Eicosanoid Metabolites Associated with Type-1 Interferon Production from Plasmacytoid Dendritic Cells Stimulated with Toll-like Receptors

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Abstract

Common among several autoimmune diseases is a prominent interferon (IFN) signature caused by aberrant IFN-α production from plasmacytoid dendritic cells (pDC). This uncontrolled response occurs when pDCs are activated through endosomal toll-like receptors (TLR). Within the endosomal compartment, TLR 7/8 and 9 are stimulated through nuclear immune complexes. The objective of this work was to determine the eicosanoids associated with IFN-α production upon TLR stimulation of murine bone marrow derived dendritic cells.

Female C57BL/6Ncrl mouse bone marrow was cultured in FLT3-ligand (FMS-like tyrosine kinase 3) for 8 days to generate dendritic cell subsets. On day 8, TLR ligands were added. Supernatant was analyzed for eicosanoids by liquid chromatography- mass spectrometry and cell pellets were analyzed for phospholipid fatty acid content by gas chromatography- mass spectrometry and IFN-α by ELISA.

Murine pDCs stimulated with TLR 7 ligand Poly U (GU-rich ssRNA40), TLR 8 ligand R848 (resiquimod), and TLR 3 ligand Poly I:C (polyinosinic-polycytidylic acid) produced no observable IFN-α production. Upon stimulation with TLR 9 ligand ODN1585 CpG, IFN-α was significantly increased as compared to unstimulated no target samples. Targeted lipidomics analysis of eicosanoids showed that in cultures stimulated with TLR9 ligand, pro-inflammatory eicosanoid production of arachidonic acid mediators derived via cyclooxygenase, such as prostaglandin A_2, B_2, D_2, and E_2 and thromboxane B_4, were increased while decreases were seen in lipoxygenase derived mediators. Phospholipid fatty acid analysis revealed low levels of α-linoleic acid, dihomo-γ-linolenic acid, and arachidonic acid and high levels of EPA, DPA, and

DHA within the phospholipid membranes. Understanding the role of fatty acids associated with IFN-α production in pDCs may provide therapeutic and mechanistic targets for autoimmune diseases with an IFN signature.

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This thesis is dedicated to my baby girl, my darling angel Madelyn, who has been an endless inspiration throughout all of this.

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Literature Review

Autoimmune diseases (AD) affect 23.5 million individuals in the U.S. (NIH, 2006) and are one of the top 10 leading causes of mortality in women under 65 years (Walsh & Rau, 2000). The chronic and life-threatening nature of many autoimmune diseases are debilitating and disabling, increasing financial and economic burdens on individual constituents (Tobias, 2010). While the exact trigger to autoimmune disease development is unknown, an individual's exposome, or lifetime exposures, is a key factor in the development of autoimmune diseases, such as lupus.

Affecting more than 5 million people worldwide and being one of the most commonly diagnosed autoimmune diseases annually, Lupus is a prevalent and problematic worldwide disease. Approximately 16,000 new cases are diagnosed each year (Danchenko et al., 2006). In the United States, Lupus is more prevalent among African Americans and Asians as compared Caucasians (Danchenko et al., 2006; Cervera et al., 2009; Vasudevanand et al., 2010; Osio-Salido & Manapat-Reys, 2010; Jakes et al., 2012). Lupus affects females more than males at a 9:1 ratio, indicating possible female hormone involvement in the pathogenesis of the disease (Elkton and Stone., 2011). Factors contributing to the development of Lupus include a combination of genetics, hormones, environment, and immunoregulation. Individuals diagnosed with lupus experience many life-altering and debilitating symptoms. These clinical symptoms encompass pain, extreme fatigue, cognitive issues, hair loss, and other major physical impairments. While Lupus presents with a relatively low mortality rate, if left untreated it could become fatal (Klarquist et al., 2016).

Lupus manifests itself with a loss of self-tolerance to nuclear autoantigens and uncontrolled autoantibody and immune complex formation. This occurs with a lack of clearance

of dead and dying cells (Berkel et al, 1997; Shoshan et al, 2001) in conjunction with abnormal apoptosis (Herrmann et al, 1998; Lorenz and Anders, 2015). When these cells become apoptotic, they release their nuclear material into the surrounding environment, promoting the formation of immune complexes that induce the loss of self-tolerance and production of inflammatory cytokines. Abnormal autoantibody production due to loss of self-tolerance leads to aberrant production of genes encoding inflammatory agents, specifically type 1 interferon (IFN) production by plasmacytoid dendritic cells (pDCs), causing systemic inflammation, tissue, and organ damage (Lorenz et al., 2016). Serum and tissue samples of lupus patients have shown significant increases in apoptotic cells (Herrmann et al, 1998) and increased levels of type 1 interferons (Kwok, et al., 2008), which can be correlated to active disease (Elkton and Stone, 2011). Dendritic cells are important mediators in the immunological response as they are the cellular bridge between innate immunity and adaptive immunity (Gallo and Gallucci, 2013). Under homeostatic conditions, the main functions of dendritic cells are to clear necrotic and dying cells through efferocytosis, to present processed antigens to naïve T lymphocytes to induce regulatory T lymphocyte development (Jongbloed et al., 2010), and inhibition of autoreactive T lymphocyte development to maintain immunological self-tolerance (Goubier et al., 2008). Dendritic cell subsets vary in their cell surface markers, production of cytokines, and their ability to initiate immune responses. (Gallo and Gallucci, 2013).

Plasmacytoid dendritic cells are a specialized subset of $CD11c^{+}$ dendritic cells that are relatively long living (Shortman and Naik, 2006). While the exact cause of lupus is unknown, previous research shows that pDCs are involved in both the formation and progression of lupus (Yeste et al, 2012). Plasmacytoid dendritic cells become activated when autoantigens bind to the FCγRIIa receptor on the surface of the cell, allowing the internalization of the autoantibody

complex into an endosomal compartment (Bave et al, 2003). Once inside the endosomal compartment, toll-like receptors (TLR) 3, 7/8 and 9 become activated. Toll-like receptors (TLRs) are a family of innate immune pattern recognition receptors (PRR) (Keating et al, 2011). These TLRs recognize foreign invading pathogenic material called pathogen-associated molecular patterns (PAMPs). TLR3 recognizes double-stranded viral RNA. TLR3 signals through the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) transcription factors and cytoplasmic toll-IL-1 domain (TIR domain) containing IFN-β (TRIF) to produce IFN-β in response to the viral pathogen (Siednieko et al., 2010; Okahira et al., 2005). Toll-like receptors 7/8 and 9 are nearly identical in signaling to produce pro-inflammatory cytokines, however they differ in their recognition of PAMPs. TLRs 7/8 recognizes single-stranded RNA and elicits and antiviral response upon stimulation, while TLR9 recognizes ssDNA, and upon recognition initiates an antibacterial immune response. This activation creates a cascade of signaling events facilitated by adaptor proteins MyD88/TRAF3/IRF7, leading to translocation to the nucleus and transcription of genes encoding the production of inflammatory cytokines and type 1 IFN (Yang et al., 2005; Bao and Liu, 2012; Lorenz, et al., 2016; Kawai et al., 2004; Lee et al., 2003). pDCs respond to intracellular pathogens by producing type-1 IFN.

Type 1 IFN is clinically important in the pathogenesis of lupus (Hooks et al., 1979). Many different cell types are capable of producing IFN-β; however, hematopoietic pDCs are unique in their ability to produce 10-100 times more IFN-α than any other cell type (Elkton & Stone, 2011). In response to the activation of TLR 7/8 and 9 by nuclear material, aberrant production of type-1 IFN occurs. Once pDCs begin secreting type-1 IFN into the surrounding environment, the exogenous type-1 IFN stimulates further production in an autocrine and paracrine fashion by binding to heterodimeric transmembrane INFAR 1/2 on the surface of the

pDC (Wu et al., 2016). Signaling through this pathway involves members of the JAK-STAT pathway to induce the expression of numerous modulatory IFN response genes (ISGs) (Crow et al., 2014; Wu et al., 2016). Aberrant IFN-α production leads to both the over production of other inflammatory cytokines and tissue damage (Gkirtzimanaki et al, 2018). Intervening in the self-perpetuating production of type-1 IFN provides a pleiotropic target for the reduction of lupus development and disease severity as well as the reduction of other acute and chronic systemic inflammatory diseases that are otherwise difficult to treat (Ah Kioon et al, 2018; Ou Jin et al, 2008). Therefore, much interest has been shown in inhibiting type-1 IFN production in pDCs (Zhuang et al., 2015; Lee et al., 2003; Mathian et al., 2015). Current immunotherapy techniques may be just as harmful as helpful and over time, treatments become less effective and many patients may look to more alternative methods to conventional pharmaceuticals in facilitating treatment of disease progression (Pestka et al., 2014).

