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Novel Role of Intestinal Lipid Transport in Food Allergy and Cholesterol Homeostasis

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NOVEL ROLE OF INTESTINAL LIPID TRANSPORT IN FOOD ALLERGY AND CHOLESTEROL HOMEOSTASIS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

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Lexington, Kentucky

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Lexington, Kentucky

2015

ABSTRACT OF DISSERTATION

NOVEL ROLE OF INTESTINAL LIPID TRANSPORT IN FOOD ALLERGY AND CHOLESTEROL HOMEOSTASIS

The small intestine is the main organ for food digestion and nutrient absorption. It is constantly exposed to antigen and immunomodulatory agents from diet and commensal microbiota. Thus, the intestine is the largest compartment of the immune system in the body. Peanuts and many other allergen resources contain triglycerides, which may affect the antigen absorption through the intestine, but their effects on sensitization and anaphylaxis are unknown. We found that medium chain triglycerides (MCT) promoted antigen absorption into Peyer's Patches, rather than into the blood directly. Both gavage and feeding of MCT plus peanut protein induced spontaneous allergic sensitization. MCT-sensitized mice experienced the IgG-dependent anaphylaxis from systemic challenges and the IgE-dependent anaphylaxis from oral challenges. Furthermore, MCT alone had direct pharmaceutical effect on enterocytes, like stimulating Jejunal-epithelial Th2 cytokine responses compared with what was seen in the long chain triglycerides (LCT) treated group. Moreover, the oral challenges conducted with peanut protein in MCT significantly exacerbated anaphylaxis compared with the LCT challenges.

The intestine also plays an important role in whole body cholesterol homeostasis due to its exclusive function in cholesterol absorption. The researchers found that the intestine function in cholesterol secretion and elimination, but it has not been proven directly until recently. This pathway that facilitates the cholesterol secretions through intestine was named the Transintestinal Cholesterol Efflux (TICE) and has not been well studied yet.

To find the possible transporter candidates involved in TICE, we compared both biliary and intestinal cholesterol excretion rates in wild-type (WT) and G5G8 deficient (KO) mice of both sexes. All mice were maintained on a plant-sterol free diet beginning at weaning to prevent the development of secondary phenotypes associated with Sitosterolemia. We found that WT mice had higher biliary cholesterol excretion rates compared to their G5G8 KO littermates as previously reported. However, this difference is significantly greater in females compared to males. Interestingly, intestinal cholesterol excretions increased in female KO mice compared to their WT littermates, a difference not observed in males. This data suggests a sexually dimorphic adaptive mechanism to maintain cholesterol elimination in the absence of G5G8. Whereas male mice maintain a greater level of biliary output in the absence of G5G8, female mice upregulate an alternate intestinal elimination route.

To determine the origin of intestinally secreted cholesterol, we compared both hepatobiliary and intestinal cholesterol secretion rates in male wild-type (WT) and CETP transgenic (CETP TG) mice at the age of 12 weeks. Cholesteryl ester transfer protein (CETP) facilitates the transport of cholesteryl esters and triglycerides between lipoproteins in plasma and alters the lipoprotein distribution of plasma cholesterol. We found that WT and CETP TG mice did not differ in either biliary or intestinal cholesterol secretion rates when maintained on a standard chow diet. However, CETP TG mice showed increased biliary cholesterol secretion rates and decreased intestinal cholesterol secretion rates compared to the WT group in response to a Western diet. We next determined the effect of CETP on the delivery of radiolabeled HDL-cholesterol ester to bile and intestinal lumen. Unlike bulk cholesterol secretions, HDL-derived cholesterol esters were preferentially delivered to the intestine in CETP TG mice. This data suggests that CETP alter the routes of total and HDL cholesterol elimination from the body in mice.

Jianing Li

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DISSERTATION

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CHAPTER I. BACKGOUND

1.1 Small intestine and its physiological functions

1.1.1 Small intestine[1]

The small and large intestines represent the continuous tube system connecting the outlet of the stomach to the anus. The small intestine contains the part from the pylorus to the ileocaecal valve, which is the entry point to the large intestine. The small intestine can be divided into three main segments. The duodenum is the closest to the stomach, followed by the jejunum and the ileum. The small intestine is characterized by villi, finger-like projections which extend into the lumen and increase the active digestive epithelium surface area. In the opposite, caecum and colon lack villi and their surfaces are flat. In all parts of intestine, the surface epithelium is renewed continuously by multipotent stem cells arising from crypts of Lieberkühn. These cells are comprised of absorptive enterocytes, but also Paneth cells, goblet cells and neuroendocrine cells. Paneth cells migrate downwards to the base of the crypt, while the other newly formed cells move from bottom of the crypt to the tip of the villus, followed by extrusion after 4-5 days. During this process, the epithelial cells acquire the majority of enzymes and other properties needed for digestion and absorptive functions as they reach the base of the villus.

