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Could Dietary Peroxidized Lipids provoke an Intestinal Inflammatory Response?

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COULD PEROXIDIZED LIPIDS PROVOKE AN INTESTINAL INFLAMMATORY RESPONSE?

by

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ABSTRACT

Inflammatory Bowel Disease and Crohn's disease represent chronic intestinal inflammatory diseases. It is suspected that bacterial infection is one of the causes of gut inflammation. Studies from others as well as from our laboratory have indicated that peroxidized lipids and their decomposition products are pro-inflammatory. As we consume considerable amounts of dietary oxidized lipids (arising from deep frying of vegetable oils), we hypothesize that dietary peroxidized lipids may also lead to intestinal inflammation. To test this hypothesis, intestine from C57BL/6 mice were collected and used in this study. The intestinal epithelial tissue as well as intestinal lymphoid tissues [Peyer's Patches (PP)] were identified and harvested. Both the tissue samples were incubated with 13-Hydroperoxyoctadecadienoic acid (HPODE, a simple form of peroxidized fatty acid) or oxidized phosphatidyl choline (Ox-PL) or minimally modified LDL (mmLDL) or bacterial lipopolysaccharide (LPS) at 37°C. After 6 hours of incubation, RNA was extracted and RT-PCR analysis was performed to determine inflammatory markers using mouse primers for the gene expression of cytokines. We noted an increased basal gene expressions of inflammatory cytokines in PP tissues as opposed to the epithelial tissue. An increase in inflammatory cytokines gene expression was observed in LPS/POL treated intestinal tissues as compared to untreated tissues. Overall, our findings might suggest additional potential sources of gut inflammation as well as an active participation of epithelial cells in the inflammatory process. These might also offer novel targets for the control of inflammation of the gut in patients suffering from gut inflammatory diseases.

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LIST OF ACRONYMS

CD: Crohn's Disease

GAPDH: Glyceraldehyde phosphate dehydrogenase

HPODE: Hydroperoxyoctadecadienoic acid

IBD: Inflammatory Bowel Disease

IE: Intestinal Epithelial Tissue

IL: Interleukin

LMB: Leucomethylene Blue Assay

LPS: Lipopolysaccharide

MCP-1: Monocyte chemoattractant protein-1

mmLDL: minimally modified LDL

Ox-PL: Oxidized Phospholipid

POL: Peroxidized Lipids

PP: Peyer's patches

RT-PCR: Real Time Polymerase Chain Reaction

TNF-α: Tumor Necrosis Factor-alpha

UC: Ulcerative Colitis

Un-ox PL: Unoxidized Phospholipid

CHAPTER-1: INTRODUCTION

1.1 Physiology of Small Intestine

The small intestine is divided into three parts duodenum, jejunum and ileum. The wall of small intestine is composed of four layers of tissues, serosa, muscularis propria, submucosa and mucosa (DeSesso, J. M., & Jacobson, C. F., 2001, Jaladanki, R. N., & Wang, J. Y., 2011). Mucosa, the innermost tissue layer is the site for absorption. It has three layers. The first layer faces the intestinal lumen and is formed by a single layer of epithelial cells attached to basement membrane. The second layer is the lamina propria, lies underneath the first layer. It is formed by subepithelial connective tissue and lymph nodes. The deepest mucosal layer is called as muscularis mucosae and constitutes smooth muscle cells. Intestinal villi are the finger like projections of the mucosa and helps in transport of substances from lumen to the vascular system. Submucosa comprised of inflammatory cells, lymphatics, nerve fibers and ganglion cells which provides support to the mucosa. Muscularis propria lies beneath the submucosa and consists of muscle layers, inner layer of circular muscles, intermuscular space and outer layer of longitudinal muscles. It is surrounded by outermost layer serosa. The layers are shown in Figure 1.

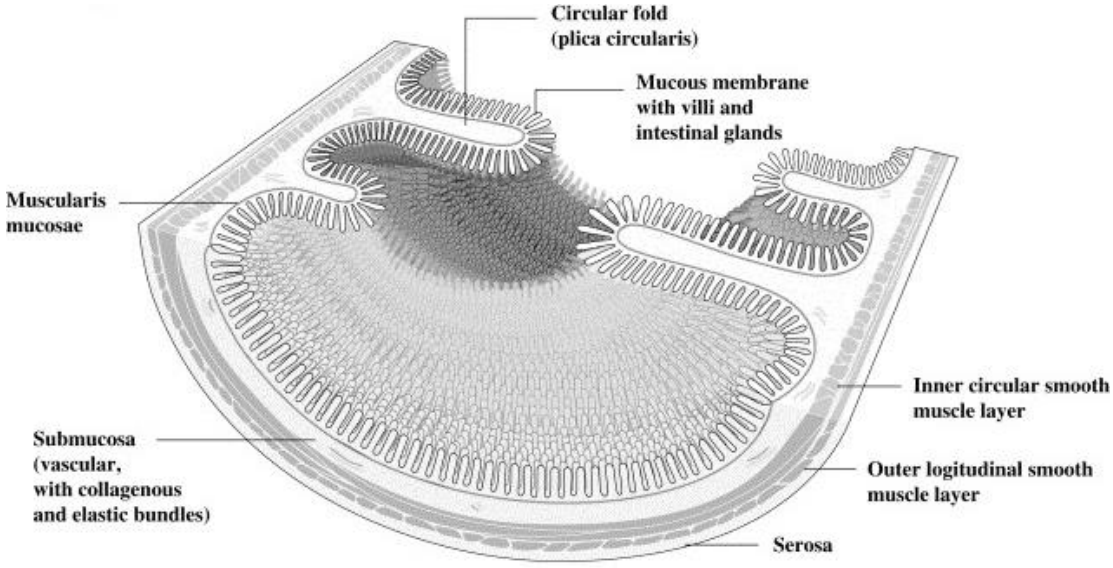

Figure 1: Different layers of the human proximal Small Intestine

This figure shows different layers of intestine. These are serosa, submucosa, muscularis propria, and mucosa (Adapted from DeSesso, J. M., & Jacobson, C. F., 2001)

1.2 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the intestine (Kraft SC, 1971, Malik, Talha A, 2015). It is most prevalent in northern parts of Europe, the United Kingdom and North America. Evidence suggests that incidence of this disease is rising in other parts of the world (Loftus, E.V, 2004).

Intestinal inflammation is a major etiological factor found in IBD patients. Crohn's disease (CD) and Ulcerative Colitis (UC) constitutes the two most common forms of IBD. The distinguishing features of CD and UC are shown in Table 1. The symptoms of IBD involve abdominal cramps, diarrhea, abdominal pain etc. IBD patients might suffer with complications such as ulceration of the intestine, rupture and bleeding from ulcers and obstruction of the

gastrointestinal tract. Both CD and UC have an increased risk of colon cancer. IBD gets worsen with time and results in serious gastrointestinal problems which affects the quality of life of IBD patients severely (Centers of Disease Control and Prevention, (CDC)). The distinguishing features of these two diseases are mentioned in Table1.

According to CDC guidelines, IBD is currently affecting about 1-1.3 million people in the United States. The exact cause of this disease is still not known and there is not a standard criteria for its diagnosis which underlines the importance of research on IBD to find a cure or prevention of this disease.

Table 1: Distinguishing features of CD and UC (CDC guidelines)

CROHN'S DISEASE	**ULCERATIVE COLITIS**
It may affects all layers of the intestine	It may affects only top layer of the intestine
It can involve any part of the intestine	Colon is usually affected
Surgery may be required in some cases	No surgery is required in most of the patients with UC.
Granuloma formation occurs (Marc D Basson, 2015)	No granuloma formation is detected (Marc D Basson, 2015)

1.3 Etiology of IBD

The inflammatory damage of intestinal mucosa is believed to be caused by interplay between host genetics (Table 2), host microbiota, and environmental factors (Sekirov, I., Russell 2010). Some of the histo-pathological findings observed in IBD includes lesions in intestinal epithelium, loss of integrity of mucosal barrier, pro-inflammatory cytokines induction and fibrotic scarring (Sekirov, I., Russell 2010). The general functional changes involved in IBD are genes regulating immune function, inflammation, integrity of mucosal barrier and clearance of microbes (Sartor, R. B, 2006).

Table 2: Some of the genes and its functions associated with IBD and experimental colitis. (Adapted from Sartor, R. B, 2006)

Gene	**Chromosome (human)**	**Function**
CARD15	16	Activation and/or regulation of NFKB, killing of intracellular pathogens, function of Paneth cells such as production of α-defensin.
SLC22A4 & SLC22A5	5	Carnitine and organic cation transporters.
DLG5	10	Scaffolding protein maintaining integrity of epithelium
PPARG	3	Intracellular inhibitor of NFKB and cellular activation
MDR1	7	Acts as an efflux transporter of drugs and possibly xenobiotic compounds from cells
Abbreviations: CARD15, caspase recruitment domain family, member 15 (formerly NOD2); DLG5, discs large homolog 5 (Drosophila); MDR1; multidrug resistance 1; PPARG, peroxisome proliferative- activated receptor gamma; SLC22A4 and SLC22A5, solute carrier family 22 members 4 and 5.		

Chronic intestinal inflammation can be caused in a susceptible individual by an alteration in beneficial and aggressive species of microbes. Studies have suggested an association of CD with an increase in pathogenic Adherent/invasive strain of E. coli (AIEC) (Sartor RB, 2008, Packey CD and Sartor RB, 2009, Sekirov, I. et al, 2010) and a decrease in the number of bacteria *Faecalibacterium prausnitzii*, responsible for increasing secretion of anti-inflammatory cytokine, IL-10 and decreasing secretion of pro-inflammatory cytokines in both *in-vivo* and *in-vitro* conditions (Sokol H. et al, 2008, Willing, B. et al, 2009, Sekirov, I. et al, 2010). There is a positive correlation between CD and Mycobacterium avium paratuberculosis (MAP) species (Naser S.A, et al, 2004). Studies (Duchmann, R. et al, 1995) suggested that CD patients may suffer from autoimmune responses against their own intestinal commensal bacteria which might be due to loss of barrier function of intestinal epithelial tissue.

1.4 Cytokines

Cytokines are small proteins secreted by cells. It includes interleukins, chemokines, lymphokines, and monokines (Zhang, J. M, & An, J. (2007). The action of cytokines can be through autocrine (acting on cells which secrete them), paracrine (acting on nearby cells) or endocrine (acting on distant cells). T helper lymphocytes cells and macrophages are the predominant cell types forming cytokines. Generally cytokines are divided into pro- and anti-inflammatory in nature. However, the context is important as a pro-inflammatory cytokine could act as a negative factor and vice versa. Pro-inflammatory cytokines such as IL-1beta, IL-6 and TNF-alpha are mainly produced by activated macrophages and takes part in upregulation of inflammatory reactions (Zhang, J. M, & An, J. (2007). Monocyte chemoattractant protein (MCP-1) is a chemotactic cytokine or chemokine and mainly involved in activation and migration of leukocytes. Anti-inflammatory cytokines control the response of pro-inflammatory cytokines (Zhang, J. M, & An, J. (2007). An anti-inflammatory cytokine, IL-10 functions by controlling the expression of IL-1, TNF alpha and IL-6 by activated macrophages. IL-4, produced by Th2 cells is considered to have anti-inflammatory actions (Hart, P. H. et al, 1989).