Treatment options differ from individual to individual based on severity and organ involvement of the disease. Currently, treatment options for lupus are designed towards controlling the damaging inflammatory process of the disease. Treatment options for lupus include immunosuppressive drugs such as corticosteroids, NSAIDS (non-steroidal anti-inflammatory drugs), and hydroxychloroquine (Urowitz et al., 1976). Although useful in the suppression and treatment of symptoms, these treatment options have many adverse side effects that include secondary infections and organ damage. Safety concerns associated with many of these potent immunomodulatory treatments outweigh any significant clinical benefit (Yildrim-Toruner and Diamond., 2011). Therefore, treatment of lupus presents itself as an important area of research to scientists of many different disciplines.

Polyunsaturated fatty acids (PUFAs) are fats that contain more than one carbon-carbon double bond in the backbone of their aliphatic chain. PUFAs are important biological compounds that are structurally diverse with varying chain lengths and degrees of unsaturation (Holuab & Kuksis, 1978). The varying amount of unsaturation plays a crucial role in membrane functions such as membrane protein function (Spector & Yorek, 1985). PUFAs can then be classified as ω-3 or ω-6 based on the position of the double bond to the first carbon of the methyl group in the aliphatic chain. Dietary fats, such as PUFAs, have a strong influence on the immunological state and function of the immune system. Dietary fish oil (rich in ω-3 fatty acids) reduces and/or ameliorates the immunological effects of system wide inflammation in autoimmune disease by acting on numerous aspects of the uncontrolled immune response (Bates et al., 2016). Although humans and mammals require essential PUFAs to maintain cellular homeostasis and membrane structure and integrity, they are unable to synthesize them *in vivo* (Kong et al., 2010). Therefore, it is necessary to have a diet balanced in PUFAs.

The addition of dietary anti-inflammatory ω-3 fatty acids (such as DHA or EPA) pleiotropically modulates eicosanoid production in different ways. Dietary ω-3 fatty acids can modulate eicosanoid production from arachidonic acid by increasing the ratio of ω-3: ω-6 phospholipid fatty acids, competitively displacing arachidonic acid as a substrate for eicosanoid production. Those metabolites produced from the enzymatic oxidation of DHA/EPA produce pro-resolving mediators, such as resolvins, protectins, maresins, and lipoxins (Shaikh et al., 2011; Calder 2016; Rajnavolgyi et al., 2014; Nastasi et al., 2015; Pestka, 2010; Carlsson et al., 2015; Kong, 2011).

Previous studies using NZBWF1 murine models have been primarily focused on pro-inflammatory effects of omega-6 fatty acids (linoleic acid) and anti-inflammatory effects of

omega-3 fatty acids (docosahexaenoic acid) on T lymphocytes (Nataraj et al, 2001), B lymphocytes (Rockett et al, 2010), and monocytes (Bingzhong et al, 2012) and the ability of omega-3 fatty acids to ameliorate SLE symptoms and progression in murine models (Pestka et al., 2014; Kong, 2011). Results of these studies show that cellular treatment of ω-3 fatty acids causes both a direct and indirect decrease in aberrant B and T lymphocyte activation and proliferation, cellular cytotoxicity, cellular chemotaxis, upregulated MHC II expression, and antigen presentation (Pestka et al., 2014; Kong et al., 2010; Kong, 2011). Pestka et al (2014) and Bates et al (2016) have shown strong evidence that nutritional supplementation dietary ω-3 fatty acids ameliorate lupus symptoms and disease progression in murine models. However, there is a lack of understanding of the effects that ω-3 fatty acids have on pDCs, especially on pro-inflammatory IFN-α production of pDCs. Although studies have shown that the addition of anti-inflammatory ω-3 PUFAs alters the phospholipid membrane structure and fluidity of lymphocytes, research is lacking as to whether ω-3 fatty acids affect phospholipid membrane fluidity, intracellular signaling, and anti-inflammatory eicosanoid production in pDCs. The focus of our laboratory is the investigation of both intrinsic and extrinsic effects that ω-3 fatty acid supplementation has on murine bone marrow-derived pDC phenotype and function, fatty acid metabolism and eicosanoid production, and TLR signaling that result in type-1 IFN production.

Methods

Mice

C57BL/6NCrl female mice were purchased from Charles River Laboratories at 5-6 weeks of age. Mice were housed four per cage in a clean animal facility at the University of Michigan-Flint. They were allowed free access to both 18% protein Teklad irradiated chow diet (Envigo 2918) feed and water. The animal facility was kept at a constant temperature and humidity and the mice were subjected to 12-hour light/dark cycles. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Michigan according to federal guidelines. The mice were sacrificed via CO_2 asphyxiation with secondary cardiac puncture and heart removal. The tibia, fibula, and femur were collected for bone marrow aspiration. Bone marrow cells were flushed with RPMI. Cells were lysed with ACK lysis buffer (155nM ammonium chloride, 10mM potassium bicarbonate, 0.1mM EDTA) and plated at a density of $1.0*10^6$ cells/mL in 12 well plates with complete RPMI-1640 [10% fetal bovine serum (Hyclone), 1x Penicillin/Streptomycin, 1x non-essential amino acids, 1x sodium pyruvate, 50μM beta mercaptoethanol] supplemented with 200ng/mL recombinant murine FLT3-L (PreproTech, Rocky Hill, NJ) and incubated at 37℃, 5% CO_2 for 9 days.

TLR Agonist Stimulation

On day 8 of the culture, the cells were stimulated with TLR8 agonist Poly(U) (InvivoGen) at 5μg/mL, TLR3 agonist Polyinosinic-polycytidylic acid (Poly I:C, InvivoGen, San Diego, CA) at 1μg/mL, TLR9 agonist ODN 1585 CpG oligonucleotide (InvivoGen, San Diego, CA) at 1μM,

and TLR7/8 agonist Resiquimod (R848, InvivoGen, San Diego, CA) at 1μg/mL and incubated at 37℃, 5% CO_2 for 24 hours.

Immunofluorescent Intracellular Staining and Flow Cytometry

Surface immunophenotyping of cell culture with fluorochrome-conjugated monoclonal antibodies and intracellular staining for IFNα protein were performed to identify cell-specific intracellular IFNα production. For intracellular staining, the cells were stimulated with TLR ligands as described above and incubated for 16 hours with the addition of 1μl/mL of GolgiPlug (Brefeldin A; BD Biosciences, San Jose, CA) for the final 8 hours of the 24-hour stimulation. Cells were harvested by vigorous pipetting and washing with Ca++/Mg++ free fluorescence activated cell sorting (FACS) buffer. Cells were blocked with anti-CD16/anti-CD32 (Fc Block, BD Biosciences, San Jose, CA) for 10 minutes on ice. Subsequently, cells were surface labeled with anti-IFNαR-PE (Leinco Technologies, Fenton, MO), anti-CD11c-APC (BD Biosciences, San Jose, CA), anti-B220-PerCp-eFluor710 (BioLegend, San Diego, CA). Cells were washed with FACS buffer, fixed with paraformaldehyde, and permeabilized with saponin according to manufacturer instructions (BD Biosciences, San Jose, CA). Fixed and permeabilized cells were stained intracellularly with fluorochrome-conjugated anti-IFNα-FITC (PBL assay science, Piscataway, NJ). The samples were acquired via flow cytometric analysis using a BD Biosciences FACS Calibur 4 color analysis. FlowJo software was used for post-acquisition data analyses.

Quantification of IFNα

To confirm and quantify the presence of IFN-α, cell cultures (without GolgiPlug) were centrifuged at 320xg for 10 minutes at 4℃ to separate cells from the supernatant. The supernatant was used to perform an ELISA (PBL Assay Science, Piscataway, NJ) to detect and quantify IFN-α production. In following the manufacturer's protocol, the standard and appropriate sample dilutions were made, and the diluted detection antibody solution was added. The plate incubated at 25℃ while on a plate shaker at 200RPM for 2 hours and was stored at 4℃ for 20-24 hours. The plate was washed four times with diluted wash solution and HRP was added. The plate was incubated on a plate shaker at 25℃ for 2 hours. After incubation, TMB substrate (PBL Assay Science, Piscataway, NJ) was added to each well, followed by 15-minute incubation and the addition of stop solution. The absorbance was read at 450nm on a microplate reader and a standard curve was generated.

