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Pro- and anti-inflammatory effects of sulforaphane on placental cytokine production

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ABSTRACT

Placental inflammation increases the risk of adverse pregnancy outcomes and possibly neurodevelopmental disorders in the offspring. Previous research suggests it may be possible to modulate the placental immune response to bacteria to favor an anti-inflammatory phenotype with dietary factors. Sulforaphane (SFN) is a dietary supplement with known anti-inflammatory activities, however, its effects on placental cytokine production are unclear. Therefore, we evaluated the effects of SFN on biomarkers of inflammation and neurodevelopment under basal conditions and a setting of mild infection. Placental explant cultures were established and treated with up to 10 μM SFN in the presence and absence of 10^7 CFU/ml heat-killed *E. coli*. Concentrations of IL-1β, TNF-α, IL-6, sgp130, HO-1 and BDNF in conditioned medium were quantified by immunoassay. SFN increased antioxidant HO-1 expression in the absence, but not the presence, of infection. SFN inhibited IL-1β and IL-10, but tended to promote, TNF-α production by bacteria-stimulated cultures. IL-6 and BDNF were inhibited by SFN irrespective of co-treatment with *E.coli*. A negative regulator of IL-6 signaling, sgp130, was increased by SFN under basal conditions, but not in *E. coli*-stimulated cultures. These results suggest that SFN has mixed effects on the placenta inhibiting both pro-inflammatory (IL-1β) and anti-inflammatory factors (IL-10) but promoting regulators of oxidative stress and inflammation (HO-1 and sgp130) in an infection-dependent manner.

1. Introduction

During pregnancy, inflammation at the maternal-fetal interface is suppressed to allow for the survival of the fetal allograft. Systemic or ascending infections from the lower genital tract can disrupt this process and increase the risk of adverse pregnancy and/or neurodevelopmental outcomes (Gibbs et al., 1992; Dammann and Leviton, 1997; Meyer et al., 2006; Atladottir et al., 2010; Zerbo et al., 2017).

Dietary factors may regulate oxidative stress responses by tissues at the maternal fetal interface and enhance their ability to control inflammation. Polyphenols such as luteolin, kaempherol, curcumin, apigenin, and naringenin reduce the production of inflammatory biomarkers by gestational tissues (Lim et al., 2013; Wall et al., 2013; Wijesuriya and Lappas, 2018). However, other dietary factors have been shown to have adverse effects on oxidative stress and inflammation. Palmitic acid reduced total anti-oxidant capacity, enhanced bacterial-stimulated peroxide and lipid-peroxidation, and reduced the production of heme oxygenase-1 (Manuel et al., 2018; Zhang et al., 2018) (HO-1) by trophoblast cultures. HO-1 is produced in response to oxidative stress via activation of the Nuclear Regulatory-2 (Nrf-2) transcription factor. It is thought to prevent oxidative stress-induced inflammation and may be essential for normal pregnancy by limiting the consequences of infection on pregnancy outcome. Mice lacking HO-1 had significant placental inflammation, reduced intra-uterine growth, and high rates of fetal resorption (Zenclussen et al., 2011). These effects of HO-1 deficiency on pregnancy outcomes were prevented by the administration of the enzyme's product, carbon monoxide (CO) (El-Mousleh et al., 2012) that has potent anti-inflammatory properties at low concentrations. In previous studies, we found that CO inhibited bacteria-induced production of proinflamatory cytokines by the fetal membranes (Klimova et al., 2013) and placenta (Peltier et al., 2013a). Furthermore, it prevented bacteria-induced preterm birth in mice and

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improved neonatal survival (Peltier et al., 2013c).

Sulforaphane (SFN) is a non-nutritive component of broccoli and other cruciferous vegetables that is a popular dietary supplement due to its anti-inflammatory and anti-oxidant properties (Zhao et al., 2006). It activates Nrf-2 to increase the transcription of HO-1 (Zhao et al., 2006) and has been used to prevent tissue injury and biomarkers for inflammation in a variety of models of infection-induced oxidative stress (Yang et al., 2007; Brandenburg et al., 2010; Yehuda et al., 2012; Eren et al., 2018). However, its effects on HO-1 production by the placenta are unknown as are its effects on cytokines produced in response to infection. Therefore, we examined the effects of SFN on HO-1 production and an array of cytokines associated with adverse pregnancy and neurodevelopmental outcomes by the placenta under normal conditions, and when stimulated with bacterial components.