Studies by Sartor, R.B, (2006) have shown that there is increased production of pro-inflammatory cytokines and chemokines in IBD. Some of these cytokines includes TNF alpha, IL-1 beta and IL-6 increased in both CD as well as UC (shown in Table 3). Studies (Cappello M, et al, 1992, Isaacs K Let al, 1992, Ligumsky M, et al,, 1990) have found increased production of IL-1 in the colorectal mucosa of the patients in the active disease phase of UC. Studies have shown the involvement of IL-1 in the development of colitis using Dextran Sodium Sulphate

(DSS) induced colitis mice model which resembles human UC patients based on symptoms and thus down regulation of IL-1 may be useful for treatment of UC (Arai, Y. et al, 1998). IL-10 is a regulatory cytokine and its deficiency in mice leads to intestinal inflammation (Berg DJ et al, 1996, Spencer SD et al, 1998). IL-10 knock out mice model is used as a model of experimental colitis (Macdonald, T. T. (1994), Rennick, D. M. et al, (1997)). Studies by Lindsay, J., et al (2002), used 2, 4, 6 trinitrobenzene sulphonic acid (TNBS) mouse colitis model which is similar to CD in many histological and immunological findings. This study showed that adenovirus mediated IL-10 gene transfer resulted in prevention of severe TNBS colitis development. Although IL-4 is a Th2 cytokine, studies by Specht S, et al (2006) have shown that IL-4 is important for colitis development. They found development of a significantly lower degree of colitis in IL-4/IL-10 double deficient mice when compared to IL-10 deficient mice which developed severe colitis.

Table 3: Association of some of the cytokines with IBD.

This table (Adapted from Sartor R.B, 2006) shows upregulation of cytokines in both CD and UC expressed by activated cells in innate immune response. The last row (Van Kampen, 2005) indicates the association of IL-4 with colitis.

Cytokine	IBD
IL-1β	↑
TNF	↑
IL-6	↑
IL-8[a]	↑
IL-18	↑
IL-12	↑ in CD but Normal in UC
IL-4 alone could induce transient colitis, the extent of which was similar to IL-12 induced colitis	

Bacterial infection is believed to be one of the contributing factors of gut inflammation in IBD patients. However, the exact cause of IBD is not clear. Due to its high incidence rate and serious complications, there is immense need to conduct more research in order to prevent the disease or finding a cure for this disease or relieve patient's symptoms suffering from this disease.

1.5 Bacterial Lipopolysaccharide

Lipopolysaccharide (LPS) was used as a positive control in this study. LPS is an endotoxin and present on the outer membrane of gram negative bacteria. Evidence (Martin, H. M. et al, 2004, Seksik, P. et al, 2003) suggests that there is an increase in gram negative bacteria in IBD patients. Studies by IM, E. et al (2012), introduced LPS directly in the mice gut by rectal enema and found that elevated LPS in the colon can initiate gut inflammatory responses. This observation was supported by studies showing intra colonic administration of LPS in rabbits can induce colitis (Hotta, T. et al, 1986)

1.6 Dietary Oxidized Lipids

Most dietary lipids mainly consist of triglycerides and small proportion (3-6%) of phospholipids (Thomson A. et al, 1989). In the small intestine, mixed micelles are formed by emulsification of dietary fats in the presence of bile salts. Triacylglycerol are hydrolyzed into monoglycerides, diglycerides, glycerol and free fatty acids by lipases present in the intestine. The fatty acids and other released products are taken up by the intestinal mucosa and re-esterified into triacylglycerol which are incorporated into chylomicrons and enter the blood (Nelson, D. L. et al, 2008).

Western diets are known to be rich sources of polyunsaturated fatty acids (PUFA). Processes, such as deep frying, long term exposure to air, cause oxidation of PUFA to form oxidized fatty acids. Linoleic acid is an omega-6 fatty acid and is the most abundant PUFA found in plants and mammals (and hence in the diet)) (Bergström, S. U. N. E., 1945) and its oxidation yields hydroperoxy octadecadienoic acid (HPODE) as a major oxidation product. Therefore, in this study we used oxidized fatty acids as a model of peroxidized fat. Previous studies from our laboratory has shown that 13-HPODE incubated at physiological temperature gets decomposed into aldehydes and carboxylic acids (Figure 2).

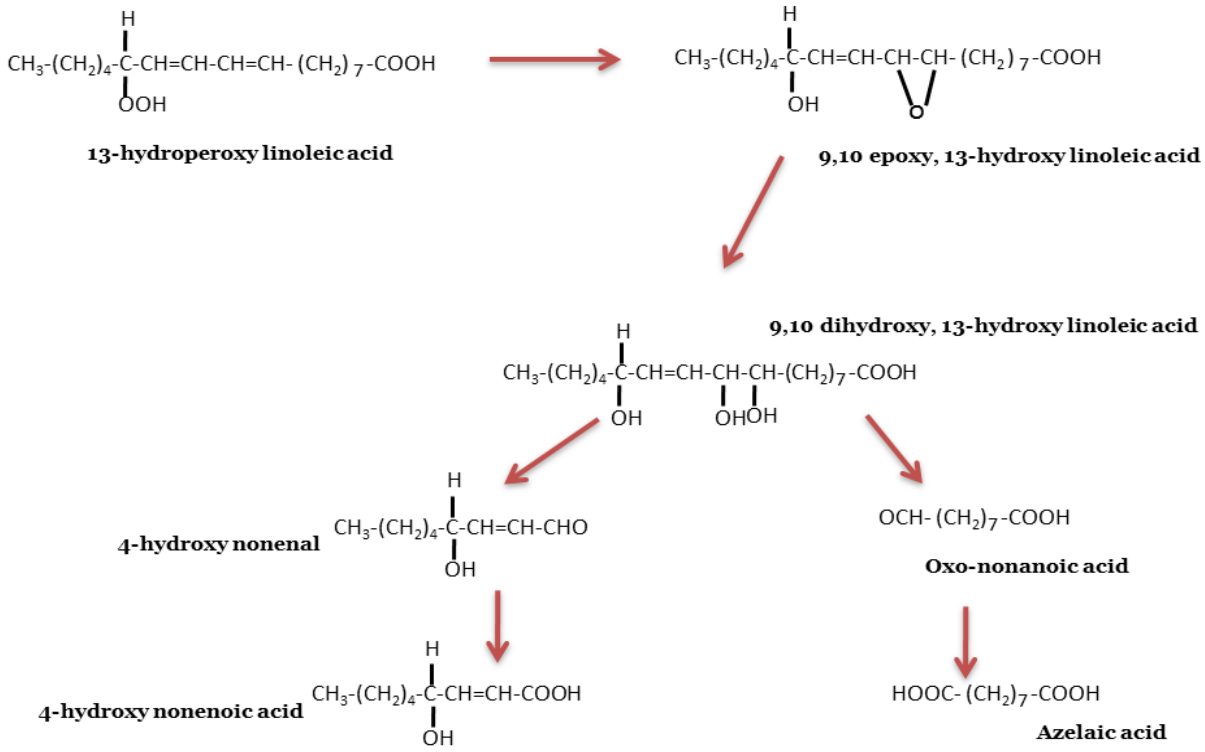

Figure 2: Decomposition products of 13-hydroperoxy linoleic acid

This figure shows the decomposition products of 13-HPODE. At 37°C, 13-HPODE is degraded into aldehydes and carboxylic acids (Adapted from Raghavamenon et al, 2009) upon prolonged exposure.

1.7 Peyer's patches and Intestinal epithelial tissues

We used Peyer's patches (PP) and Intestinal epithelial tissues (IE) for this study. Gut associated lymphoid tissues consists of isolated and aggregated lymphoid follicles (Jung, C., et al (2010)). PP are aggregated lymphoid follicles and structurally divided into three domains, the central part is germinal center surrounded by subepithelial dome which is further surrounded by the outermost domain facing the lumen, follicle associated epithelium. According to Jung, C., et

al (2010), the cell composition of a PP of a mouse consists of around 60% of B-lymphocytes, 25% T-cells, 10% dendritic cells, and less than 5% of macrophages. The follicle associated epithelium also contains specialized microfold (M cells) which samples the luminal antigens to the underlying lamina propria immune cells (Abreu, M. T., 2010). Studies by Sangari F.J et al (2001) and Momotani E. et al (1988) have demonstrated the interaction of enterocytes and M-cells present on PP with MAP show the involvement of enterocytes and M-cells in enteritis.

Peroxidized lipids (POL) are considered to be pro-inflammatory in other cell systems. Previous studies (Khan, B. et al, (1995), O'Brien et al, (1993), Springer, T. A. (1990)) have shown that hydroperoxy form of fatty acid (HPODE) increases TNF-alpha mediated cellular adhesion molecules expression (VCAM-1 and ICAM-1) in endothelial cells. Evidence (Dwarakanath, R. S. et al, 2004) suggests that 13-HPODE can induce a chemokine monocyte chemoattractant protein-1 (MCP-1) in vascular smooth muscle cells which shows the inflammatory nature of HPODE. Studies by Awada M et al (2012), compared inflammatory markers in plasma of mice fed high fat diet with oxidized or unoxidized n-3 PUFA. They found significantly increased concentrations of inflammatory markers, IL-6 and the chemokine MCP-1 in oxidized fed group as compared to unoxidized fed group. Studies by Mickel HS and Horbar J, (1974) have shown that peroxidized arachidonic acid caused a greater degree of platelet aggregation than arachidonic acid.

Studies by Parthasarathy, S. et al, (1989), Steinberg D, (1997), has shown that oxidized lipoproteins plays an important role in atherosclerosis development. Studies (Gerrity, R. G., et al, 1979, Faggiotto, A. et al, 1984, Jerome, W. G., & Lewis, J. C. (1984), Ross, R. (1986)) have found that increased monocytes recruitment into the arterial sub-endothelium is an important

early event in atherogenesis. Studies (Quinn, M. T. et al., 1987) have shown the chemotactic activity of oxidatively modified LDL which might be mainly due to its peroxidized lipid components. Studies by Cushing S.D et al, 1990, by using cultures of aortic endothelial cells and smooth muscle cells independently or their co-cultures together, showed that minimally modified LDL (mmLDL) acts as a potent inducer of MCP-1, a chemotactic factor in these cell types. This might suggest that mmLDL might be involved in the recruitment of monocytes into the sub-endothelium during the early stages of atherogenesis.

We consume large quantities of fat in diet and cooking process especially deep frying causes oxidation of fat forming peroxidized lipids. Here, we aim to study that dietary POL might contribute to induction of inflammatory response in intestinal tissues.