Lipidomics Analysis

Pellets of cells stimulated with TLR agonists were collected and stored at -80C until submission to the University of Michigan Regional Comprehensive Metabolomics Resource Core (Ann Arbor, MI) for analysis of phospholipid fatty acids (palmitic acid, palmitoleic acid, stearic acid, oleic acid, and arachidonic acid). The lipids were extracted from the sample matrix, transesterification phospholipids to methyl esters occurred, and then analyzed via GCMS.

Metabolomics Analysis

Supernatant samples of cells stimulated with TLR agonists were collected and sent to the University of Michigan Regional Comprehensive Metabolomics Resource Core (Ann Arbor, MI) eicosanoid analysis. Using solid phase extraction, eicosanoids are extracted, concentrated, dried and re-suspended for separation by LCMS/RPLC.

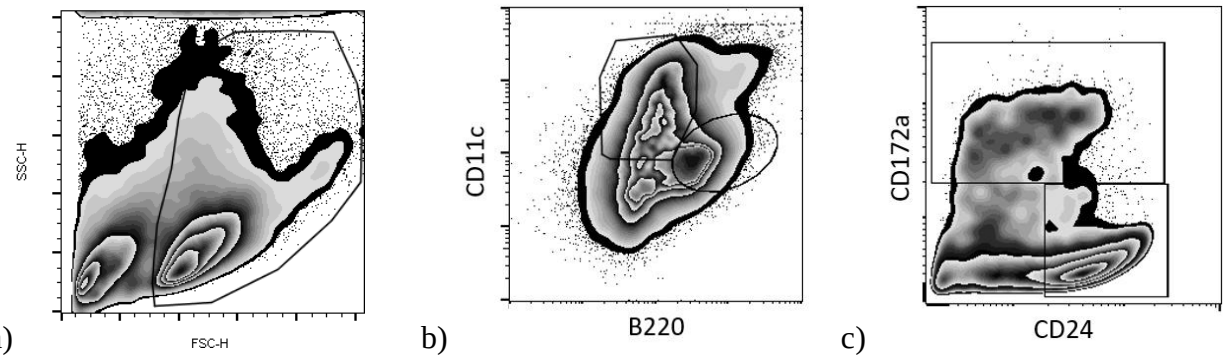
Data Analysis

Duplicate lipidomic samples were averaged and mean no target values were subtracted. A heat map was generated based on relative percent changes across stimulations.

Results

From two C57BL/6NCrl female mice, $3.88*10^7$ hematopoietic bone marrow cells were obtained and plated in 12 well culture plates at a density of $1.0*10^6$ cells/mL and supplemented with 200ng/mL of recombinant murine FLT3-L for 9 days to induce dendritic cell expansion of myeloid and lymphoid dendritic cell populations. The cells were blocked via Fc block and surface labeled with anti-CD11c-APC, and anti-IFNαR-PE (data not shown). Phenotypic analysis of the cell culture was determined via flow cytometric analysis of cells (Figure 1).

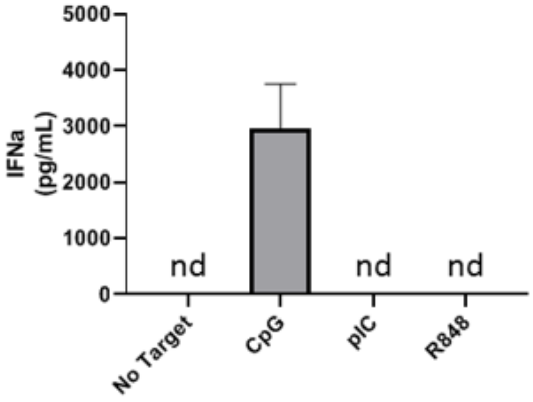
Figure 1- Phenotypic Analysis of FL3-L Induced Myeloid and Lymphoid Dendritic Cell Populations.



A sequential gating strategy used to identify FLT3-L induced myeloid and lymphoid dendritic cell populations from culture. a) Forward scatter (FSC) and side scatter (SSC) of cell populations to gate out lymphocytic cell population. b-c) Gating strategy to identify cDCs and pDCs expressing specific cell markers. In figure b, cells that are $CD11c^{+}/B220^{-}$ are cDC cell population. 38.6% +/- 0.867% [Standard error of the mean (SEM)] of cells in figure b are $CD11c^{DIM}/B220^{+}$ are pDC cell population. Figure c is the remaining gating strategy to identify 13.6% +/- 2.27% CD8-like cDCs and 2.87% +/- 0.48% CD11b-like cCDs. The CD8-like cDCs are $CD11c^{+}/B220^{-}/CD24^{+}$ $CD172a^{-}$. The CD11b-like cDCs are $CD11c^{+}/B220^{-}/CD24^{-}/CD172a^{+}$.

On day 8, the cells were stimulated with TLR 3 agonist Poly(I:C) or TLR 9 agonist ODN 1585 CpG for 24 hours. To confirm IFN-α production, intracellular staining was performed and analyzed via flow cytometry (data not shown). The supernatant of the cell cultures was collected and used to perform a murine specific IFNα ELISA to detect and quantify IFN-α present (Figure 2).

Figure 2- Quantification of IFN-α Production across TLR Stimulations



An ELISA was performed using the supernatant of the cell cultures of cells stimulated through TLRs to detect and quantify the inducible IFN-α production. In the cells lacking TLR stimulation (No target) IFN-α was not detectable. Cells were further stimulated via TLR 9 agonist CpG, TLR 3 agonist pIC, and TLR 7/8 agonist R848. From the stimulation, quantifiable amounts at 2963 ±798.0 pg/mL (n=3) of IFN-α was produced only through stimulation through TLR 9 via ODN1585 CpG.

Cells were pelleted and sent to the University of Michigan Regional Comprehensive Metabolomics Resource Core for phospholipid fatty acid analysis. Supernatant of the stimulated cell cultures was collected and also sent to the University of Michigan Regional Comprehensive Metabolomics Resource Core for analysis of lipid mediators/eicosanoids that are involved in IFN-α production and stimulation through various TLRs (Table 1).

Table 1- Phospholipid Fatty Acid Lipidomic Changes due to TLR Stimulation

PL-FA Profile	NT-A	(%) NT-A	NT-B	(%) NT-B	pIC-A	(%) pIC-A	pIC-B	(%) pIC-B	CpG-A	(%) CpG-A	CpG-B	(%) CpG-B
Name	Amt (n mol)	percent (%)	Amt (n mol)	percent (%)	Amt (n mol)	percent (%)	Amt (n mol)	percent (%)	Amt (n mol)	percent (%)	Amt (n mol)	percent (%)
14:0	0.03	0.47	0.04	0.40	0.01	0.18	0.02	0.19	0.06	0.25	0.02	0.20
14:1 (n-5)	0.41	5.61	0.40	4.28	0.48	6.19	0.36	2.86	0.47	2.10	0.43	3.45
16:0	1.39	19.21	0.89	9.63	0.75	9.65	1.66	13.19	1.78	8.04	1.19	9.46
16:1 (n-7)c	0.02	0.29	0.06	0.62	0.05	0.66	0.09	0.73	0.04	0.17	0.13	1.02
18:00	3.03	41.78	3.94	42.73	3.92	50.73	5.75	45.62	11.53	52.10	4.88	38.76
18:1(n-7)	0.17	2.39	0.24	2.65	0.27	3.49	0.34	2.67	0.51	2.31	0.41	3.27
18:1(n-9)	0.48	6.59	0.50	5.45	0.55	7.06	0.68	5.38	1.58	7.14	0.70	5.58
18:2 (n-6)	0.14	1.90	0.15	1.57	0.17	2.21	0.19	1.53	0.33	1.48	0.25	1.96
18:3 (n-3)	0.02	0.23	0.07	0.75	0.02	0.30	0.00	0.00	0.03	0.14	0.02	0.17
18:3 (n-6)	0.09	1.20	0.10	1.10	0.07	0.89	0.03	0.24	0.20	0.91	0.08	0.64
20:0	0.08	1.14	0.00	0.00	0.00	0.00	0.05	0.40	0.24	1.09	0.00	0.00
20:1	0.00	0.00	0.00	0.00	0.03	0.44	0.00	0.00	0.05	0.24	0.00	0.00
20:2	0.00	0.00	0.00	0.00	0.05	0.65	0.09	0.71	0.11	0.48	0.06	0.44
20:3 (n-6)	0.03	0.48	0.00	0.00	0.07	0.95	0.09	0.68	0.00	0.00	0.15	1.18
20:4 (n-6)	0.08	1.04	1.13	12.27	0.03	0.34	1.52	12.09	2.15	9.72	2.06	16.36
20:5 (n-3)	0.06	0.83	0.09	0.98	0.13	1.64	0.10	0.83	0.24	1.09	0.05	0.43
21:0	0.07	1.03	0.11	1.21	0.06	0.77	0.07	0.54	0.29	1.30	0.08	0.63
22:0	0.20	2.78	0.19	2.07	0.07	0.93	0.36	2.83	0.14	0.65	0.10	0.83
22:1	0.25	3.47	0.26	2.76	0.24	3.07	0.05	0.36	0.34	1.56	0.31	2.48
22:2 (n-6)	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.33	0.00	0.00	0.08	0.65
22:4 (n-6)	0.16	2.16	0.28	3.01	0.24	3.17	0.37	2.91	0.67	3.02	0.47	3.72
22:5 (n-3)	0.03	0.35	0.08	0.82	0.11	1.44	0.17	1.35	0.19	0.87	0.25	1.96
22:6 (n-3)	0.02	0.30	0.11	1.14	0.15	1.96	0.15	1.21	0.25	1.13	0.27	2.15
24:0	0.26	3.56	0.12	1.29	0.00	0.00	0.19	1.48	0.26	1.16	0.26	2.08
24:1	0.23	3.20	0.48	5.25	0.25	3.29	0.24	1.87	0.68	3.06	0.32	2.57
Sum (n mol)	7.26	100.00	9.23	100.00	7.73	100.00	12.60	100.00	22.13	100.00	12.59	100.00