2. Materials and methods

The placental culture system used for this experiment is standard for our lab and details have been published elsewhere (Peltier et al., 2013c) but are repeated here for the benefit of the reader.

2.1. Materials

Synthetic SFN was purchased from LKT pharmaceuticals through Fisher Scientific (Atlanta, GA), Phosphate Buffered Saline, Dulbecco's Minimal Essential Medium (DMEM), Dimethyl Sulfoxide, antibiotic solution (100X Penicillin-Streptomycin), tetrazolium salts, isopropanol, Nutrient Broth, Nutrient Agar, and Fetal Bovine Serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Atlanta, GA). SFN was prepared at 4 mM stock in DMSO.

We prepared stocks of heat-killed bacteria to model the effects of ascending infection on the host immune response by the placenta. Bacteria contain a number of proinflammatory molecules that include lipoproteins and CpG-modified DNA in addition to lipopolysaccharide (LPS) that is more commonly used for this purpose. A low-passage stock culture of *E. coli* strain J5 was purchased from the American Type Tissue Collection (Manassas, VA) and cultivated in nutrient broth to late log phase at 37 °C. Bacteria were then pelleted by centrifugation at 10,000 *g* for 30 min and suspended in culture medium (DMEM + 10% FBS). Quantitative cultures were then established to determine the CFU/ml and the bacteria were killed by heating at 75 °C for 1 h. The concentrated stocks of the heat-killed bacteria were then stored at −80 °C until use.

2.2. Placental cultures

Placental tissues from women who underwent elective Cesarean sections at term (typically for breech presentation or due to previous Cesarean section) but who were not in labor were transferred to the investigators after release for disposal by the attending surgeon. This is in compliance with NYU-Winthrop policies for using anonymous tissues and with oversight by the NYU Winthrop Institutional Review Board. No information regarding patient characteristics is available but they are expected to reflect the demographics of Nassau County, NY (˜74% White, ˜17% African-American, ˜10% Asian).

Upon arrival to the lab, tissues were washed extensively with sterile PBS and blood clots removed by gentle blotting with sterile gauze. Small (1 cm^3) segments of the villous placenta were isolated by sharp dissection and washed with PBS. These were then chopped on a McIllwain tissue chopper (Mickle Engineering, CO, Surrey, UK) set to cut at 0.1 mm increments for 3–6 passes. Chopped tissues were washed with culture medium (DMEM + 10% FBS + 100 U/ml Penicillin G + 60 ug/ml Streptomycin). Tissues were then plated at 100 mg/well in 12-well plates and suspended in culture medium. Bacteria (0 or 10^7 CFU/ml) and SFN (0, 2.5, 5 or 10 mM) were then added to final concentrations in a final volume of 1.0 ml. DMSO was added as needed so that the final concentration was 0.25% (v/v) for all treatments. Concentrations of SFN were based on previously studies performed with microglia and macrophages (Lin et al., 2008; Akhtar et al., 2012; Foresti et al., 2013). Tissues were then incubated overnight (16 h) in a humidified incubator under 5% (v/v) CO_2. Conditioned medium was then harvested and stored at −80 °C until immunoassay.

2.3. Viability assays

Potential effects of SFN on viability of the cultured tissues was ascertained using a variant of the MTT assay as previously described (Peltier et al., 2013a, b; Arita et al., 2018). Briefly, a subset of cultures were treated exactly as described above except that at the end of the culture, 5 mg/ml Thiazolyl Blue Tetrazolium Bromide (MTT) in PBS was added to the final concentration at 0.5 mg/ml into the placenta cultures. After 30 min of incubation at 37 °C, the conditioned medium was removed and tissues were washed with PBS. Tissues were collected by centrifugation at 2000 *g* and insoluble formed formazan was extracted with 10 ml isopropanol. $Aborbance_{540\text{-}495}$ was then determined using a microplate spectrophotometer.