1.8 Rationale for the study

Previous studies have shown that the peroxidized lipids, particularly the peroxidized fatty acids and the peroxidized phospholipids are pro-inflammatory (Böhm, T. et al, 2013, Henry, P. D., & Chen, C. H., 1993). Studies by Shoda et al 1996, have found a positive correlation between high consumption of fat and CD. Based on these observations, we propose that, in addition to bacteria, dietary oxidized lipids may also cause inflammation and thus may contribute to the gut inflammatory diseases. These studies are expected to shed light on CD and other IBD.

CHAPTER-2: MATERIALS AND METHODS

2.1 Isolation of Intestinal epithelial tissues and Peyer's patches

Normal C57BL/6J mice were purchased from Jackson Laboratory and used in this study. Animals were maintained and all procedures were performed following the guidelines of Animal Care and Use Committee of the University of Central Florida, USA. Mice were allowed to acclimatize. For experimental procedures, mice were anesthetized with 1-2% isoflurane, blood samples were collected into vaccutainer tubes by heart puncture. The serum was separated and stored at -80°C and mice were euthanized using carbon dioxide asphyxia. For experiments under fasting conditions, mice were fasted overnight before the experimental procedures. Their intestines were harvested. Peyer's patches and Intestinal epithelial tissues were collected in separate petri dishes. Tissue samples were washed with PBS and used for experimental procedures.

2.2 Experimental conditions

For treatment experiments, IE and PP tissues were incubated with 25 nmoles/ml of hydroperoxy octadecadienoic acid (HPODE) or 25 nmoles/ml of oxidized phosphatidyl choline (Ox-PL) or 1 µg/ml of lipopolysaacharide (LPS) or 25 µg/ml of minimally modified LDL (mmLDL) at 37°C for 6 hours The reagents were prepared in Hanks' balanced salt solution (HBSS). After incubation, tissues were collected in Trizol reagent for RNA isolation and medium was stored at -80°C. Basal level expressions of cytokines were determined from IE and PP without any treatment. Tissue samples were lysed in Trizol reagent using polytron tissue

homogenizer. RNA was isolated, quality and quantity was assessed by nanodrop spectrophotometer (Thermoscientific). After confirming the quality and quantity, cDNA was synthesiszed and RT-PCR analysis was performed to detect the specific markers [tumor necrotic factor alpha (TNF-α), interleukins (IL-1β, IL-4, IL-6, and IL-10). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the reference gene. For experiments with mmLDL or Ox-PL treatment, Monocyte Chemoattractant Protein (MCP-1) gene expression was also determined.

2.3 Preparation of linoleic acid hydroperoxide

13-HPODE was prepared from 200 mM Linoleic acid (Sigma #W338001-25G) stock solution with the enzyme 10 U of Soyabean Lipoxygenase (SLO) (Sigma #L6632-1MU) in phosphate buffered saline (PBS with pH 7.4) solution. Linoleic acid stock solution was prepared in ethanol and protected from light at 4°C. The reaction was carried out at room temperature for two hours. The final concentration of HPODE used for the experiments was 25nmoles/ml prepared with HBSS. HPODE is a hydroperoxy molecule containing conjugated dienes. The formation of HPODE was monitored by measuring absorption spectrum between 200 nm and 300 nm in a spectrophotometer (Jenway 6850 UV/Visible double beam spectrophotometer) where PBS was used as a blank. The conjugated dienes present in HPODE gives maximum absorbance at 234 nm. The hydroperoxy group present in HPODE was tested by Leuco-methylene blue (LMB) assay (Auerbach, B. J., et al. 1992). In LMB assay, hydroperoxy group oxidizes N-benzoyl leucomethylene blue to methylene blue in the presence of hemoglobin which was measured spectrophotometrically at 660 nm wavelength. 13-HPODE was freshly prepared and used immediately for the experiments.

2.4 Detection of conjugated dienes in oxidized linoleic acid

The formation of HPODE was monitored by measuring absorption spectrum between wavelength range 200 to 300 nm in a spectrophotometer (Jenway 6850 spectrophotometer). The presence of conjugated dienes in HPODE was observed with an increase in absorbance at 234nm (Figure 3). PBS was used as a blank.

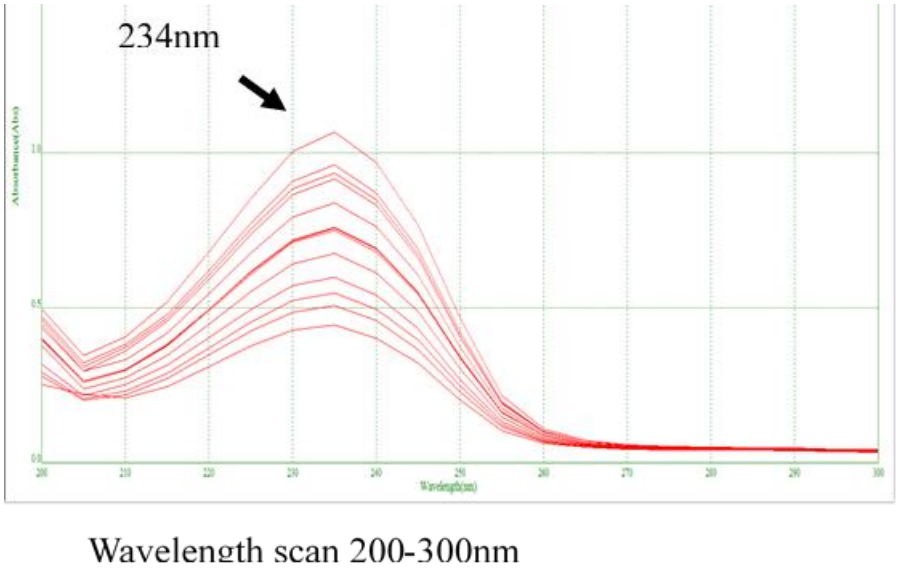

Figure 3: Absorption spectrum of HPODE between wavelength range 200 to 300 nm with a peak at 234 nm.

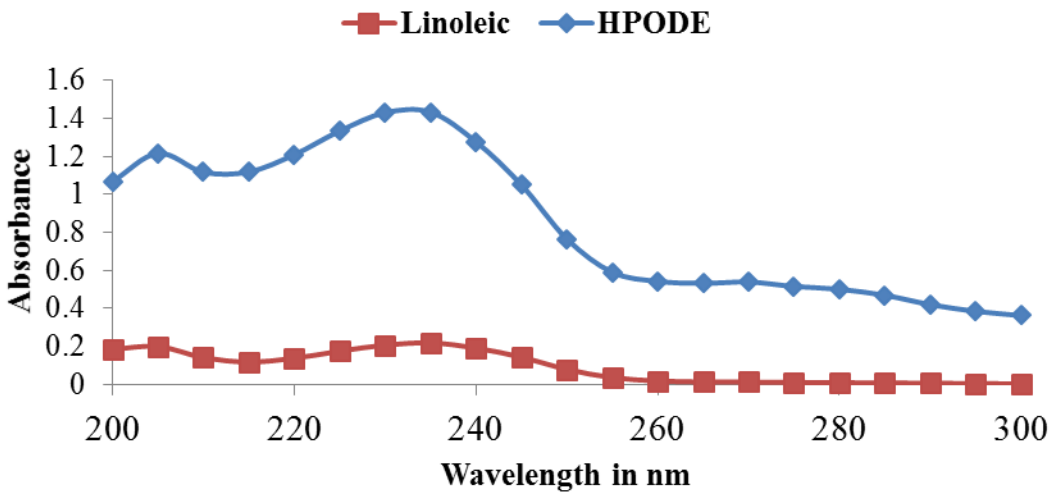

Figure 4: Absorption spectrum of HPODE (peak at 234 nm) and linoleic acid

The absorption spectrum of linoleic acid and HPODE formed from linoleic acid with the addition of SLO was observed (Figure 4) in a spectrophotometer. HPODE showed an increase in absorbance with a peak at 234 nm.

2.5 Detection of peroxide in oxidized linoleic acid

13-HPODE was prepared by adding 10 U of SLO V to linoleic acid in PBS. The peroxide present in 13-HPODE was confirmed by LMB assay (Figure 5). Peroxides present in 13-HPODE oxidized N-benzoyl leucomethylene blue to methylene blue in the presence of hemoglobin, which was measured spectrophotometrically at 660 nm wavelength (Auerbach, Kiely et al. 1992). PBS was used as a blank.

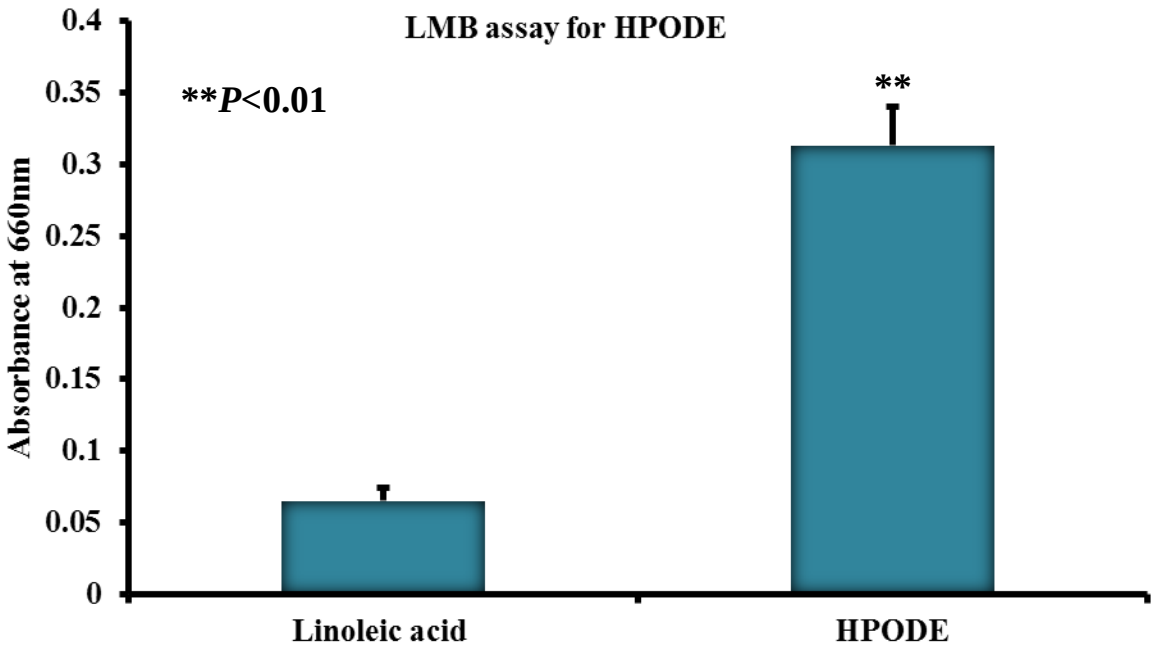

Figure 5: LMB assay showing increased absorbance in the presence of peroxides in oxidized linoleic acid as compared to linoleic acid

2.6 Synthesis of oxidized phospholipid

Oxidized phospholipid (Ox-PL) stock solution was prepared by dissolving 7.5 mg of 2-linoleyl phosphatidyl choline in 1ml solution containing 1:1 proportion of chloroform and methanol. 20µl of this solution was vacuum dried and reconstituted with 100 µl of BHT free ether. 1ml of 0.2M borate buffer (to maintain pH) containing 10mM of Sodium deoxycholine (NaDOC) was added to this mixture. Sodium deoxycholine was used to keep phospholipid in solubilized form. The tube was kept in warm water for 10 minutes to evaporate the ether followed by addition of SLO. The final concentration of Ox-PL used for the experiment was 25 nmoles/ml prepared with HBSS. The formation of oxidized phospholipid was monitored by measuring absorption spectrum between 200 and 300nm in a spectrophotometer (Jenway 6850

Double beam UV/Visible spectrophotometer) where borate buffer was used as a blank. The conjugated dienes present in oxidized phospholipid gives maximum absorbance at 234 nm. The synthesis of ox-PL was confirmed by LMB assay measuring absorbance at 660 nm.