Results of lipidomics analysis of 25 tested phospholipid fatty acid concentration (n mol) per stimulation. Cells were cultured and stimulated in duplicates. NT-A and NT-B consist of bone marrow-derived dendritic cell pellets of cells that were cultured in RPMI-1640 and were lacking stimulation through TLR agonists. Duplicate stimulations of pIC and CpG consist of cell pellets of cells cultured in RPMI-1640 and stimulated with TLR3 (poly(I:C)) or TLR9 (CpG) agonists for 24 hours. 14:0-myristic acid, 14:1- Myristoleic acid, 16:0- Hexadecenoic acid, 16:1-Palmitoleic acid, 18:0- Octadecanoic acid, 18:1 (n-7)- Vaccenic acid, 18:1 (n-9)-oleic acid, 18:2-linoleic acid, 18:3 (n-3)-α-linolenic acid, 18:3 (n-6)-γ -linoleic acid, 20:0-Arachididic acid, 20:1-Eicosanoic acid, 20:2- Eicosadienoic acid, 20:3- dihomo-γ-linolenic acid, 20:4- Arachidonic acid, 20:5- Eicosapentaenoic acid (EPA), 21:0- Heineicosanoic acid, 22:0- Eicosanoic acid, 22:1-Eruic acid, 22:2- Docosadienoic acid, 22:4- Docosatetraenoic acid, 22:5- Docosapentaenoic acid, 22:6- Docosahexaenoic acid (DHA), 24:0-Tetracosanoic acid, 24:1-Nervonic acid. An increase is seen in total phospholipid fatty acid concentrations in CpG-A.

Table 2- Average Concentration and Average Relative Percent of Phospholipid Fatty Acids due to TLR Stimulation

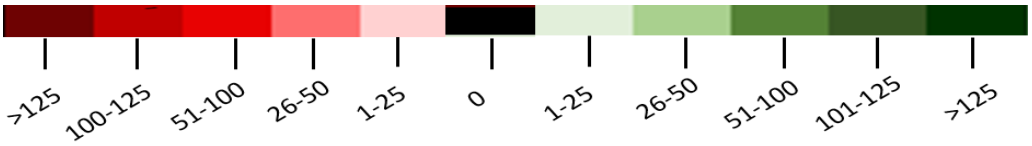
PL-FA Profile	Average NT	(%) Ave Percent NT	Average pIC	(%) Ave Percent pIC	Average CpG	(%) Ave Percent CpG
Name	Amt (n mol)	percent (%)	Amt (n mol)	percent (%)	Amt (n mol)	percent (%)
14:0	0.04	0.42	0.02	0.15	0.04	0.23
14:1 (n-5)	0.41	4.87	0.42	4.13	0.45	2.59
16:0	1.14	13.70	1.21	11.85	1.49	8.56
16:1 (n-7)c	0.04	0.48	0.07	0.69	0.09	0.26
18:00	3.49	41.89	4.84	47.57	8.21	47.28
18:1(n-7)	0.21	2.46	0.31	3.00	0.46	2.65
18:1(n-9)	0.49	5.89	0.62	6.05	1.14	6.57
18:2 (n-6)	0.15	1.74	0.18	1.77	0.29	1.67
18:3 (n-3)	0.05	0.54	0.01	0.10	0.03	0.14
18:3 (n-6)	0.10	1.14	0.05	0.49	0.14	0.81
20:0	0.04	0.48	0.03	0.25	0.12	0.69
20:1	0.00	0.00	0.02	0.15	0.03	0.14
20:2	0.00	0.00	0.07	0.69	0.09	0.49
20:3 (n-6)	0.02	0.18	0.08	0.79	0.08	0.43
20:4 (n-6)	0.61	7.27	0.78	7.62	2.11	12.13
20:5 (n-3)	0.15	1.80	0.12	1.13	0.15	0.84
21:0	0.09	1.08	0.07	0.64	0.19	1.07
22:0	0.20	2.34	0.22	2.12	0.12	0.69
22:1	0.26	3.06	0.15	1.43	0.33	1.87
22:2 (n-6)	0.00	0.00	0.02	0.20	0.04	0.23
22:4 (n-6)	0.22	2.64	0.31	3.00	0.57	3.28
22:5 (n-3)	0.06	0.66	0.14	1.38	0.22	1.27
22:6 (n-3)	0.07	0.78	0.15	1.48	0.26	1.50
24:0	0.19	2.28	0.10	0.93	0.26	1.50
24:1	0.36	4.27	0.25	2.41	0.50	2.88
Sum (n mol)	8.32	100.00	10.17	100.00	17.36	100.00

Fatty acid concentrations (n mol) among duplicate cultures were averaged for each individual fatty acid present in the lipidomics analysis. The average total among duplicate cultures was also calculated. From the average fatty acid concentrations for each individual fatty acid and the average total, the average percent of each fatty acid was calculated by taking the calculated average fatty acid concentration, dividing it by the average total concentration, and multiplying that value by 100 to get a percent of each fatty acid as a part of the total. 14:0-myristic acid, 14:1- Myristoleic acid, 16:0- Hexadecenoic acid, 16:1- Palmitoleic acid, 18:0- Octadecanoic acid, 18:1 (n-7)- Vaccenic acid, 18:1 (n-9)-oleic acid, 18:2-linoleic acid, 18:3 (n-3)-α-linolenic acid, 18:3 (n-6)-γ -linoleic acid, 20:0-Arachididic acid, 20:1- Eicosanoic acid, 20:2-Eicosadienoic acid, 20:3- dihomo-γ-linolenic acid, 20:4- Arachidonic acid, 20:5-Eicosapentaenoic acid (EPA), 21:0- Heineicosanoic acid, 22:0- Eicosanoic acid, 22:1- Eruic acid, 22:2- Docosadienoic acid, 22:4- Docosatetraenoic acid, 22:5- Docosapentaenoic acid, 22:6-

Docosahexaenoic acid (DHA), 24:0-Tetracosanoic acid, 24:1-Nervonic acid. Average total fatty acid concentrations increase along with 20:4, 22:3, 22:6 due to stimulation through TLR9 agonist CpG. Conversely, decreases were seen in the average percent fatty acids of 16:1, 18:3(n-3), 18:3(n-6), 20:5, and 24:1.