2.4. Immunoassays

Concentrations of HO-1, Interleukin(IL)-1β, Tumor Necrosis Factor (TNF)-α, IL-6, soluble glycoprotein (sgp)130, IL-10 and brain-derived neurotrophic factor (BDNF) in conditioned medium were quantified by ELISA using reagents purchased from Enzo (Farmingdale, NY for HO-1), eBiosciences (San Diego, CA, for IL-1β, IL-6, TNF-α, IL-10) or R&D systems (Minneapolis, MN) for sgp130 and BDNF. IL-1β and TNF-α were selected because together they are both necessary and sufficient to induce preterm birth in rodent models (Hirsch and Wang, 2005). IL-10 was selected because it regulates IL-1β and TNF-α and can inhibit infection-mediated preterm birth in animal models (Robertson et al., 2006). IL-6 was quantified because it is increased in vivo by infections and because administration of IL-6 to pregnant mice results in autism-like behaviors in the offspring (Smith et al., 2007). Sgp130 was measured because it is a natural inhibitor of IL-6 bioactivity binding to IL-6 and preventing its interactions with gp130 and sIL-6RC. BDNF was quantified because it is produced by the placenta, stimulates the development of dopaminergic neurons (Portmann-Lanz et al., 2010) and is known to be dysregulated in children with ASD (Ricci et al., 2013).

2.5. Statistical analyses

Log-transformed biomarker concentrations in conditioned medium were evaluated using linear mixed effects models with the lme4 package of the R statistical programing language (www.r-project.org) as previously described (Bates et al., 2015). Effects due to patient were considered random and effects due to SFN, *E coli*-treatment and interaction of *E. coli* with SFN were considered fixed. Models where checked for compliance with the assumptions of parametric techniques (normality, identity and independence or errors). Preplanned comparisons between individual treatments were compared using the esticon function and exponentiated (back-transformed) for clarity of presentation. Results are presented as fold-difference (95% CI) from control (0 μM SFN) for each bacterial treatment. The effects of bacterial stimulation itself and point estimates (least-squares means) for the biomarker concentrations are also presented in Tables 1 and 2.

3. Results

As expected, bacterial stimulation alone (0 SFN) significantly reduced the viability of the cultures by about 20% (P = 0.017; Table 1) and increased the production of IL-1β, TNF-α, IL-6 and IL-10. It also resulted in a small increase (P = 0.024) in placental HO-1 production but had no detectible effect on BDNF or sgp130 production (Table 1).

Table 1
Effect of *E. coli*-stimulation on Biomarker levels.

Biomarker	Control	*E. coli*	Fold-Difference (95% CI)	P-value
MTT	0.478	.385	0.80 (0.67,0.96)	0.017
HO-1	30	50	1.65 (1.06, 2.55)	0.024
IL-1β	14	14262	1042 (387,2804)	< 0.001
TNF-α	< 5	10316	Not Estimated	Not Estimated
IL-6	149764	1763385	11.88 (10.59,13.20)	< 0.001
sgp130	20220	22507	1.11(0.88,1.40)	0.360
IL-10	< 50	840	Not Estimated	Not Estimated
BDNF	20	14	0.96 (0.72,1.28)	0.797

Table 2
Effect of SFN on placental biomarkers for unstimulated and *E. coli*-stimulated cultures. Shown are least-squares means for biomarker concentrations in conditioned medium for experiments performed on tissues from 12 different women. Please refer to figures for comparisons between individual treatments where patient-to-patient variability is accounted for.