2.7 Isolation of LDL and preparation of minimally modified LDL (mmLDL)

The protocol was approved by the University of Central Florida Institutional Review Board and informed consent was obtained from subjects. LDL was isolated from 50 ml of plasma by using potassium bromide (0.989g/2ml) and then aliquoted to 5ml centrifuge tubes (Beckman tube cat# 362248). This KBr/plasma layer was overlaid with ~2.25 ml of 0.15M sodium chloride gently without disturbing the lower layer. The tube was sealed with the Beckman sealer and spinned at 100,000 rpm for 60 minutes in a Beckman tabletop ultracentrifuge. LDL band was identified as the clear yellow band in the upper middle part of the tube and removed carefully under dim light so as to prevent oxidation. The above steps were repeated with isolated LDL to remove any contamination of albumin or other lipoproteins during the first LDL isolation. The LDL layer was separated and dialyzed against 0.3 mM EDTA in 1X PBS (pH 7.4) at 4°C for a minimum of 6 hours and filtered sterilized. The amount of protein was measured by Bio-Rad Protein kit Assay (Bio-Rad DC™ Protein Assay Cat. #500-0114). After dialysis, the LDL sample was set immediately for oxidation. 100µg/ml of LDL was oxidized with 5µM copper in 1ml of PBS at 37°C. The conjugated dienes formation was monitored by measuring optical density at 234 nm for 90 minutes in a spectrophotometer (Jenway 6850 spectrophotometer). The extent of this oxidation of LDL generates minimally modified LDL (at the end of initial lag phase) (Chandrakala, A. N., 2012) which was collected and the hydroperoxy group was detected by LMB assay.

2.8 Bio-Rad Protein Assay

The amount of protein was measured according to supplier's instructions. 25 µl of an alkaline copper tartrate solution containing reagent was added to 5 µL of standards or sample in a 96 well microtiter plate. 200 µL of folin reagent was added to the mixture in each well. The plate was incubated for 15 minutes followed by mesuring absorbance at 750 nm. (Bio-Rad DC™ Protein Assay Cat. #500-0114).

2.9 LMB reaction

The peroxides were detected by Leucomethylene blue (LMB) assay (Auerbach, Kiely et al. 1992). LMB reagent was prepared by adding 5.5mg hemoglobin and 1.4gm Triton X-100 to 80 ml of 0.05M phosphate buffer. 5 mg of benzyl-leucomethylene blue dissolved in 8 ml of dimethyl formamide was added to the solution. The total volume of the solution was made up to 100 ml with phosphate buffer. LMB reagent was aliquoted and stored at -20°C until further use. LMB assay was carried out by adding 100µL of LMB reagent and 100µL of peroxide containing samples in a 96 well plate followed by incubation for 15-20 minutes at room temperature. All experiments were run in triplicates. After incubation, absorbance was measured at 660 nm in a microtiter plate reader (Bio-Rad Benchmark Plus).

2.10 Confirmation of Oxidized Phospholipid (Ox-PL) and minimally modified LDL (mmLDL) by LMB assay

To determine if IE tissues and PP tissues response to other peroxidized lipid molecules, Ox-PL and mmLDL were used to treat IE and PP tissues similarly to HPODE. The conversion of 2-linoleyl phosphatidyl choline into its oxidized form was confirmed by detection of peroxides in

Ox-PL by LMB assay as previously mentioned. Figure 6 represents the increase in absorbance at 660 nm in the presence Ox-PL.

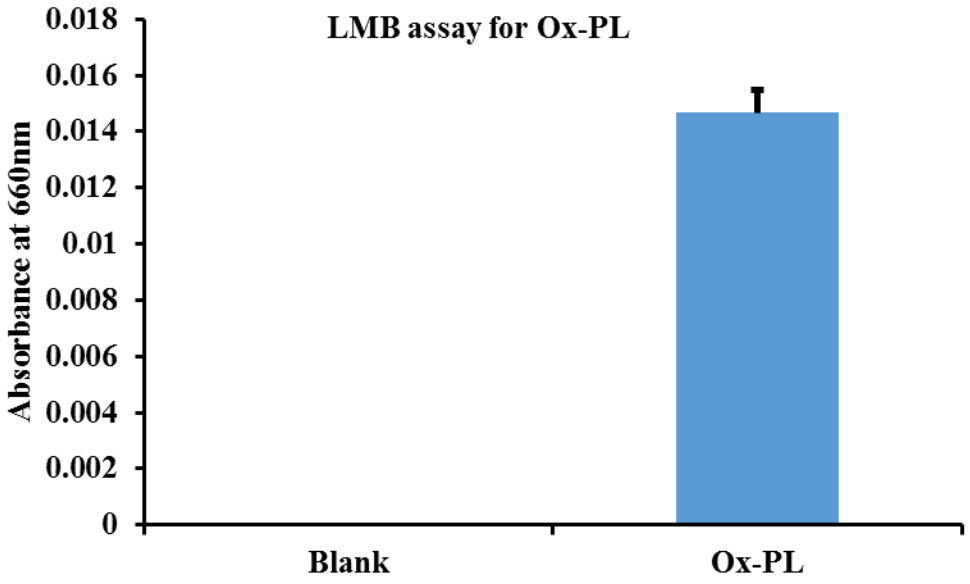

Figure 6: Detection of peroxides in Oxidized Phospholipid by LMB assay

Similarly, the generation of mmLDL was confirmed with LMB assay. The peroxides present in mmLDL resulted in increase in absorbance at 660nm when compared to native LDL (Figure 7)

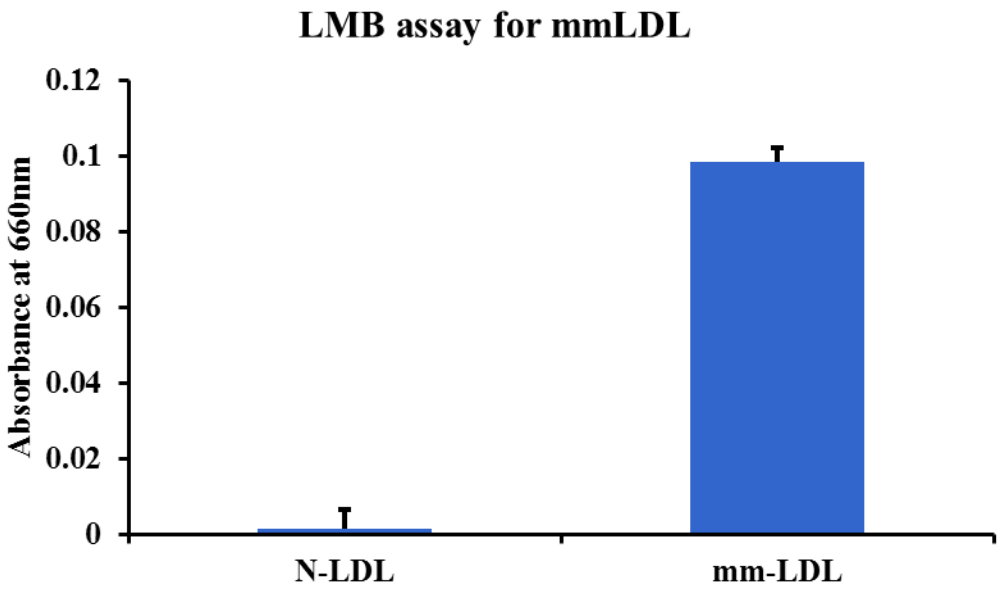

Figure 7: Detection of peroxides in mmLDL by LMB assay

2.11 RNA isolation

Total RNA was isolated from tissues using TRIzol® reagent (Invitrogen #15596018), a monophasic solution of guanidine isothiocyanate, phenol and other proprietary components. 1 ml of TRIzol was added to each 1.5 ml eppendorf tube containing tissue samples. Tissue samples were homogenized in Trizol using polytron tissue homogenizer. After homogenization, the samples were incubated for 5 minutes to allow for complete dissociation of nucleoprotein complex. It was followed by adding 200 µL chloroform in each tube. The tubes were capped securely and vigorously shaken for 15 seconds. The tubes were kept at room temperature for 2 to 3 minutes and then centrifuged at 12000 x g for 15 minutes at 4°C thus resulting the solution to form into three layers. The topmost aqueous layer containing RNA was carefully pipetted out into new tubes while the lower organic layer was stored for at -80°C for protein isolation. This was followed by addition of 500 µl of 100% isopropanol to the separated aqueous phase. The

tubes were kept for incubation for 10 minutes at room temperature followed by centrifugation for 10 minutes at 12000 x g at 4°C. Isopropanol was used to precipitate RNA from the aqueous phase. It was followed by aspiration of the supernatant and addition of 1ml of 75% ethanol for washing of the RNA. The tubes were vortexed for some time and then centrifuged for 5 minutes at 7500 x g at 4°C. This washing step was repeated three times followed by air drying of RNA for 10 minutes and then resuspension in 20-50 µl of RNase free water. RNA was isolated and its quality and quantity was assessed by nanodrop spectrophotometer (ThermoScientific). 1µg of total RNA was used for reverse transcription to cDNA synthesis which was further used for RT-PCR reaction.

2.12 Complimentary DNA (cDNA) synthesis

cDNA was synthesized using Superscript® III First Strand Synthesis supermix for real time quantitative RT-PCR (qRT-PCR) (Invitrogen, Carlsbad, CA). The supermix for qRT-PCR kit was purchased from Invitrogen (Life Technologies # 11752-050). Components of kit are 2X RT Reaction Mix, RT Enzyme Mix and E.Coli RNase H. 2X RT Reaction Mix includes oligo$(dt)_{20}$ (2.5 µM), random hexamers (2.5 ng/µL), 10 mM MgCl2, and dNTPS. RT Enzyme Mix includes SuperScript ® III RT and RNaseOut™. The mixture of reagents was prepared by combining the following kit components in a tube placed on ice (Table 4).