The different regions of intestine have distinct physiological functions. The surface of absorptive epithelial cells in the small intestine is covered by a layer of microvilli where the majority of digestive enzymes are located, as well as nutrient transporters (Figure 1). The

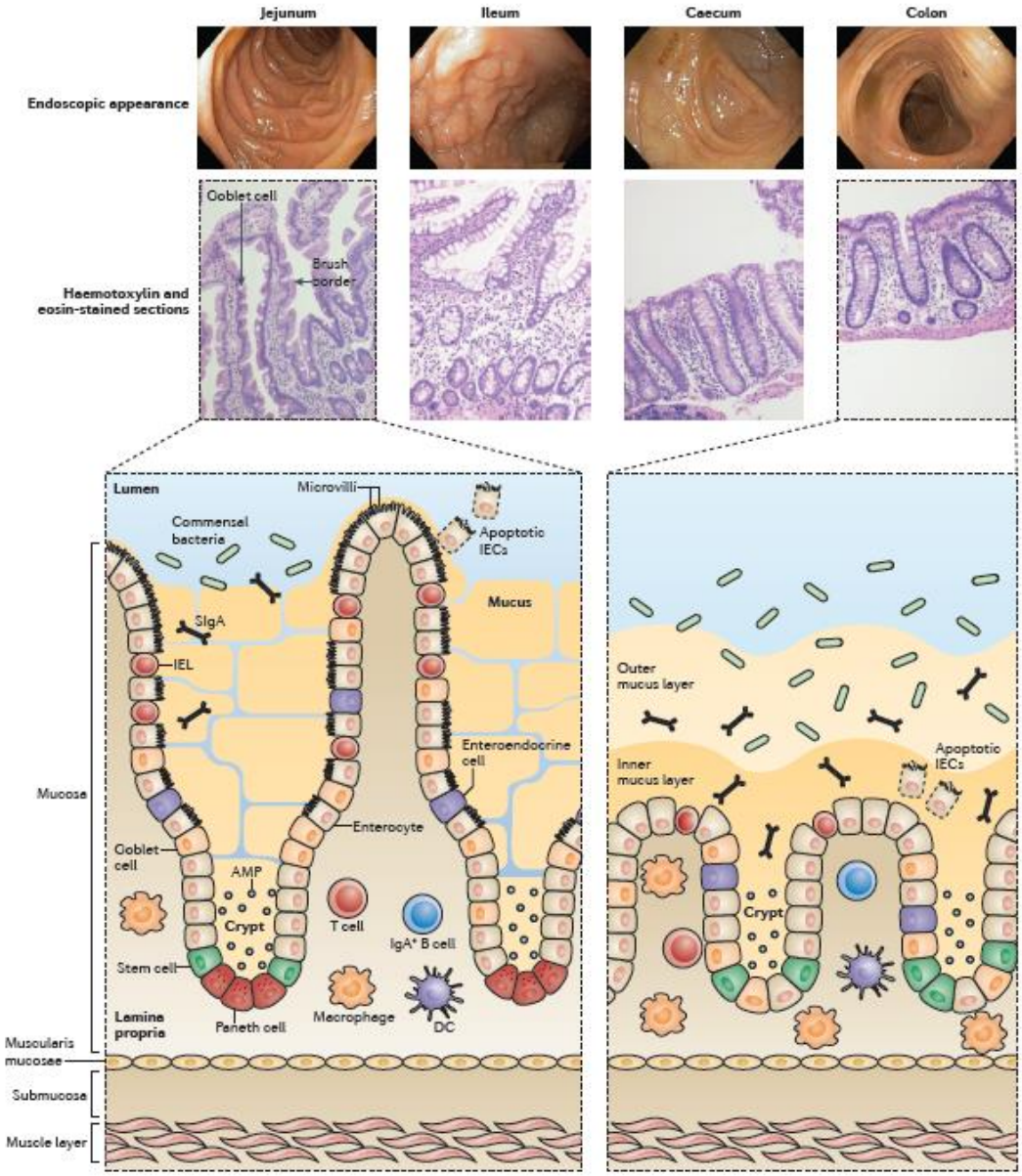

Figure 1.1. Anatomy of the intestinal mucosa and its immune apparatus[1].