Biomarker	SFN (μM)	Unstimulated	*E. coli*-Stimulated
MTT	0	0.478	0.385
MTT	2.5	0.424	0.363
MTT	5	0.458	0.406
MTT	10	0.466	0.436
HO-1	0	30	50
HO-1	2.5	48	59
HO-1	5	73	54
HO-1	10	68	67
IL-1β	0	14	14262
IL-1β	2.5	10	9317
IL-1β	5	11	1702
IL-1β	10	10	400
TNF-α	0	< 5	10316
TNF-α	2.5	< 5	22408
TNF-α	5	< 5	17878
TNF-α	10	< 5	21570
IL-6	0	149164	1763385
IL-6	2.5	152959	1643820
IL-6	5	145915	1569884
IL-6	10	131106	1294178
sgp130	0	20220	22507
sgp130	2.5	28195	20424
sgp130	5	32120	26013
sgp130	10	36875	22239
IL-10	0	< 50	840
IL-10	2.5	< 50	484
IL-10	5	< 50	282
IL-10	10	< 50	66
BDNF	0	20	19
BDNF	2.5	20	14
BDNF	5	16	15
BDNF	10	15	11

The effects of SFN on production of the placenta biomarkers in the presence and absence of heat-killed *E. coli* are summarized in Figs. 1–4. Panels A and C document the effect of SFN alone and panels B and D show the effects of SFN on *E. coli*-stimulated cultures. No effect on MTT activity was observed for cultures treated with SFN alone (Fig. 1A) or those co-treated with 10^7 heat-killed *E. coli* (Fig. 1B). Antioxidant HO-1 production was significantly enhanced by SFN in a dose-dependent manner at 2.5 to 10 μM (Fig. 1C). However, co-treatment of SFN with heat-killed bacteria showed no effect on HO-1 production relative to bacteria alone (Fig. 1D). SFN had no effect on IL-1β production in the absence of bacterial products (Fig. 2A), however, it significantly reduced IL-1β production by cultures stimulated with heat-killed *E. coli* in a dose-dependent manner with results being statistically significant at 5 and 10 μM (Fig. 2B). Although TNF-α was undetectable (< 5 pg/ml) in the placental cultures not treated with bacteria and unaffected by SFN, bacteria-stimulated TNF-α production tended to be increased by SFN but results did not reach statistical significance (Fig. 2D). SFN

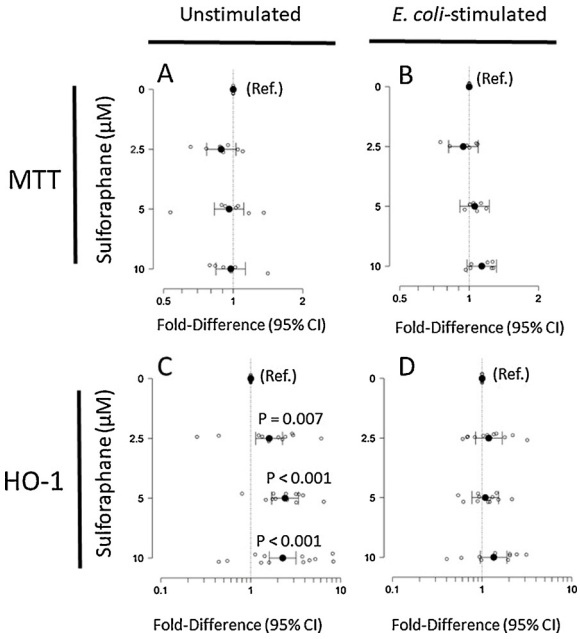

Fig. 1. Effect of SFN on placental culture viability (MTT activity) and HO-1 expression in the absence (Panels A and C) and presence (Panels B and D) of 10^7 CFU heat-killed *E. coli.* Shown are differences from control for each placenta (small open circles) as well as mean differences from control (large filled circle) with 95% confidence intervals for each level of bacterial stimulation. Treatments with bars crossing the dotted line are not statistically different from control. Results are from experiments using placental tissues from 7 (MTT activity) or 12 (HO-1) different women.