Table 3- Heat Map Representing Relative Percent Change in Phospholipid Fatty Acid Across TLR Stimulations

PL-FA Profile	pIC-A	pIC-B	CpG-A	CpG-B
Name	% Change	% Change	% Change	% Change
14:0	69.34	62.26	36.32	62.26
14:1 (n-5)	26.43	41.78	56.84	30.58
16:0	29.85	4.75	41.85	31.66
16:1 (n-7) c	31.96	46.39	62.89	112.37
18:0	19.97	7.95	41.28	78.33
18:1(n-7)	40.87	8.61	7.48	107.56
18:1(n-9)	19.81	9.14	20.14	53.39
18:2 (n-6)	25.07	14.72	15.29	13.13
18:3 (n-3)	52.81	100.00	76.19	70.67
18:3 (n-6)	20.01	79.17	21.89	45.31
20:0	100	19.59	122.68	100
20:1	39.00	0.00	23.00	0.00
20:2	64.00	50.00	71.00	48.00
20:3 (n-6)	400	290.1	100	553.85
20:4 (n-6)	94.82	64.35	90.73	122.95
20:5 (n-3)	84.62	13.19	18.68	57.14
21:0	28.57	48.72	19.96	42.31
22:0	61.95	20.93	73.36	66.60
22:1	204.59	63.30	42.84	125.69
22:2 (n-6)	0.00	32.00	0.00	64.00
22:4 (n-6)	161.92	9.82	13.19	39.81
22:5 (n-3)	112.89	100.9	28.94	198.35
22:6 (n-3)	14.619	51.02	42.132	171.574
24:0	100	91.75	88.72	88.72
24:1	94.19	94.43	84.2	92.57



A heat map was generated from the lipidomics data comparing relative percent change in phospholipid fatty acids across differing TLR stimulations. NT samples are duplicate cell cultures of FLT3-L induced bone marrow-derived dendritic cells cultured in RPM-1640 lacking

TLR agonistic stimulation. Poly(I:C) and CpG duplicate cultures are cultured in RPMI-1640 stimulated with TLR agonists. Decreasing percentages from the control value are depicted in varying shades of red while increasing percentages are depicted in varying shades of green. Those values that remained unchanged or undetected are shown in black. To calculate the percent change, the average phospholipid fatty acid concentration for each fatty acid was determined for NT. The average NT total concentration was also calculated. From these calculations, the total percent of each NT was calculated. The percent of each individual fatty acid was calculated for both poly(I:C) stimulations and for both CpG stimulations. Finally, to determine the relative change in percent of each phospholipid fatty acid, the percent of the whole fatty acid of each stimulation was subtracted from the total percent of each NT fatty acid, divided by the total percent of each NT fatty acid, and multiplied by 100% to yield the relative percent change of each phospholipid fatty acid from the NT.

Lipidomics analysis shows a 100% decrease in dihomo-ɣ-linolenic acid (20:3) in CpG-A stimulation and a marked 554% increase in CpG-B stimulation. A 91% decrease in arachidonic acid (20:4) was noted in CpG-A stimulation, while a 123% increase occurred in CpG-B. Analysis shows a 19% increase in EPA (20:5) with CpG-A stimulation while there was a 57% decrease in CpG-B. In CpG-A, DHA (22:6) increased 42%, while in CpG-B there was a dramatic 172% increase. Palmitoleic acid (16:1) decreased 63% upon stimulation with CpG-A, however increased 112% in CpG-B. While DPA (22:5) increased throughout all stimulations, it was not as increased in CpG-A.

Table 4- Omega-3: Omega-6 Ratio of Average Fatty Acid Concentrations Across Stimulations

Fatty Acid	NT	pIC	CpG
18:3 n-3 (ALA)	0.05	0.10	0.03
20:5 n-3 (EPA)	0.15	1.13	0.15
22:5 n-3 (DPA)	0.06	1.38	0.22
22:6 n-3 (DHA)	0.07	1.48	0.26
TOTAL n-3	**0.33**	**4.09**	**0.66**
18:2 n-6 (LA)	0.15	0.18	1.67
20:3 n-6 (DGLA)	0.02	0.08	0.08
20:4 n-6 (AA)	0.61	0.78	2.11
22:4 n-6	0.22	0.31	0.57
TOTAL n-6	**1.00**	**1.35**	**4.43**
OMEGA-6 /OMEGA-3	**3.03**	**0.33**	**6.71**

The ω-6: ω-3 ratio was calculated from the average concentrations (n mol) of each of the listed unesterified ω-6 and ω-3 fatty acids. Average concentrations were summed for ω-3 fatty acids and ω-6 fatty acids and then the ω-6 average total concentration (n mol) was divided by the average total ω-3 (n mol) across each stimulation and the NT. NT samples are duplicate cell cultures of FLT3-L induced bone marrow-derived dendritic cells cultured in RPM-1640 lacking TLR agonistic stimulation. Poly(I:C) and CpG duplicate cultures are cultured in RPMI-1640 stimulated with TLR agonists. ω-3 and ω-6 unesterified fatty acids used in calculating ω-6: ω-3 ratio include: 18:3 n-3: α-linolenic acid, 20:5 n-3: eicosapentaenoic acid (EPA), 22:5 n-3: docosapentaenoic acid (DPA), 22:6 n-3: docosahexaenoic acid (DHA), 18:2 n-6: linoleic acid, 20:3 n-6: dihomo-γ-linolenic acid, 20:4 n-6: arachidonic acid, 22:4 n-6: docosatetraenoic acid. In the culture cells lacking TLR stimulation (NT), the ω-6: ω-3 ratio value is 3.03. Upon stimulation with Poly(I:C), the ω-6: ω-3 ratio is 0.33, indicating the omega-6:omega-3 ratio

shifted toward omega-3 PUFAs. Conversely, upon stimulation with TLR9 agonist CpG, the ω-6: ω-3 ratio was 6.71. This was greater than a two-fold increase as compared to NT cell unesterified ω -6 and ω -3 fatty acid concentrations and indicated that the ratio shifted towards ω-6 PUFAs.

Table 5- Lipid Mediator Production due to Differing TLR Stimulation

Eicosanoids	NT-A	(%) NT-A	NT-B	(%) NT-B	pIC-A	(%) pIC-A	pIC-B	(%) pIC-B	CpG-A	(%) CpG-A	CpG-B	(%) CpG-B
Name	Amount (µM)	%	Amount (µM)	%	Amount (µM)	%	Amount (µM)	%	Amount (µM)	%	Amount (µM)	%
11_HETE	1.27	0.63	1.25	0.87	1.26	0.72	0.92	1.22	1.25	0.87	1.82	0.76
12oxoETE	11.95	5.89	2.95	2.07	14.67	8.41	3.75	4.99	0.20	0.14	0.21	0.09
12s HETE	1.03	0.51	0.74	0.52	1.30	0.74	1.00	1.33	0.79	0.55	1.19	0.49
13S-HODE	7.02	3.46	6.57	4.59	8.10	4.64	6.71	8.93	7.63	5.29	9.15	3.79
15s HETE	4.07	2.01	2.67	1.87	2.84	1.63	2.13	2.83	2.37	1.64	3.23	1.34
20S HETE	10.92	5.39	3.35	2.34	8.79	5.04	4.53	6.04	6.20	4.30	3.28	1.36
5oxoETE	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5s HETE	6.36	3.14	3.15	2.21	6.29	3.60	3.72	4.95	2.68	1.86	2.26	0.94
6k PSG F1a	1.82	0.90	2.67	1.87	2.47	1.42	1.95	2.60	2.02	1.40	2.16	0.90
8_HETE	2.06	1.02	1.46	1.02	1.05	0.60	0.31	0.41	0.72	0.50	3.07	1.27
9_HETE	0.11	0.05	0.00	0.00	2.09	1.20	0.00	0.00	0.80	0.55	1.29	0.53
9S-HODE	138.95	68.52	102.03	71.38	106.21	60.89	28.70	38.21	97.74	67.75	194.55	80.63
LKT B4	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PSG_D2	1.40	0.69	1.15	0.81	1.78	1.02	1.59	2.12	1.50	1.04	1.58	0.65
PSG E2	2.37	1.17	2.12	1.48	3.01	1.73	2.70	3.59	2.99	2.07	2.53	1.05
PSG F2a	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
PSG_A2	1.73	0.84	1.61	1.13	3.10	1.78	2.56	3.41	3.12	2.17	1.20	0.50
PSG B2	1.82	0.90	1.76	1.23	1.84	1.05	1.87	2.49	2.05	1.42	1.93	0.80
RSV_D1	7.72	3.81	7.05	4.93	7.17	4.11	10.23	13.62	9.82	6.80	9.62	3.99
RSV_D2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
RSV_E1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TB B2	2.16	1.07	2.40	1.68	2.46	1.41	2.45	3.26	2.39	1.66	2.22	0.92
Total	202.75	100.00	142.93	100.00	174.42	100.00	75.10	100.00	144.25	100.00	241.29	100.00