Table 4: Components of cDNA Kit

Component	**Amount per Reaction**
2X RT Reaction Mix	10 µL
RT Enzyme Mix	2 µL
RNA (1µg)	x µL
DEPC-treated water	Volume made up to 20 µl
Total Volume	20 µL

The reagents were thawed before mixing in a tube. The composition of RT Reaction mix are Oligo (dT) (2.5 µM), random hexamers (2.5 ng/µl), 10mM of MgCl and dNTPs. RT Enzyme mix consists of Superscript III RT and RNaseOUT. The tube contents were gently mixed followed by incubation at 25°C for 10 minutes and then followed by incubated at 50°C for 30 minutes. The reaction was then terminated at 85°C for 5 minutes and the tubes were kept on ice. These steps were carried out in a thermocycler (VWR). It was followed by addition of 1 µl (2U) of E. coli RNase H supplied in the kit and further incubation was done for 20 minutes at 37°C in a thermocycler. After these steps were done, samples were stored at -20°C until further use.

2.13 Real Time PCR

Real Time PCR was performed using SYBR GreenER^{TM}. qPCR SuperMix for iCycler (Invitrogen # 11761-500). The reaction was carried out in an instrument Bio-Rad IQ5 Multicolor Real Time PCR Detection System using a 96 well PCR plate (Bio-Rad # 2239441). The mouse primers used in this study are mentioned in Table 5.

Table 5: Mouse forward and reverse primer sequences used in this study

Target	Forward Primer	Reverse Primer
GAPDH	5'-ACCCAGAAGACTGTGGATGG-3'	5'-ACATTGGGGGTAGGAACAC-3'
IL-1 β	5'-AACCTGCTGGTGTGTGACTTC-3'	5'-CAGCACGAGGCTTTTTTGT-3'
IL-4	5'-GCGACAAAAATCACTTGAGAG-3'	5'-CCTTGGAAGCCCTACAGAC-3'
IL-6	5'-AGTTGCCTTCTTGGGACTGA-3'	5'-TCCACGATTTCCCAGAGAAC-3'
IL-10	5'-AACCTGCTGGTGTGATTC-3'	5'-CAGCACGAGGCTTTTTTGT-3'
TNF-α	5'-ACACTCAGATCATCTTCCAAAA-3'	5'-GCAATGACTCTAAGTAGACCTGC-3'
MCP-1	5'-CAGCAAGATGATCCCAATGA-3'	5'-TGGTTCCGATCCAGGTTTT -3'

Master Mix (MM) for each gene was prepared by mixing the following components in a tube (Table 6).

Table 6: Components of a MasterMix (MM)

Component	**Amount per reaction tube**
2X SYBR GreenER Supermix	10 µL
Forward Primer, 10 µM	1 µL
Reverse Primer, 10 µM	1 µL
DEPC-treated water	7 µl
Total Volume	19 µL

Each well of the PCR plate was loaded with 19 µl of MM and 1 µl of cDNA. After loading, the PCR plate was sealed with micro seal optical adhesive film (Bio-Rad # MSB 1001). The plate was then centrifuged at 1200rpm for 10 minutes at 4°C. Then the plate was kept in the CSF iCycler instrument. The programming of the machine was set as 1 cycle at 50°C for 2 minutes followed by 1 cycle at 95°C for 8 minutes and 30 seconds which is followed by 40

cycles each at 95°C for 15 seconds and 60°C for 1 minute. Melt curve analysis was carried out at 95°C for 1 minute then 55°C for 1 minute followed by 80 cycles at 55°C ±0.5°C /cycle for 10 seconds till the temperature rises to 95°C. When the run was complete, Ct values and melt curves were analyzed using Bio-Rad CFX optical system software. The mRNA expression of the target gene was normalized to the corresponding GAPDH gene expression levels using above mentioned mouse primers.

2.14 ELISA

The Enzyme Linked Immunosorbent Assay (ELISA) kits for mouse cytokines (TNF-α, IL-1β, IL-4, IL-6 and IL-10) were purchased from R & D systems Quantikine ELISA and experiments were carried out according to manufacturer's protocol. Briefly, 50µL of cytokine standards or tissue incubated medium was added to each pre-coated well loaded with 50 µL of assay diluent followed by incubation for 2 hours at room temperature. After incubation, plate was washed 5 times by adding 400 µl of wash buffer in each well every time. It was followed by adding 100 µl of anti-mouse cytokine polyclonal antibody-horseradish peroxidase Conjugate to each well. The plates were incubated for 2 hour at room temperature followed by washing 5 times as previously mentioned. It was followed by adding 100µl of chromogen substrate solution to each well. Incubation was done for 30 minutes. The reaction was stopped then by adding 100 µl of stop solution and measured the absorbance at 450 nm. To avoid optical imperfections in the plate, correction absorbance was measured at 570 nm.

2.15 Statistical significance evaluation

Student t-test was used to evaluate statistical significance for differences in values. These differences were considered as significant if $p<0.05$. All data were analyzed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA, USA).

CHAPTER-3: RESULTS

3.1 Basal level expression of cytokines in intestinal epithelial tissue and Peyer's patches under *fasting* conditions

Basal level gene expression (n=9) of various cytokines were measured in intestinal IE and PP tissues of C57BL/6J mice after overnight *fasting*. Tissue samples were homogenized in TRIzol reagent, RNA was isolated, cDNA was synthesized and RT-PCR was performed to determine cytokines (IL-1β, IL-4, IL-6, TNF-α, and IL-10) gene expression. GAPDH was used as a reference gene. As shown in Figure 8, basal cytokine expression was higher in PP as compared to IE tissues. The cytokines TNF alpha, IL-1beta and IL-4 were significantly higher in PP as compared to IE while there was not much difference found in IL-6 and IL-10 gene expression between IE and PP.

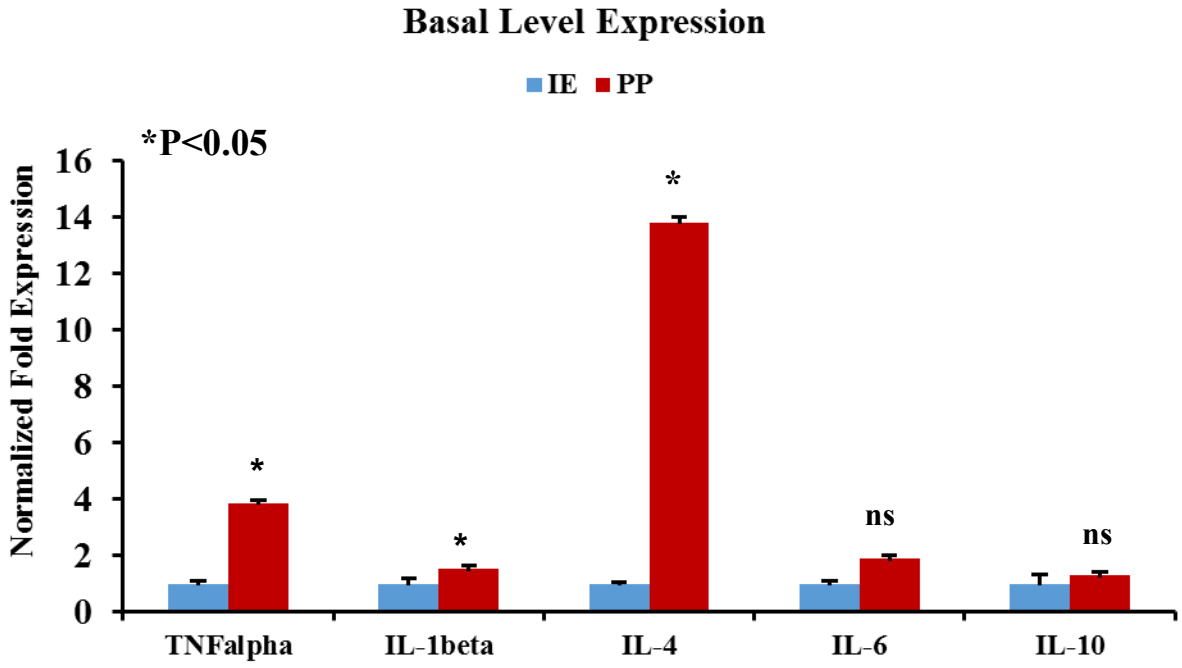

Figure 8: Basal level expressions of cytokines in intestinal epithelial and Peyer's patches tissues of C57BL/6J mice after overnight *fasting*.

3.2 Basal level expression of cytokines in intestinal epithelial tissue and Peyer's patches under *fed* conditions

Similarly, as mentioned above, the basal level gene expression (n=3) of cytokines in IE tissues and PP tissues of C57BL/6J mice under fed state were analyzed. As shown in Figure 9, no significant difference in cytokine expressions were observed between IE and PP except for IL-4 which remains significantly elevated in PP even after feeding.

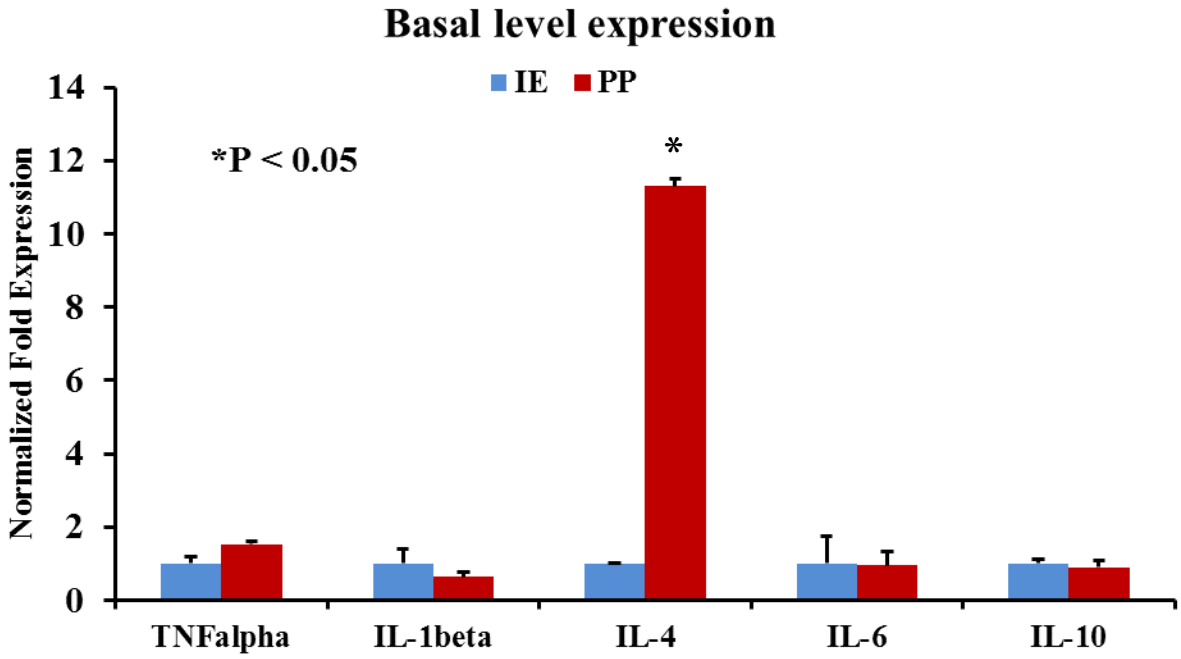

Figure 9: Basal level expression of cytokines in intestinal epithelial tissue and Peyer's patches under *fed* conditions

At this point, it can only be speculated whether feeding suppresses some of the inflammatory genes studied. Subsequent experiments were done under *fasting* conditions.

