of functions including acute and chronic inflammation. They can be loosely grouped depending on whether they act on the cells of the adaptive immune system, are pro-inflammatory, or are anti-inflammatory [71]. More specifically, the cytokines we assessed can be grouped as follows: adaptive immune response (IL-2, GM-CSF); pro-inflammatory (IL-8, IL-1β, IFN-γ, IL-6, TNF-α); anti-inflammatory (IL-10, IL-12p70). Metal exposure suggests a subtle decrease in cytokine response regardless of specific metal. For most metals, exposure to DEET, PB, and PM prior to metal exposure prevented the decrease in cytokines IL-2, IL-8, IL-12p70 and IL-6, although this effect was blunted in Cu and or Zn with PB and PM, but not DEET. PB had a synergistic effect with Ni and Mn to increase TNF-α, and PM synergistically increased IL-8, IL-1β, and IFN-γ when given prior to DU exposure compared with DU metal exposure alone. Interestingly, PM was the only organic compound to have any effect on cytokine expression, increasing IL-2. Overall, sub-toxic doses of all three organic compounds investigated, along with sub-toxic doses of most metals, had little to no effect on cytokine response, with several exceptions. Both PB and PM in combination with Cu or Zn lowered the response of several pro- and anti-inflammatory cytokines. Conversely, DU increased the levels of several pro-inflammatory cytokines when co-exposed with PM. Previously we showed that DU had the most impact on human BMEC oxidative stress response compared with the other metals and organic compounds tested [62]. Therefore, DU continues to be a metal to watch both in single as well as combined exposure scenarios.

Military personnel are not the only people facing health effects from exposure to metals and pesticides; contamination of food and water [72-76] or inhalation of metals and other chemicals from e-cigarettes and air pollution [77-79] can affect populations all over the world. Understanding how exposure interactions can affect health in addition to understanding the consequences of exposure to any individual metal or compound is necessary to improve the health and well-being of people in many circumstances.

CONCLUSION

Our hypothesis that combined exposures of military-relevant organic compounds and metals would lead to synergistic negative effects on human BMEC and human astrocyte cultures and disrupt their function in a BBB model was simple and straightforward, but the effects we found are more complicated. Overall there do seem to be some synergistic effects of organics and metals on the functionality of the BBB, particularly PM with multiple metals and DU alone and with multiple organics, but functional changes are subtle. Future studies using more adaptive models of BBB such as organ-on-a-chip technologies that incorporate flow simulation and more cell types may be better suited to parsing out clinically relevant changes from exposure to these compounds.

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expressed in the paper are those of the authors and do not reflect the official policy or position of the Armed Forces Radiobiology Research Institute, Uniformed Services University, Department of Defense, or US Government.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analysis, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

ABBREVIATIONS

BBB	:	blood brain-barrier
BMEC	:	brain microvascular endothelial cells
DEET	:	N,N-diethyl-*m*-toluamide
DU	:	depleted uranium
GWI	:	Gulf War Illness
PB	:	pyridostigmine bromide
PM	:	permethrin
TEER	:	trans-epithelial electrical resistance

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