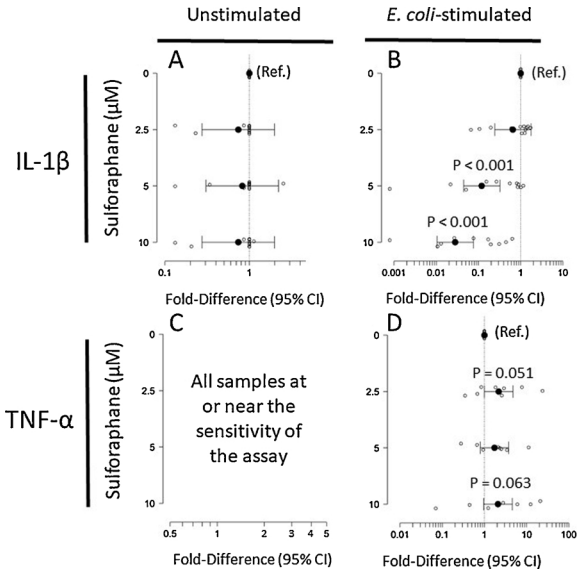

Fig. 2. Effect of SFN on expression of the proinflammatory cytokines IL-1β and TNF-α in the absence (Panels A and C) and presence (Panels B and D) of heat-killed *E. coli.* Shown are differences from control for each placenta (small open circles) as well as mean differences from control (large filled circle) with 95% confidence intervals for each level of bacterial stimulation. Treatments with error bars crossing the dotted line are not statistically different from control. Results are from experiments using placental tissues from 8 (TNF-a) or 12 (IL-1β) different women.

significantly reduced IL-6 production at 10 μM in the absence of bacteria. A similar trend for SFN was found for *E. coli*-stimulated IL-6 production with achieving statistical significance at 5 and 10 μM (Fig. 3B). Sgp130 production was significantly increased by SFN in a

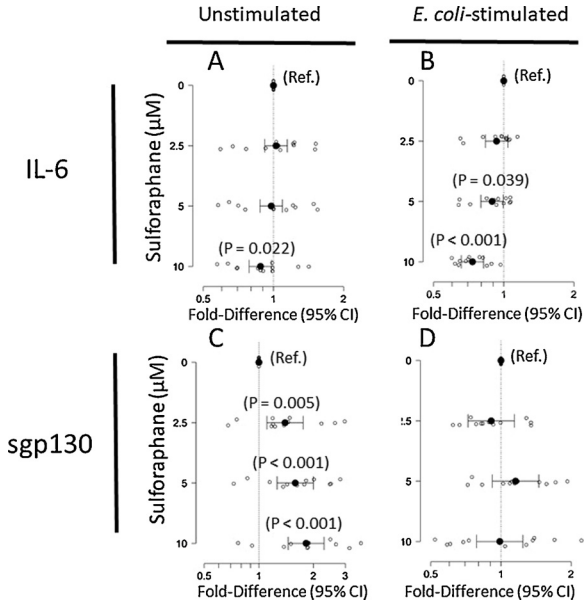

Fig. 3. Effect of SFN on expression of IL-6 and its natural inhibitor, sgp130 in the absence (Panels A and C) and presence (Panels B and D) of heat-killed bacteria. Shown are differences from control for each placenta (small open circles) as well as mean differences from control (large filled circle) with 95% confidence intervals for each level of bacterial stimulation. Treatments that have error bars crossing the dotted line are not statistically different from control. Points with bars crossing the dotted line are not statistically significant. Results are from experiments using placental tissues from 12 different women.