3.3 Increase in cytokine gene expression in intestinal epithelial tissue and PP on treatment with LPS

LPS was used as a positive control to check whether tissues respond to inflammatory stimulus. LPS treated (n=9) IE tissues, as shown in Table 7, showed increased expression of cytokines IL-1β, IL-6, IL-4 and IL-10 as compared to basal level IE expression of cytokines

Table 7: LPS treatment of intestinal epithelial tissues increased cytokines gene expression as compared to basal levels.

Epithelial Tissue	IL-1Beta	IL-6	IL-4	IL-10
Basal	1	1	1	1
LPS	↑(1.5)	↑*(2.47)	↑**(6.35)	↑(1.45)

Similarly, LPS treated (n=9) PP tissues, as shown in Table 8, showed increased expression of cytokines IL-1beta, IL-6, IL-4 and IL-10 as compared to the basal level PP expression of cytokines (no difference was observed with TNF-alpha).

Table 8: LPS treatment of Peyer's patches tissues increased cytokines gene expression as compared to basal levels.

Peyer's Patches	IL-1Beta	IL-6	IL-4	IL-10
Basal	1	1	1	1
LPS	↑*(1.58)	↑*(1.83)	↑*(2.71)	(1.09)

3.4 Cytokine induction in intestinal IE and PP in presence of HPODE

To determine whether HPODE induces inflammatory responses in Intestinal tissues, IE and PP samples were incubated with 25nmoles/ml HPODE in HBSS for 6 hrs at 37ºC. After 6 hours of incubation, samples were lysed in Trizol. RNA was isolated, cDNA was synthesized and RT-PCR analysis was performed to determine the gene expression of cytokines (IL-1β, IL-4, IL-6, TNF-α, and IL-10). GAPDH was used as the reference gene. The cytokines expression in HPODE treated (n=9) IE tissues were compared with the IE tissues basal level cytokines expressions. Similarly, HPODE treated (n=9) PP tissues were compared with the PP tissues basal level cytokines expressions.

We observed (Figure 10) a great significant increased expression of cytokines IL-1beta, IL-6, and IL-4 as compared to the basal level IE expression of cytokines. HPODE treated PP tissues (Figure 11), showed significantly increased expression of cytokines IL-1beta, IL-6, and IL-4 as compared to the basal level PP expression of cytokines while not much change was observed in IL-10 gene expression between HPODE treated intestinal tissues and basal level tissues (no difference in TNF-α expression was observed).

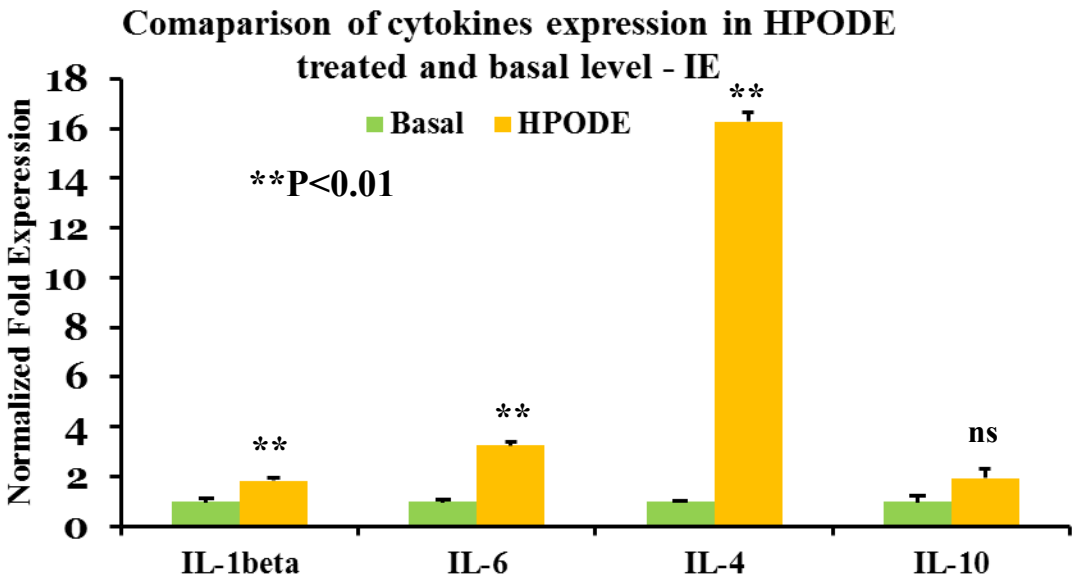

Figure 10: Significantly increased cytokine gene expression was observed in IE tissue in the presence of HPODE as compared to basal level expression.

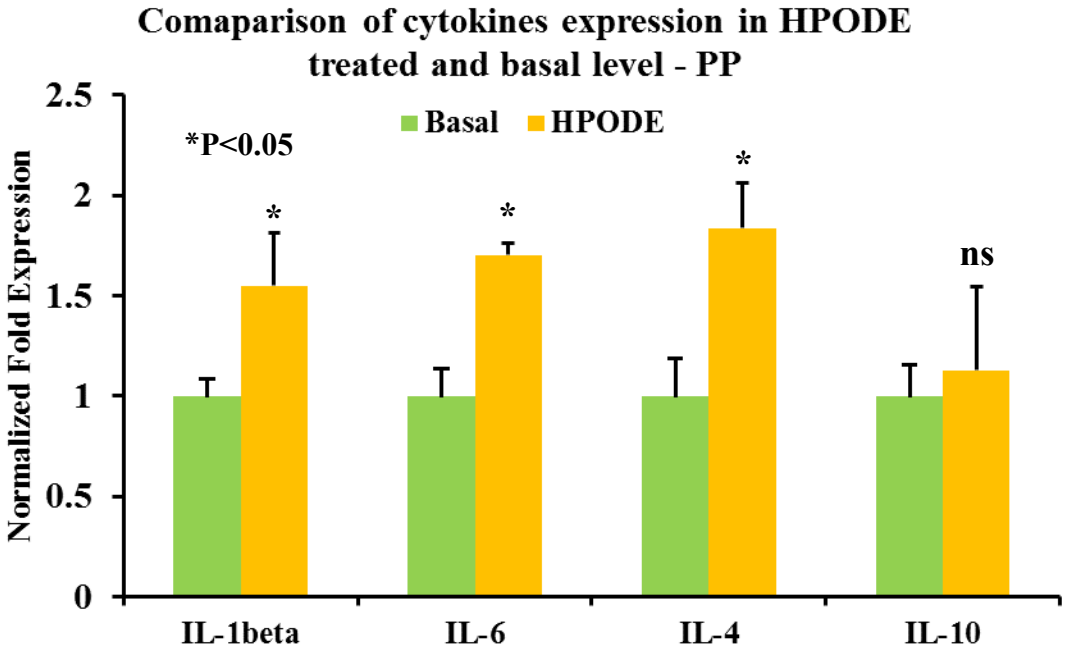

Figure 11: Significant increase in inflammatory gene expression was observed in PP tissue in the presence of HPODE as compared to basal level PP cytokines expression.

Although the basal level cytokines expression was higher in PP than IE (Figure 8), it was observed that the induction of cytokines expression after HPODE treatment when compared to basal levels, was higher in IE (Figure 10) as compared to PP (Figure 11). This might suggest an active participation of IE tissues in the inflammatory process.

3.5 Cytokine secretion by IE and PP in presence of HPODE: ELISA

The IE and PP tissues were incubated with HPODE (25 nmoles/ml in HBSS) for 6 hours at 37ºC. After 6 hours of incubation, the IE tissue incubated medium was used for the detection of cytokines secreted to the medium. Respective controls were maintained. The levels of cytokines were measured from HPODE treated intestinal tissues incubated medium and compared with respective control medium treated similarly to HPODE treated tissues. As shown in Figure 12, HPODE treated IE tissues incubated medium showed increased levels of cytokines IL-1beta and IL-6 as compared to control medium. Similarly, HPODE treated PP tissues incubated medium showed increased cytokines IL-1beta as compared to control cytokines IL-1beta (Figure 13). (TNF-α, IL-4 and IL-10 were undetectable in the medium possibly due to time-lapse in secretion of the protein).

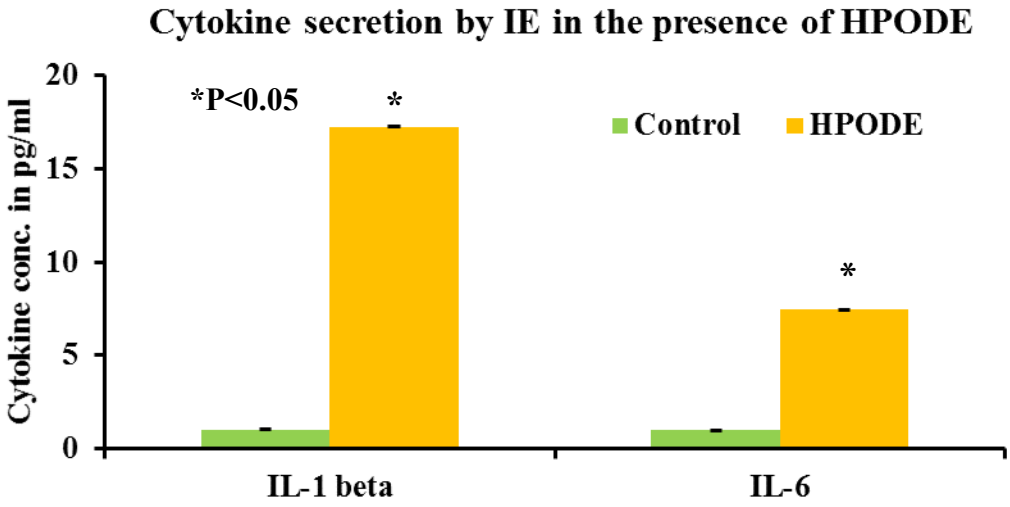

Figure 12: Cytokine concentration in HPODE treated IE tissues incubated medium compared to control IE incubated medium.