‘brush border’ increases the surface area for digestion on the basis of long villi in the duodenum and jejunum. Most protein, carbohydrates and lipids digestions occur in this upper part of intestine. Damage to the upper small intestine leads to severe malabsorption, protein leakage and malnutrition. Compared to upper intestine, ileum mainly absorb bile salts and vitamin B12, contributing less to nutrition absorption and has remarkably shorter villi and lower levels of brush border enzymes[1].

1.1.2 Role of small intestine in immunology[1] and food allergy

1.1.2.1 Role of small intestine in immunology

The epithelium, the underlying lamina propria and the muscularis mucosa, a thin muscle layer below the lamina propria, comprise the mucosa, where most immunological processes occur (Figure 1). Lamina propria consists of connective tissues, blood supply, lymph drainage, nervous supply for the mucosa and also many cells from innate and adaptive immune systems. The lamina propria and epithelium form very distinct immunological compartments. The organized structures of the Gut Associated Lymphoid Tissue (GALT) that comprises subepithelia lymphoid and lies in the mucosa and the mesenteric lymph nodes are the primary locations for priming adaptive immune cell responses in the intestine. Effector immune cells are distributed throughout the lamina propria.

GALT is characterized by being covered by follicle-associated epithelium which contains Microfold cell (M cells). M cells uptake and transport particular antigens from lumen into subepithelial dome (SED) region which is rich in dendritic cell (DC), where they can be presented to adaptive immune cells by antigen presenting cells (APC). Peyer’s patches are the best-characterized tissues in GALT and locate on the outside of small intestine. Peyer’s patches are found mostly in the ileum, less in jejunum and barely found in duodenum[2]. There are great amount of B cell lymphoid follicles surrounded by smaller T cell areas in Peyer’s patches.

Different from lymph nodes, Peyer's patches do not have complete capsule structures and contain germinal centers always, indicating of continual immune stimulation mainly in response to antigen from intestinal lumen. With the same reason, the intestinal (mesenteric) lymph nodes draining are the largest in the body.

The lamina propria and epithelium are the main distribution site for effector cells in the intestinal immune system and their compartments are very distinct. The lamina propria is rich in B cells, T cells and great amount of innate immune cells such as DCs, macrophages, eosinophils and mast cells. The epithelium contains T cells primarily. In summary, lamina propria and epithelium contain the largest population of innate immune cells in the body[1].

1.1.2.2 Role of intestine in food allergy

As mentioned above, the varieties of intestinal compartments made intestine a good barrier for nutrients absorption and exclude the unwanted components, such as pathogens or allergen. Under normal conditions, after the consumption of nutrients, most of food proteins are hydrolyzed under the action of gastric, pancreatic and small intestinal brush border proteases such as pepsin, trypsin and chymotrypsin, to a mixture of free amino acids, di and tri-peptides and other proteolysis products for absorption[3]. There are still around 10% of the proteins that escape from proteolysis crossing intestinal barrier intact[3]. Husby et al. detected intact ovalbumin in human blood after egg white consumption by using a combination of HPLC and ELISA[4]. It is believed that those intact proteins and peptides may contain intact epitopes to be recognized by antigen presenting cells and induce sensitization or to elicit an allergic reaction. It is shown that 40% of the hydrolyzed protein have an 1100 Da molecular weight, which is still big enough to be recognized by MHC-II molecules (minimum length: 18-20 amino acids)[5]. Besides, 2% of intact proteins are estimated to reach mesenteric lymph nodes and portal circulation under normal conditions[6].

How do these non-fully hydrolyzed proteins absorbed as intact? Firstly, besides not fully hydrolysis through proteolysis, other trans-cellular pathway can also enhance antigen uptake by enterocytes and absorbed as intact. One of the believed mechanisms is the IgE coating theory. The luminal antigen-specific IgE forms a complex with antigen which are transported by CD23 receptor, which are overexpressed in the sensitized persons, trans-cellularly without lysosomal degradation across the intestinal membrane[7].