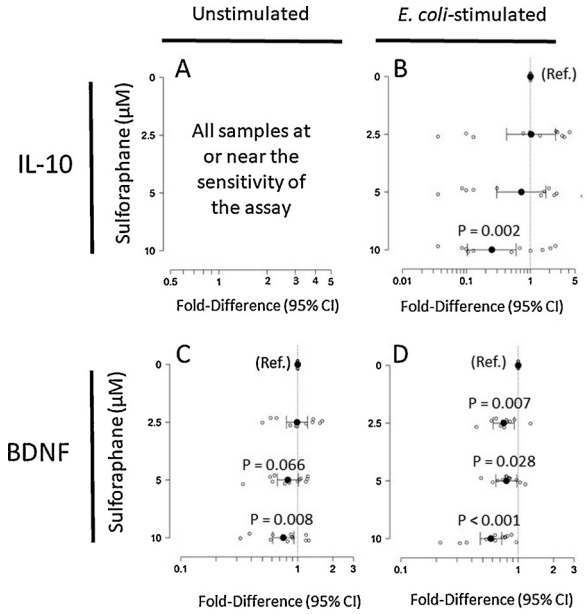

Fig. 4. Effect of SFN on expression of the anti-inflammatory cytokine, IL-10 and BDNF in the absence (Panels A and C) and presence (Panels B and D) of heat-killed bacteria. Shown are differences from control for each placenta (small open circles) as well as mean differences from control (large filled circle) with 95% confidence intervals for each level of bacterial stimulation. Treatments that have error bars crossing the dotted line are not statistically different from control. Points with bars crossing the dotted line are not statistically significant. Results are from experiments using placental tissues from 12 different women.

dose-dependent manner (Fig. 3C), however, SFN had no effect on spg130 production (Fig. 3D) by *E. coli*-stimulated cultures. IL-10 was undetectable in the absence of bacterial stimulation and unaffected by SFN (Fig. 4A). However, IL-10 production by *E. coli*-stimulated cultures co-treated with SFN was inhibited in a dose-dependent manner (Fig. 4B). BDNF was significantly inhibited by SFN in a both the absence (Fig. 4C) and presence (Fig. 4D) of heat-killed *E. coli*. However, SFN was more potent at inhibiting BDNF production by bacteria-stimulated cultures, with results as low as 2.5 μM being statistically significant (Fig. 4D).

4. Discussion

Previous studies have demonstrated that HO-1 and its enzymatic product, CO, may have important roles for the survival of the fetal allograft. Mice deficient in HO-1 had extensive placental inflammation that was accompanied by greater rates of fetal wastage (Zenclussen et al., 2011) that could be reversed by administration of CO (El-Mousleh et al., 2012). We have also found that the CO inhibits the production of proinflammatory cytokines, such as IL-1β, IL-6 and TNF-α by the placenta (Peltier et al., 2013a) and fetal membranes (Klimova et al., 2013) and it prevents infection-mediated preterm birth in a mouse model (Peltier et al., 2013c). Other investigators have demonstrated that CO may also prevent preeclampsia (Venditti et al., 2014; Venditti and Smith, 2014) that can result from placental inflammation. Although, endogenous and therapeutic doses of CO are well below toxic levels of ˜50 ppm/8 h, the reputation that CO has as poisonous gas makes it difficult to implement in a clinical setting. However, induction of the enzyme that makes CO, HO-1, is possible with SFN that is already available as a nutrient supplement. Therefore, we tested the effects of SFN on placental production of HO-1 as well as a series of biomarkers that regulate inflammation and neurodevelopment.

We found that SFN had no effect on viability of the placental cultures examined by MTT assay at the concentrations up to 10 μM, suggesting that it is not overtly toxic. Like macrophages (Lin et al., 2008), vascular endothelial (Shan et al., 2010) and liver cells (Jeong et al., 2005), the placenta responds to SFN with increased production of HO-1 in a dose-dependent manner. This response, however, was conditional on the presence of bacteria as no enhancement of HO-1 production by SFN in bacteria-stimulated cultures was observed. It is possible that HO-1 production, already increased slightly by bacteria alone (Table 2), could not be further augmented by SFN because the response was already maximal.

To determine how HO-1 induction via SFN may affect cytokine production, we quantified an array of cytokines whose dysregulation has been implicated in preterm birth and ASD, conditions that are also associated with infections during pregnancy (Gibbs et al., 1992; Peltier, 2003; Atladottir et al., 2010; Zerbo et al., 2013; Lee et al., 2015; Zerbo et al., 2015). We found that in the absence of infection, SFN inhibited the production of IL-6 but enhanced its negative regulator, sgp130. This suggests that SFN may lower bioactive concentrations of IL-6 at the maternal fetal interface. This result differs from what Park and Loch-Caruso found with a first-trimester placental cell line where they reported no effect of SFN on IL-6 production (Park and Loch-Caruso, 2014). It is possible that first and third trimester trophoblast cells respond differently to SFN, however, it is more plausible that these discordant results are due to our use of explant cultures containing many different cells types (e.g. endothelial cells, stroma and macrophages). In a clinical trial, consumption of broccoli for 70 days reduced plasma IL-6 levels in volunteers (Lopez-Chillon et al., 2018), suggesting that our results may approximate what occurs in vivo.