11-HETE- 11S-hydroxy-5Z,8Z,11E, 14Z-eicosatetraenoic acid; 12-oxoETE- 12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid; 5s-HETE-5-hydroxyeicosatetraenoic acid; 12s-HETE- 12-hydroxyeicosatetraenoic acid, 13s-HODE- 13-hydroxyoctadecadienoic acid; 15s-HETE- 15-hydroxyeicosatetraenoic acid; 20s-HETE- 20-hydroxyeicosatetraenoic acid; 5oxoETE- 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid; 6kPSGF1a- 6-keto-prostaglandin F1 alpha; 8-HETE- 8-hydroxyeicosatetraenoic acid; 9-HETE- 9-hydroxyeicosatetraenoic acid; 9s-HODE- 9-hydroxyoctadecadienoic acid; LKTB4- leukotriene B_4; PSG_D2- prostaglandin D_2; PSG E2- prostaglandin E_2; PSG F2a- prostaglandin F_2 alpha; PSG_A2- prostaglandin A_2; PSG B2- prostaglandin B_2; RSV_D1- resolvin D1; RSV_D2- resolvin D2; RSV_E1-resolvin E1; TB B2- thromboxane B_2. An increase in total eicosanoid concentration is seen in CpG-B. Concentration amounts shown in µM. Percentages shown are percent of each eicosanoid present based off of the total of each stimulation. Culture and stimulations were duplicated (A and B). No target (NT) samples are cells cultured in RPMI-1640 lacking stimulation with TLR agonists, whereas pIC

and CpG samples are cells cultured in RPMI-1640 with TLR3 (pIC) and TLR9 (CpG) agonists. Culture supernatant was sent to Michigan Regional Comprehensive Metabolomics Core Facility post 24- hour stimulation for eicosanoid analysis.

Table 6- Average Concentration and Average Relative Percent of Eicosanoid Production due to Differing TLR Agonists

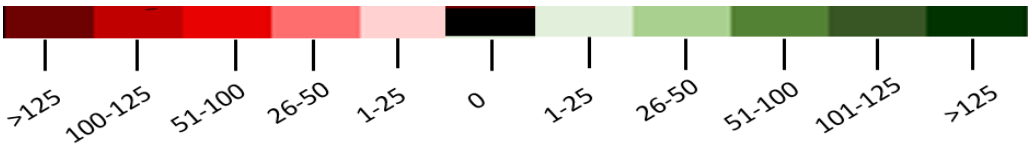
Eicosanoids	Ave NT	Ave (%) NT	Ave pIC	Ave (%) pIC	Ave CpG	Ave (%) CpG
Name	Amount (uM)	Percent (%)	Amount (µM)	Percent (%)	Amount (uM)	Percent (%)
11_HETE	1.26	0.73	1.09	0.87	1.54	0.80
12oxoETE	7.45	4.31	9.21	7.38	0.20	0.11
12s HETE	0.89	0.51	1.15	0.92	0.99	0.51
13S-HODE	6.80	3.93	7.40	5.93	8.39	4.35
15s HETE	3.37	1.95	2.48	1.99	2.80	1.45
20S HETE	7.14	4.13	6.66	5.34	4.74	2.46
5oxoETE	0.00	0.00	0.00	0.00	0.00	0.00
5s HETE	4.76	2.75	5.00	4.01	2.47	1.28
6k PSG F1a	2.25	1.30	2.21	1.77	2.09	1.08
8_HETE	1.76	1.02	0.68	0.54	1.89	0.98
9_HETE	0.05	0.03	1.04	0.84	1.04	0.54
9S-HODE	120.49	69.71	67.45	54.07	146.14	75.81
LKT B4	0.00	0.00	0.00	0.00	0.00	0.00
PSG_D2	1.28	0.74	1.69	1.35	1.54	0.80
PSG E2	2.24	1.30	2.85	2.29	2.76	1.43
PSG F2a	0.00	0.00	0.00	0.00	0.00	0.00
PSG_A2	1.67	0.97	2.83	2.27	2.16	1.12
PSG B2	1.79	1.04	1.85	1.49	1.99	1.03
RSV_D1	7.39	4.27	8.70	6.97	9.72	5.04
RSV_D2	0.00	0.00	0.00	0.00	0.00	0.00
RSV_E1	0.00	0.00	0.00	0.00	0.00	0.00
TB B2	2.28	1.32	2.45	1.96	2.31	1.20
Total	172.84	100.00	124.76	100.00	192.77	100.00

Eicosanoid values for duplicate cultures were averaged. The total eicosanoids produced (µM) in duplicate cultures were also averaged. From these values, the average percent of each individual eicosanoid produced was calculate from the average total by taking the average total eicosanoid per stimulation, dividing that value by the average total for that stimulation, and multiplying that value by 100 to generate the average percentage of each eicosanoid as part of the total concentration of eicosanoid per stimulation. NT samples are cultures of murine FLT3-L induced bone marrow-derived dendritic cells cultured in RPMI-1640 without any TLR agonist. Conversely, pIC and CpG are cultures of FLT3-L induced bone marrow-derived dendritic cells cultured in RPMI-1640 for 9 days with a 24-stimulation with TLR3 (pIC) or TLR (TLR9) agonist. After the 24-hour stimulation, cell culture supernatant was sent to Michigan Regional

Comprehensive Metabolomics Core Facility for eicosanoid analysis. 11-HETE- 11S-hydroxy-5Z,8Z,11E, 14Z-eicosatetraenoic acid; 12-oxoETE- 12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid; 5s-HETE-5-hydroxyeicosatetraenoic acid; 12s-HETE- 12-hydroxyeicosatetraenoic acid, 13s-HODE- 13-hydroxyoctadecadienoic acid; 15s-HETE- 15-hydroxyeicosatetraenoic acid; 20s-HETE- 20-hydroxyeicosatetraenoic acid; 5oxoETE- 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid; 6kPSGF1a- 6-keto-prostaglandin F1 alpha; 8-HETE- 8-hydroxyeicosatetraenoic acid; 9-HETE-9-hydroxyeicosatetraenoic acid; 9s-HODE- 9-hydroxyoctadecadienoic acid; LKTB4- leukotriene B_4; PSG_D2- prostaglandin D_2; PSG E2- prostaglandin E_2; PSG F2a- prostaglandin F_2 alpha; PSG_A2- prostaglandin A_2; PSG B2- prostaglandin B_2; RSV_D1- resolvin D1; RSV_D2-resolvin D2; RSV_E1-resolvin E1; TB B2- thromboxane B_2.

Table 7- Heat Map representing Relative Percent Change of Eicosanoids Across TLR Stimulations

Column1	pIC-A	pIC-B	CpG-A	CpG-B
Eicosanoids	% Change	% Change	% Change	% Change
11_HETE	1.10	19.51	67.58	150.55
12oxoETE	95.08	15.75	96.75	97.91
12s HETE	31.44	134.46	2.31	12.97
13S-HODE	18.10	127.28	34.64	3.54
15s HETE	16.41	45.13	15.89	31.80
20S HETE	44.38	46.32	4.17	67.05
5oxoETE	0.00	0.00	0.00	0.00
5s HETE	30.86	79.94	32.39	65.83
6k PSG F1a	8.46	100.00	61.54	30.78
8_HETE	40.95	59.65	50.79	25.00
9_HETE	3650.00	100.00	1618.75	1565.63
9S-HODE	12.66	45.20	40.27	15.66
LKT B4	0.00	0.00	0.00	0.00
PSG_D2	38.02	186.87	40.73	12.04
PSG E2	33.39	176.79	59.60	19.04
PSG F2a	0.00	0.00	0.00	0.00
PSG_A2	83.88	252.27	124.17	17.34
PSG B2	1.25	140.16	36.93	22.85
RSV_D1	3.82	218.75	59.14	125.18
RSV_D2	0.00	0.00	0.00	0.00
RSV_E1	0.00	0.00	0.00	0.00
TB B2	6.90	147.16	28.85	30.25



Heat Map representing Relative Percent Change of Eicosanoids Across TLR Stimulations: A heat map was generated from the data received from the Metabolomics Resource Core pertaining to eicosanoid and lipid mediator changes as a relative percent change of eicosanoids across stimulations as compared to the control. NT samples are duplicate cell cultures of FLT3-L

induced bone marrow-derived dendritic cells cultured in RPM-1640 lacking TLR agonistic stimulation. Poly(I:C) and CpG duplicate cultures are cultured in RPMI-1640 stimulated with TLR agonists. Decreasing values are depicted by varying shades of red while increasing values are depicted in differing shades of green. Those values remaining unchanged or undetected are shown in black. To calculate the percent change, the average eicosanoid concentration for each eicosanoid was determined for NT. The average NT total concentration was also calculated. From these calculations, the total percent of each NT was calculated. The percent of each individual eicosanoid was calculated for both poly(I:C) stimulations and for both CpG stimulations. Finally, to determine the relative change in percent of each eicosanoid, the percent of the whole eicosanoid of each stimulation was subtracted from the total percent of each NT eicosanoid, divided by the total percent of each NT eicosanoid, and multiplied by 100% to yield the relative percent change of each eicosanoid from the NT.