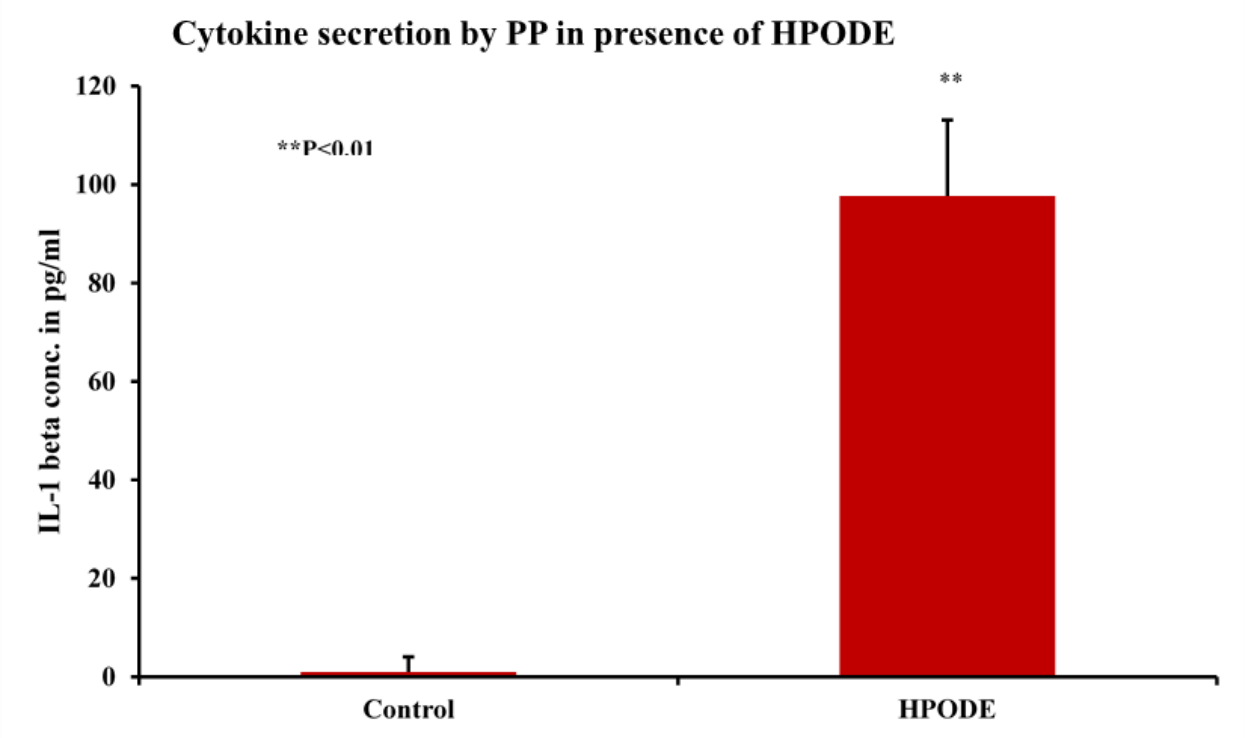

Figure 13: Cytokine level in HPODE treated PP tissues incubated medium compared to control medium.

3.6 Cytokine induction in IE tissue and PP in presence of Ox-PL

To determine whether Ox-PL induces inflammatory responses in intestinal tissues, IE and PP samples were incubated with 25nmoles/ml Ox-PL for 6 hours at 37°C. After 6 hours of incubation, samples were lysed in Trizol. RNA was isolated, cDNA was synthesized and RT-PCR analysis was performed to determine the gene expression of cytokines (IL-1 β, IL-4, IL-6, TNF-α, IL-10 and MCP-1) similarly to HPODE treatment. GAPDH was used as the reference gene. The cytokines expression in Ox-PL treated (n=3) IE tissues and PP were compared with the basal level (n=3) IE tissues and PP cytokines expressions respectively.

Ox-PL treated IE tissues (Figure 14), showed an increased expression of cytokine IL-4 as compared to basal level IE expression of cytokines. Similarly, PP treated with Ox-PL showed an increase in IL-4 cytokine expression as compared to basal level PP cytokine expression (Figure 15). (no difference was observed with TNF alpha and IL-10).

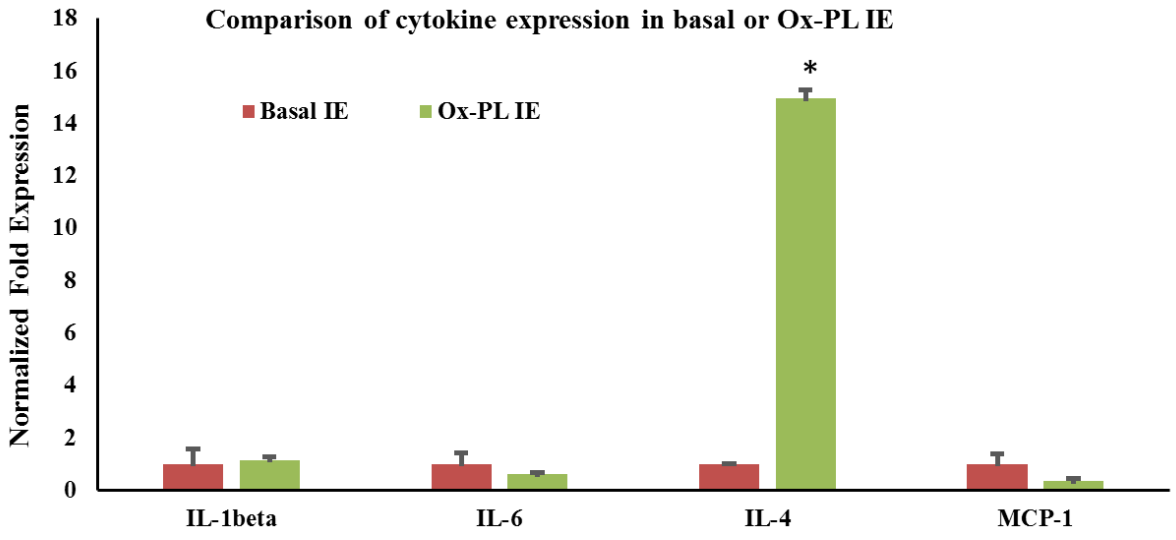

Figure 14: Comparison of cytokine expression in IE tissues for Ox-PL treated and basal levels

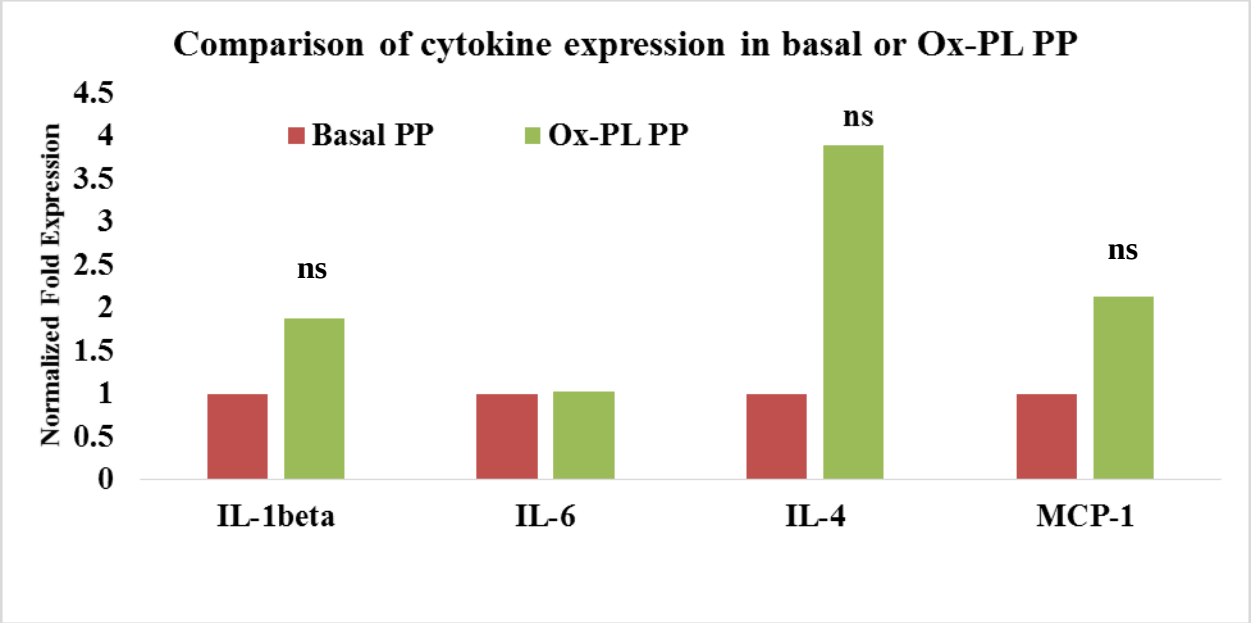

Figure 15: Comparison of cytokine expression in PP tissues for Ox-PL treated and basal levels

3.7 Cytokine induction in IE tissue and PP in presence of mmLDL

To determine whether mmLDL induces inflammatory responses in intestinal tissues, IE and PP samples were incubated with 25 µg/ml of mmLDL for 6 hours at 37°C. After 6 hours of incubation, samples were lysed in Trizol. RNA was isolated, cDNA was synthesized and RT-PCR analysis was performed to determine the gene expression of cytokines (IL-1β, IL-4, IL-6, TNF-α, IL-10 and MCP-1) similarly to HPODE treatment. GAPDH was used as the reference gene. The cytokines expression in mmLDL treated (n=3) IE tissues and PP were compared with the basal level IE tissues and PP cytokines expressions respectively.

IE tissues treated with mmLDL (Figure 16), showed an increased expression of cytokine TNF alpha and IL-4 as compared to basal level IE expression of cytokines. PP treated with

mmLDL showed an increase in MCP-1 expression as compared to basal level PP expression (Figure 17).

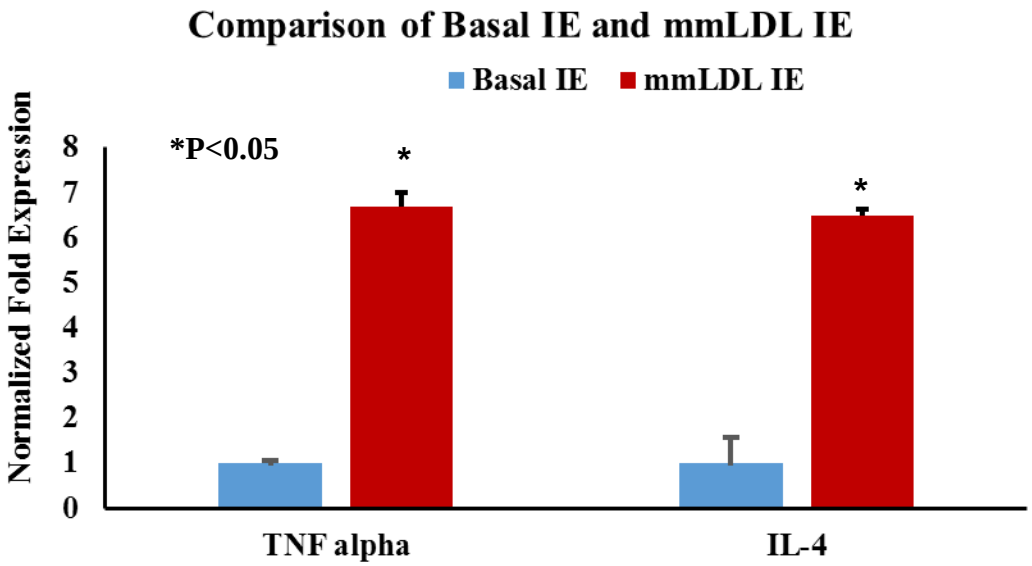

Figure 16: Comparison of cytokine expression in IE tissues for mmLDL treated and basal levels