Our finding that SFN inhibits basal BDNF production also conflicts with a previous report that studied the effects of SFN on neuroblastoma cells (Angeloni et al., 2015) where SFN increased BDNF production. The reasons for this difference between tissues is unclear but other studies have demonstrated that SFN increases BDNF expression in primary cortical neurons through an epigenetic mechanism (Kim et al., 2017) that may not be present in the placenta. BDNF is elevated in the serum of people with ASD (Ricci et al., 2013). This growth factor mediates the development of dopaminergic neurons in response to thyroid hormones

(Berbel et al., 2014) and it has been proposed that ASD is the result of dopaminergic dysfunction (Paval, 2017). Therefore, reductions of BDNF by SFN in suggest that this molecules effects on neurodevelopment may be worth further examination..

Bacteria-stimulated IL-1β, IL-6 and IL-10 production were reduced by SFN. Previous studies have reported that SFN reduces LPS-induced production of these cytokines by macrophages (Lin et al., 2008) and microglia (Yang et al., 2007). However, we observed reductions in bacteria-induced IL-10 production with our placental culture system by SFN whereas others observed an augmentation of IL-10 production by microglia *in vitro* (Yang et al., 2007). Other investigators also found that TNF-α production by LPS-stimulated microglia (Innamorato et al., 2008) and macrophages (Lin et al., 2008) was suppressed by SFN. However, we found that bacteria-stimulated TNF-α production tended to be increased by SFN in our placental culture system.

The observed changes in placental biomarker production by SFN suggest that this substance may have potential to reduce the consequences of ascending infections on pregnancy outcome. Previous studies have demonstrated that inhibition of IL-1β (Romero and Tartakovsky, 1992), TNF-α (Holmgren et al., 2008) or administration of IL-10 (Robertson et al., 2006, 2007) can prevent infection-mediated preterm birth in mice. Although we observed reductions in IL-1β, our finding, that SFN does not inhibit TNF-α or promote IL-10 expression suggests that HO-1 may not be a global inhibitor of inflammation at the maternal fetal interface and further studies are needed to determine how SFN, HO-1 and CO may differentially regulate these different cytokines in the placenta. Although IL-6 is increased in both clinical (Dammann and Leviton, 1997) and animal models of preterm birth (Dudley et al., 1996), IL-6 administration does not cause preterm birth and bacteria still induce preterm birth in mice that are knock-out for IL-6 (Yoshimura and Hirsch, 2003). However, IL-6 may play an important role in adverse neurodevelopmental outcomes that result from infections during pregnancy. Maternal infections during pregnancy increase placental production of IL-6 and this is followed by behavioral, neurological and neuroanatomical changes in the offspring that are consistent with ASD (Smith et al., 2007). These changes in behavior can be mimicked by administration of IL-6 or blocked by monoclonal antibodies to IL-6 in the models of maternal infection (Smith et al., 2007). Our finding that SFN reduces IL-6 production suggests that it may reduce the risk of neurodevelopmental disorders in pregnancies complicated by maternal infection. Previous studies have found that SFN reduced aberrant behavior checklist and social responsiveness scale scores (Singh et al., 2014) in autistic adults and part of this mechanism may be mediated through reductions in neuroinflamation (Singh and Zimmerman, 2016). However, additional studies are needed to determine if increased cruciferous vegetable consumption or SFN supplementation during pregnancy can lower the risk of neurodevelopmental disorders.

In summary, we found that SFN altered the production of placental biomarkers that are associated with ASD in children but had mixed effects on those associated with preterm birth. Further work with animal and clinical models of preterm birth and neurodevelopmental disorders are required to determine if SFN can prevent these common adverse pregnancy outcomes.

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