Due to stimulation with CpG, decreases were seen in 12-oxoETE, 5s-HETE and 6kPSGF1a, while an increase due to CpG stimulation was seen in 9s-HODE. Conversely, upon stimulation with CpG in A, an increase was seen in PSG D_2, PSG E_2, PSG B_2, and PSG A_2 as well as TB B_2, however in CpG-B, they have decreased values. Resolvin D1 is increased in CpG-A; however, it is markedly increased in CpG-B.

Discussion

The overarching objective of this study was to investigate the intrinsic and extrinsic changes associated with IFN-α production by pDCs due to various TLR agonists at the metabolomic and lipidomic levels. There were many strengths and weaknesses associated with this preliminary study. The existence of a mixed culture with other DC subsets having overlapping properties to pDCs made this a demanding project. Within a mixed culture, our cells of interest were allowed to interact with each other and with other cells *in vitro*. While this is beneficial in some respects because it mimics an *in vivo* environment, it is just as detrimental because the effects seen may be associated with cDCs and not pDCs. Further, while we were able to generate data from our experimentation, it is important to note that while we had enough cells in culture to stimulate IFN-α production, we had the minimum cell number requirement for the core facility to analyze phospholipid fatty acids, possibly attributing to the variability of our data. With a novel project, this makes data highly variable. Further, an adjustment to the stimulation protocol needs to be made as it seems there was possible inflammatory mediation occurring in CpG-B. Lastly, as previously stated, current research has focused on the effects that dietary DHA has on immune cells, such as T lymphocytes, B lymphocytes, and monocytes. Because of this, the focus of this study was to begin exploring the effects that TLR stimulation of IFN-α production of pDCs had on phospholipid fatty acids and eicosanoid production, making the data obtained from this study extremely novel and innovative. Because our laboratory was the first to explore this, we had no other results to compare our data to; therefore, data were analyzed to the best of our abilities.

In this study, we created an inflammatory environment by stimulating bone marrow derived dendritic cells through TLRs specific to pDCs. Cells were stimulated with TLR3 agonist

poly(I:C) to induce an antiviral inflammatory response and with the TLR9 agonist unmethylated CpG ODN1585 to induce an antibacterial inflammatory. A murine IFN-α ELISA was performed to confirm and quantify the production of IFN-α. From the ELISA, it was determined that IFN-α production occurred due to TLR9 stimulation with ssDNA agonist unmethylated ODN1585 CpG, backing numerous studies (Björck et al, 2011; Krung, 2001). Conversely, IFN-α production was not quantifiable or detectable through TLR3 stimulation with dsRNA agonist poly(I:C) or through TLR7/8 ssRNA agonist R848 despite research showing R848 is a potent stimulator of IFN-α production in murine pDCs (Gibson et al, 2002). Further experimentation is needed to allow for proper dosing of R848 to stimulate IFN-α production in murine pDCs.

Pellets of our stimulated, mixed cell cultures were sent to Michigan Regional Comprehensive Metabolomics Core Facility for phospholipid fatty acid analysis. While we had sufficient cells in culture to induce IFN-α production, the number of cells sent to the core facility was the minimum they were able to test, making accurate phospholipid fatty acid measures difficult. However, data showed an increase in total phospholipid fatty acids across both stimulations with ODN 1585 CpG and poly(I:C) as compared to the no target sample. Increases in both saturated and unsaturated fatty acids were seen; however, the amount of unsaturated fatty acids that increased or decreased in number across stimulations was much larger. This is important to note because changes in fatty acid content affects membrane fluidity. Through increasing the amount of saturated phospholipid fatty acids, the membrane becomes stiff and less viscous, resulting in decreased membrane fluidity. Conversely, by increasing the number of unsaturated phospholipid fatty acids, the double bonded carbon atoms of the bent fatty acid chains create difficulty for the phospholipids to pack tightly together thereby making the membrane more fluid. Altering the membrane fluidity alters membrane-regulated functions such

as phagocytosis, specific surface protein-protein interactions, and cellular signaling, further changing the dynamics and responsiveness of the cell to different types of stimuli (Boheim et al, 1980). It is also important to note the change in total phospholipid fatty acid concentration seen in CpG-A associated with IFN-α production. From these data, we can conclude that phospholipid fatty acids are increasing to begin producing inflammatory resolving mediators, as seen by the increase in total eicosanoid concentrations seen in CpG-B.

Phospholipid fatty acid analysis shows a decrease in phospholipid fatty acid concentrations of ω-6 PUFA, arachidonic acid during poly(I:C) stimulations and CpG-A stimulations. Our results also present a decrease in arachidonic acid precursors in CpG-A stimulation. Dihomo-γ-linolenic acid and γ -linoleic acid are precursors for the production of arachidonic acid from essential fatty acid linoleic acid. Dietary linoleic acid is desaturated by Δ^6 desatuase to γ-linoleic acid. γ-linoleic acid is elongated to form dihomo- γ-linolenic acid which is further desaturated through Δ^5 desaturase to produce arachidonic acid. To sustain production of these eicosanoids, arachidonic acid needs to be replaced. Depleting levels of arachidonic acid are replenished through the desaturation and elongation of these precursors (γ-linoleic acid and dihomo- γ-linolenic acid) to produce arachidonic acid. Our data suggests that γ-linoleic acid is being delpleated and elongated to dihomo- γ-linolenic acid and further desaturated to arachidonic acid to continue producing pro-inflammatory arachidonic acid derived eicosanoids in response to the inflammatory enviroment created by stimulaiton with poly(I:C) and CpG-A. Eicosanoid analysis further validates these phospholipid fatty acid changes. Data show an increase in arachidonic acid cyclooxygenase derived metabolites, such as prostaglandin D_2, prostaglandin E_2, prostaglandid A_2, prostaglandin B_2, and thromboxane B_2. Conversely, data show decreases in arachidonic acid lipoxygenase derived metabolites such as 15-HETE, 12-HETE, 12oxoETE, and

5-HETE with CpG-A stimulation. An increase in oxidized linoleic acid metabolites 13s-HODE and 9s-HODE was noted across stimultaions with poly(I:C) and CpG-A indicitave of their role in inflammation; however, both decreased in CpG-B. To further all of this, analysis of the ω-6: ω-3 ratio indicated that upon stimulation with poly(I:C), the ratio shifted towards ω-3 PUFAs (Table 4). Conversely, with CpG stimulation of IFN-α production, the ω-6: ω-3 ratio shifted towards W-6 PUFAs, showing that when IFN-α is induced, there is an increase of ω-6 PUFAs to sustain pro-inflammatory cytokine production along with the increase in pro-inflammatory cyclooxygenase eicosanoid mediator production.

A decrease in palmitoleic acid (16:1) was noted with CpG-A alone, indicative of being a possible change due to IFN-α production. Palmitoleic acid is a monosaturated fatty acid with anti-inflammatory properties. Palmitoleic acid, when present, reduces inflammatory cytokines such as NFκB and inflammatory genes of the inflammasome complex independent of PPARα, PPARγ, and AMPK (Souza et al, 2017). Our studies showed a decrease in palmitoleic acid with CpG-A, further increasing the inflammatory environment through potential dysregulated NFκB expression. We can conclude that dysregulated NFκB expression may occur due to IFN-α activation of an alternative NFκB pathway furthering the inflammatory environment (Yang et al, 2005). However, further experimentation would be needed to confirm these results.