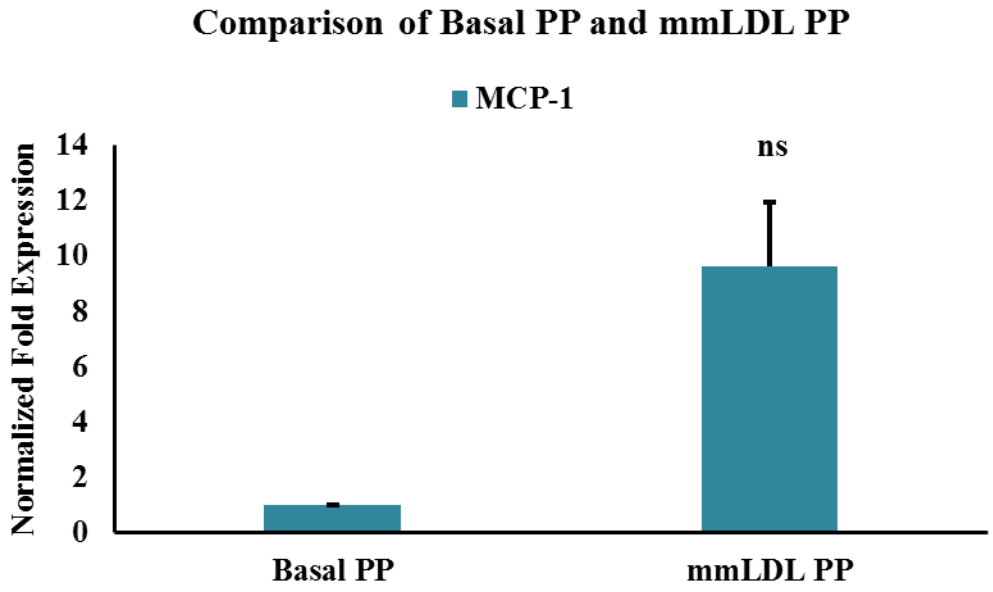

Figure 17: Comparison of cytokine expression in PP tissues for mmLDL treated and basal levels

3.8 Determining the production of cytokines in intestinal tissues by different concentrations of HPODE

In this experiment, we determined the induction of cytokines (IL-1β, IL-4, IL-6, TNF-α, and IL-10) in IE and PP after treatment with different concentrations of HPODE. The HPODE was confirmed by an increase in absorbance at 234 nm and LMB assay as previously mentioned. Different concentrations of HPODE used in this experiment were 0 -250 nmoles/ml, prepared with HBSS. IE tissues were divided into 6 groups and each group was incubated with different concentration of HPODE for 6 hours at 37ºC. Similarly, PP tissues were divided into 6 groups and each group was incubated with different concentration of HPODE. After 6 hours of incubation, samples were lysed in Trizol. RNA was isolated, cDNA was synthesized and RT-PCR analysis was performed to determine the gene expression of cytokines. GAPDH was used as the reference gene. The cytokines expression in different concentrations of HPODE treated IE tissues were compared with the HPODE (0 nmoles/ml) treated IE expression of cytokines. Similarly, the cytokines expression in different concentrations of HPODE treated PP tissues were compared with the HPODE (0 nmoles/ml) treated PP expression of cytokines. An increase in IL-4 and IL-10 gene expressions was observed with increased concentrations of HPODE treated PP tissues (Figure 18) while other cytokines did not show much difference in expression when compared to their respective controls (HPODE - 0 nmoles/ml).

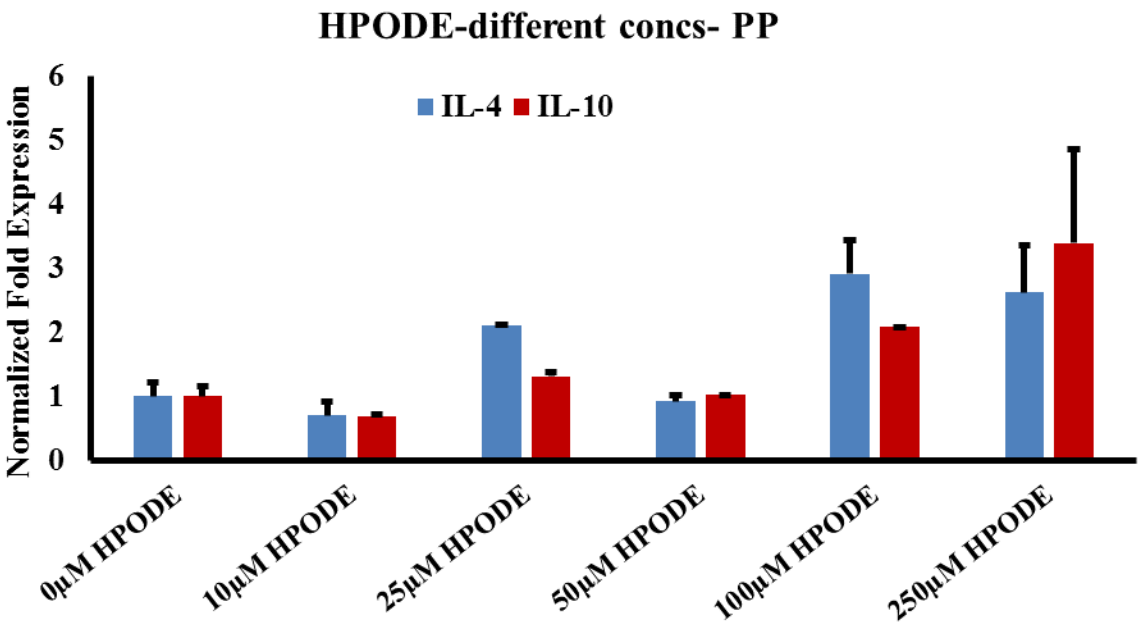

Figure 18: Cytokine induction in PP after treatment with increasing concentrations of HPODE

CHAPTER-4: DISCUSSION

There are a number of studies suggesting that peroxidized lipids in the diet might be atherogenic (Staprans, I., et al. 1993, Staprans, I. L. O. N. A., et al. 1993) and cause recruitment of inflammatory mediators in cell systems (Khan, B. et al, (1995), O'Brien et al, (1993), Springer, T. A. (1990), Dwarakanath, R. S. et al, 2004). Peroxidized fatty acids are suggested to be inflammatory. Studies (Awada M. et al 2012) demonstrated that inflammatory markers in plasma was significantly increased in mice fed oxidized n-3 PUFA with high fat diet as compared to unoxidized n-3 PUFA with high fat diet. Studies by Mickel HS and Horbar J, (1974) have shown that peroxidized arachidonic acid caused a greater degree of platelet aggregation than arachidonic acid. Studies by Staprans, I., et al (1993) have shown that the peroxide content of serum VLDL + LDL lipoproteins were significantly increased with feeding rats diet containing sucrose with high lipid peroxides as compared to diet containing sucrose with low lipid peroxide content or fat free sucrose diet. Studies (Parthasarathy, S. et al, (1989), Steinberg D (2002), Badimon L, Vilahur G (2012)), have shown that oxidation of lipoproteins may play an important role in atherogenesis and studies by Parthasarathy, S. et al, (1989), have demonstrated the involvement of cellular lipoxygenases in oxidation of LDL. According to Chen, C., & Khismatullin, D. B. (2015) studies, a significant increase was observed in release of TNF alpha in the supernatant of Ox-LDL treated THP-1 monocytes and macrophages as compared to untreated THP-1 cells as measured by ELISA. However, there are not many studies to suggest that peroxidized lipids (POL) such as peroxidized fatty acids could cause inflammatory response in mice intestinal tissues. Moreover, as studies have shown that dietary oxidized lipids can be absorbed by the intestine and incorporated into chylomicrons or

chylomicrons remnants (Staprans, I., et al., 1994), therefore we tested the hypothesis that POL could cause induction of inflammatory cytokines in IE and PP tissues.

Our results showed higher basal level expression of inflammatory cytokines in PP as compared to IE after overnight fasting. This was expected as PP are the aggregated lymphoid follicles containing immune cells. We found increased cytokines gene expression in both IE and PP tissues on treatment with HPODE as compared to basal levels IE and PP tissues respectively. However, the induction of inflammatory cytokines by HPODE treatment was more marked in IE tissues than PP tissues when compared to their basal levels respectively. This increased cytokines expression observed in IE tissue as well as PP in the presence of HPODE, was analogous to LPS suggesting that HPODE might be inducing gut inflammation like LPS. The gene expression results were consistent with protein secretion as measured by ELISA. We found increased IL-1β and IL-6 secretions in HPODE treated IE tissue incubated medium than control IE tissue medium. IL-1β secretion were also significantly increased in HPODE treated PP incubated medium than control PP tissue medium. We found increased expression of IL-4 in HPODE treated IE and PP tissues as compared to basal levels IE and PP tissues respectively. This is consistent with studies (Van Kampen, C., et al, 2005) showing IL-4 alone has the ability to act as a pro-inflammatory cytokine in the mucosa of the colon and could lead to colitis. Other studies (Specht S. et al, 2006) have also shown the involvement of IL-4 in colitis development. According to these studies, the development of colitis was of significantly lower degree in IL-4/IL-10 double deficient mice as compared to IL-10 deficient mice which developed severe colitis. These findings suggested that IL-4 might be involved in causing gut inflammation. mmLDL was used as a positive control in this study. Similarly, we observed increased IL-4

expression in mmLDL treated IE in comparison with basal levels. We also observed increased MCP-1 gene expression in mmLDL treated PP tissues. This might suggests that dietary POL might be involved in recruitment of monocytes and their differentiation into macrophages by the induction of IL-4 and MCP-1in intestinal tissues. This in turn might lead to increased TNF-α release by macrophages and correlates with the necrosis found in IBD patients. However, IL-4 levels were undetectable in HPODE treated IE and PP medium during ELISA. This might be due to time-lapse in expression of gene and secretion of the protein. IL-4 might be in transcriptional phase at the duration of the exposure of HPODE and might not be secreted into medium in that duration of treatment. Other possibility might be due to the short half-life of IL-4. Previous studies have shown that uncomplexed IL-4 has a very short half-life (<15 min) (Sato, T.A. et al, 1993, Fernandez-Botran, R. et al 1996). Other studies have shown that natural sIL-4Rα and rIL-4 complexes rapidly dissociate and the half-life of IL-4/sIL-4Rα formed from its dissociation is~2 hr (Khodoun, M.et al, 2007)

Thus, HPODE could be acting as an inflammatory agent and can induce gut inflammation. The findings of this project might suggest that restricting fatty acid consumption in Crohn's patients may be beneficial. It also suggests that IL-4 might be a target to lower inflammation in intestinal inflammatory diseases.

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