It is reported that antigens are secreted into exosomes after uptake with or without lysosomal degradation[8]. Thereafter, the antigen-containing exosomes cross basolateral membrane pore and enter circulation to reach MHC-II molecules to present the antigen to T cells[9, 10]. Inflammatory conditions may enhance these exosomes from intestinal epithelial cells[9]. However, the role of these exosomes remains debatable. Besides, IgA and IgG are involved in enterocytic antigen transport as well. The Polymeric IgA reach the intestinal lumen by polymeric IgA receptors[11]. However, different from IgE, IgA binds antigen to prevent their absorption and can lead to secretion back to intestinal lumen[3]. Therefore, antigen-specific IgA is considered to play a protective role in sensitization and allergic reactions. Polymeric IgG is secreted to intestinal lumen by Fc receptors and binds antigen as a complex as well. It is believed absorption of IgG-antigen complex in the neonate seems to be protective from sensitization, yet the role in adults remains unclear[12]. M cells, the specialized epithelial cells of Peyer's patches, have been shown to transport proteins, bacteria, viruses and other particles of size smaller than 1μm[13]. It is suggested that M cells transport proteins or antigens via a basal pocket which is rich in B and T cells, macrophages and dendritic cells[14, 15]. The exact function of the pockets remain unclear but it is assumed that the pockets may shorten the intracellular distance before antigens being seen by antigen presenting cells (APC), which then migrate to antigen-specific lymphocytes to induce T cell proliferation[3]. M cells have been confirmed to

transport both soluble and insoluble peptides. Sensitization or tolerance induction most likely depends on the particle sizes[16, 17]. Mast cells, DCs, macrophages and goblet cells are also thought to influence protein transport in the intestine yet the mechanisms remain controversial[3]. Taken these findings together, intestine is a significant organ to study the initiation of food allergy and has significant potential to works as an important therapeutic target for food allergy prevention and treatment.

1.1.3 Role of intestine in cholesterol homeostasis

Whole body cholesterol homeostasis is highly regulated by a balance of sterol uptake, absorption, de novo synthesis, fecal losses and export of intestinal lipoproteins, principally chylomicrons[18]. Intestine contributes significantly to sterol uptake, absorption and reverse cholesterol transport process.

Both dietary and biliary cholesterol are found absorbed from intestinal lumen in the proximal jejunum of small intestine. This process has been considered solely passive despite the fact that cholesterol is absorbed in a high efficiency yet the structurally similar plant sterols are not. The mechanisms of the interaction between sterols and intestinal brush-border membrane transporters still remain controversial, but it is proved that the key step of this process is to interact with *Niemann-Pick C1 like 1* (NPC1L1) by the discovery of a powerful cholesterol absorption inhibitor, ezetimibe, a drug that inhibits intestinal sterol absorption by binding to the NPC1L1 transporter and blocking its internalization with sterol via clathrin-coated vesicles[19]. NPC1L1 is expressed at the brush border membrane of intestine and NPC1L1 deficient mice showed around 70% reduction in cholesterol absorption[19]. ABCG5/G8 are heterodimers cholesterol transporter located at the canalicular membrane of hepatocytes and the brush border of enterocytes. In humans, mutations in genes encoding ABCG5 or ABCG8 were shown to cause sitosterolemia (STSL) characterized by accumulation of plant sterols in blood and tissues

due to the increased intestinal sterols absorption and decreased biliary removal[20]. G5 and/or G8 in mice were confirmed to upregulate plant sterol absorption[21] yet the effect on cholesterol absorption efficiency are limited[22]. In contrast, overexpression of G5G8 in mice leads to a dramatically reduced cholesterol absorption efficiency under pharmacological induction[23], indicating G5G8 manipulate cholesterol absorption under certain conditions. In the small intestine, SR-B1 localized both apical and basolateral phases of enterocytes. It was reported that SR-B1 deficiency only leads to a slightly increase in cholesterol absorption and decrease in fecal neutral sterol loss[24]. Similary, overexpression of SR-B1 did lead to increased cholesterol absorption, suggesting a possible role of SR-B1 in intestinal cholesterol absorption[25]. After uptake by enterocytes, cholesterol is esterified to cholesterol ester by ACAT2 at the endoplasmic reticulum (ER). ACAT-2 deficiency in mice would reduce cholesterol absorption upon high fat, high cholesterol Western diet feeding yet no clear reduction of cholesterol absorption was found in mice fed with chow diet[26]. Other proteins like microsomal triglyceride transfer protein (MTTP) and apolipoprotein B (apoB) are crucial in chylomicron formation, a critical process for cholesterol absorption. Mutations in MTTP gene leads to abetalipoproteinemia characterized by extremely low plasma cholesterol and TG levels and absence of apo-B-containing particles[27].