On the contrary, the results from CpG-B stimulation differed vastly from CpG-A. Our findings from the stimulation show a decrease in all measured arachidonic acid cyclooxygenase derived metabolites, such as prostaglandins and thromboxane B_2. This is confirmed through phospholipid fatty acid analysis showing an increase in arachidonic acid as well as arachidonic acid precursor dihomo-γ-linolenic acid. Because arachidonic acid utilization has decreased, we

see an increase in these. Also, the arachidonic acid lipoxygenase derived mediators remained decreased, indicating possible inflammatory mediation occuring.

As a compensatory mechanism to inflammatory resolution, anti-inflammatory lipid mediators such as E-and D-series resolvins, protectins, and maresins are derived from ω-3 polyunsaturated fatty acids EPA/DHA. Lipoxins are pro-resolving mediators derived from the oxidation of arachidonic acid or EPA (Serhan et al, 1984). α-linolenic acid is an essential dietary n-3 polyunsaturated fatty acid and can be enzymatically desaturated by Δ^6 desaturase to stearidonic acid (18:4n-3), elongated via elongase to eicosatetraenoic acid (20:4n-3), and further desaturated via Δ^5 desaturase to form EPA. Analysis of phospholipid fatty acids shows a decrease in α-linolenic acid and EPA and an increase in DHA and DHA-derived resolvin D1. This suggests that that α-linolenic acid was being desaturated and elongated to EPA to be oxidized to pro-resolving lipoxins via 15-lipoxygenase to aid in the mediation of the inflammatory microenvironment. Further, DPA (22:5) and DHA (22:6) are produced in lower amounts in CpG-A. However, in CpG-B, both are produced in much larger amounts in CpG-B, with DPA being slightly more than DHA. Because DPA is able to be desaturated through Δ^4 desaturase to produce DHA (Park et al, 2015), it is possible that DPA is being desaturated to produce the DHA needed for the resolution of inflammation via D-series resolvins, protectins, and maresins. While we are limited to the results of those detectible mediators from the metabolomics analysis (resolvin D1, resolvin D2, and resolvin E1), we are not able to account for other pro-resolving mediators, such as AA/EPA- derived lipoxins, or DHA-derived maresins and protectins.

Due to the protective nature of DHA, it has been a potential treatment or complementary approach in treatment of inflammatory conditions such as atherosclerosis (Van Noolen et al, 2014), colitis (Arita et al, 2005), rheumatoid arthritis (Lima-Garcia et al, 2011), and lupus (Bates et al, 2017). EPA can be retroconverted to DHA, although in minimal amounts; therefore, dietary supplementation is necessary. When taken in through dietary means or supplementation, DHA is able to incorporate into the phospholipid bilayer of cells and does so by competitively displacing arachidonic acid, lowering the ω-6 index. This further reduces the substrate needed for pro-inflammatory eicosanoid production. Research has shown that diets with excess ω-6 fatty acid consumption increases the risk of certain diseases, such as cardiovascular disease through atherosclerotic plaques. Typical Western diets have high dietary ω-6 fatty acid ratios as high as 20:1, while an optimal dietary ω-6 to ω-3 fatty acid ratio is 4:1 or lower (Pestka et al., 2014). Further studies have indicated that supplemental DHA is able to suppress and reverse the progression of inflammation, inflammatory cell involvement, inflammasome activation, and regulating clearance of necrotic cells in inflammatory and autoimmune diseases (Xiong et al, 2005).

The observations from this study provide a platform for further exploration. Going on with future experimentation will include positive or negative selection of pDCs to obtain a pure culture. Not only will this concentrate our pDCs, it will also allow us to differentiate between those eicosanoid and phospholipid fatty acid changes seen in this experiment that were associated with cDCs responding to IFN-α production. Also, because dendritic cells are a cellular bridge between innate and adaptive immunity, they are able to secrete many chemokines to attract varying immune cell types at specific times in the immune response. Some secreted cytokines include IL-6, IL-10, IL-12, and IL-23. Assaying for any of these cytokines will give us

insight as to how dendritic cells are able to polarize naïve T cells into Th1, Th2, or Treg cells during IFN-α stimulation. From there, we will be able to look for differences between eicosanoids and fatty acids due to cytokine production and IFN-α production. Going further, the TLR stimulation time course should be adjusted accordingly to sustain the inflammatory response for further analysis and to avoid pro-resolving mediation of the inflammatory response. This will allow us a clearer picture of the eicosanoid and phospholipid fatty acid changes that are being seen due to IFN-α production. From this we will also be able to determine whether IFN-α production is responsible for resolvin production or if timing due to pro-resolving mediator production is responsible. This will also give us insight into whether the pro-resolving lipid mediators are blunting the IFN-α response. Finally, IFN-α producing pDCs will be treated with DHA *in vitro* to determine DHA incorporation into phospholipid fatty acids, eicosanoids and mediators changes due to DHA treatment, modulation of IFN-α production, and effects on cytokine production.

From our studies, we were able to confirm that INF-α production in murine bone marrow-derived dendritic cells occurs through TLR9 stimulation with ODN 1585 CpG. We were able to determine that due to IFN-α production, a decrease in arachidonic acid and arachidonic acid precursors occurred along with an upregulation of arachidonic acid metabolites of the cyclooxygenase pathway, suggesting that arachidonic acid is being used to sustain pro-inflammatory eicosanoid production. Furthering this, we saw greater than a two-fold increase in ω-6: ω-3 ratio towards ω-6 PUFAs in TLR9 stimulation with CpG, showing that a shift in the ω-6: ω-3 ratio is needed to sustain the pro-inflammatory environment. Although our experimentation was novel and innovative, it was also full of many challenges. From our experimentation, we were able to gather sufficient preliminary data to warrant further

exploration into DHA and the role that it plays modulating IFN-α induced inflammatory environment.

Appendices

Appendix I: Explanation of Acronyms

ADs (Autoimmune Diseases)

pDCs (Plasmacytoid dendritic cells)

TLR (Toll-like receptor)

PAMP (pathogen-associated molecular pattern)

PRR (pattern precognition receptor)

IFN-α (interferon alpha)

IFN-β (interferon beta)

MyD88- (myeloid differentiation primary response protein 88)

RNA- ribonucleic acid

ssDNA (single-stranded deoxyribonucleic acid)

IRAK (interleukin-1 receptor (IL-1R) associated kinase 1)

IFNAR1 (Interferon alpha receptor 1)

JAK/STAT (Janus kinase/ signal transducers and activators of transcription)

ISGs (interferon stimulated genes)

NSAID (non-steroidal anti-inflammatory)

PUFAs (polyunsaturated fatty acids)

MHC II (major-histocompatability complex II)

DHA (docosahexaenoic acid)

EPA (eicosapentaenoic acid)

Poly I:C (polyinosinic-polycytidylic acid)

ELISA (enzyme-linked immunosorbent assay)

FLT3-L (FMS-like tyrosine kinase 3)

TMB- 3,3',5,5'-tetramethylbenzidine

HRP- horseradish peroxidase

ACK- ammonium-chloride-potassium

RPMI media- Roswell Park Memorial Institute

FACS- fluorescence activated cell sorting

LCMS- liquid chromatography-mass spectrometry

RPLC- reverse phase liquid chromatography

Appendix II: Table 5- Murine IFN-α ELISA Concentrations

	1	2	3	4	5
A	BLK 0.000	BLK 0.000	NT-A 0.000	NT-B 0.000	R848+pIC-A 11.688
B	STD1 400 397.537	STD1 400 402.168	NT-A 0.000	NT-B 0.000	R848+pIC-A 0.000
C	STD2 200 199.15	STD2 200 195.828	R848-A 0.000	CpG+R848-A 0.000	CpG-A 16.852
D	STD3 100 101.87	STD3 100 98.358	R848-A 0.000	CpG+R848-A 0.000	CpG-A 22.877
E	STD4 50 50.809	STD4 50 50.411	R848-B 0.000	CpG+R8848-B 0.000	CpG-B 34.503
F	STD5 25 42.296	STD5 25 24.502	R848-B 0.000	CpG+R848-B 0.000	CpG-B 54.96
G	STD6 12.5 23.576	STD6 12.5 0.000	pIC-A 0.000	pIC-A 0.000	R848+pIC-B 0.000
H	BLK 0.000	BLK 0.000	pIC-B 0.000	pIC-B 0.000	R848+pIC-B 12.489

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