As described above, intestine plays a significant role in cholesterol homeostasis as a cholesterol absorbing tissue. It is recently revealed that intestine also acts as a cholesterol secretion tissue in Reverse Cholesterol Transport (RCT) process. Details will be discussed in the paragraph 1.3.4. In summary, besides an absorptive tissue, intestine has significant potential as a therapeutic target for maintaining cholesterol homeostasis in the whole body.

1.2 Peanut Allergy and Dietary Fats

1.2.1 Peanut allergy

Food allergy is thought to cause more than 50,000 cases anaphylaxis and estimated 100 deaths annually in the U.S. The foods that induce allergy can be divided into two groups briefly: 1) the foods that commonly cause food allergy in infants and young children but rarely cause food allergy in adults and rarely cause death, such as milk and eggs. 2) the foods that cause food allergy in both children and adults and very likely to cause severe shock and death, such as fish, shellfish, nuts and peanuts[28]. Of the second category, peanuts are inexpensive and broadly consumed in unmodified form and the components of many food products, causing the largest population of peanut allergy with severe anaphylaxis and death in U.S[29].

1.2.1.1 The prevalence and pathogenesis of peanut allergy

Several studies have evaluated peanut allergy prevalence in the U.S. and Canada and determined if it is changing by time. In one study, 1.4% of subjects were reported peanut or tree nuts allergy by random telephone survey [30]. This percentage in children (less than 18 years) increased from 0.6% in 1997 to 1.2% in 2002 and 2.1% in 2007. Yet the percentage remains unchanged in adults [31]. Consistent with the increased peanut allergy prevalence in children, the data of evaluating hospitalization rates for peanut induced anaphylaxis in New York State showed a 4-fold increase from 1990 to 2006 for young subjects younger than 20 years[31]. Another study, however, reported that only 22% of the children (8 years) who were diagnosed to be peanut sensitized based on skin prick testing or plasma IgE measurements had positive response to oral peanut challenge[32], indicating that not all people who have “peanut allergy” would actually develop anaphylaxis responses following peanut consumption. Even so, some studies showed that children with asthma and peanut allergy had 2.32-fold more hospitalizations for asthma than asthmatic children without peanut allergy[33]. Furthermore, a survey found that the incidence of peanut allergy in sibling of patients with peanut allergy is 6-7 fold higher than the siblings of children without peanut allergy[34].

A British study evaluated the relationship between peanut exposure and peanut allergy development. In the study, the prevalence of peanut allergy of Jewish children in the UK are found 10% less than the Jewish children in Israel [35]. The hypothesis from this study is that the early consumption of peanut product (bamba, food made from peanut butter and puffed corn) by Israeli infants may reduce the chance to develop peanut allergy because the infants in the UK avoid peanut products. However, non-oral exposure to peanuts, especially peanut butter, is found related to increased risk of peanut sensitization[36]. These studies indicate but not proved that consumption of peanut products at early life would reduce the risk of peanut allergy development. More animal experiments and clinical trial in infants needed to be initiated to provide more evidences to test the hypothesis.

Peanut allergy shares the similar induction mechanism as other food allergy which can be broadly divided into IgE mediated or non-IgE-mediated reactions. In IgE-mediated food allergy, exposure to glycoproteins in food would cause a series reactions among antigen presenting cells (APC), T cells, B cells, and production of IgE, which resides on mast cells and basophils. This phase is named “sensitization” (Figure 2). The same glycoprotein exposed to the sensitized subjects the second time would bind the IgE residing on mast cells and basophils which induces the activation and degranulation of these cells. Activated mast cells and basophils release histamine and other inflammatory chemical mediators that cause systemic reactions such as mucous secretion, smooth muscle contraction and vasodilatation. These effects result in symptoms like rhinorrhea, itching, dyspnea and anaphylaxis (Figure 2)[37]. Non-IgE mediated food allergy is more common. Activation and recruitment of lymphocytes and eosinophils are the cause of these diseases and symptoms.

Studies based on mouse model showed that IgE, mast cells and IL-13 are required for peanut allergy development[38]. Unlike requirements of IgE and mast cells that were

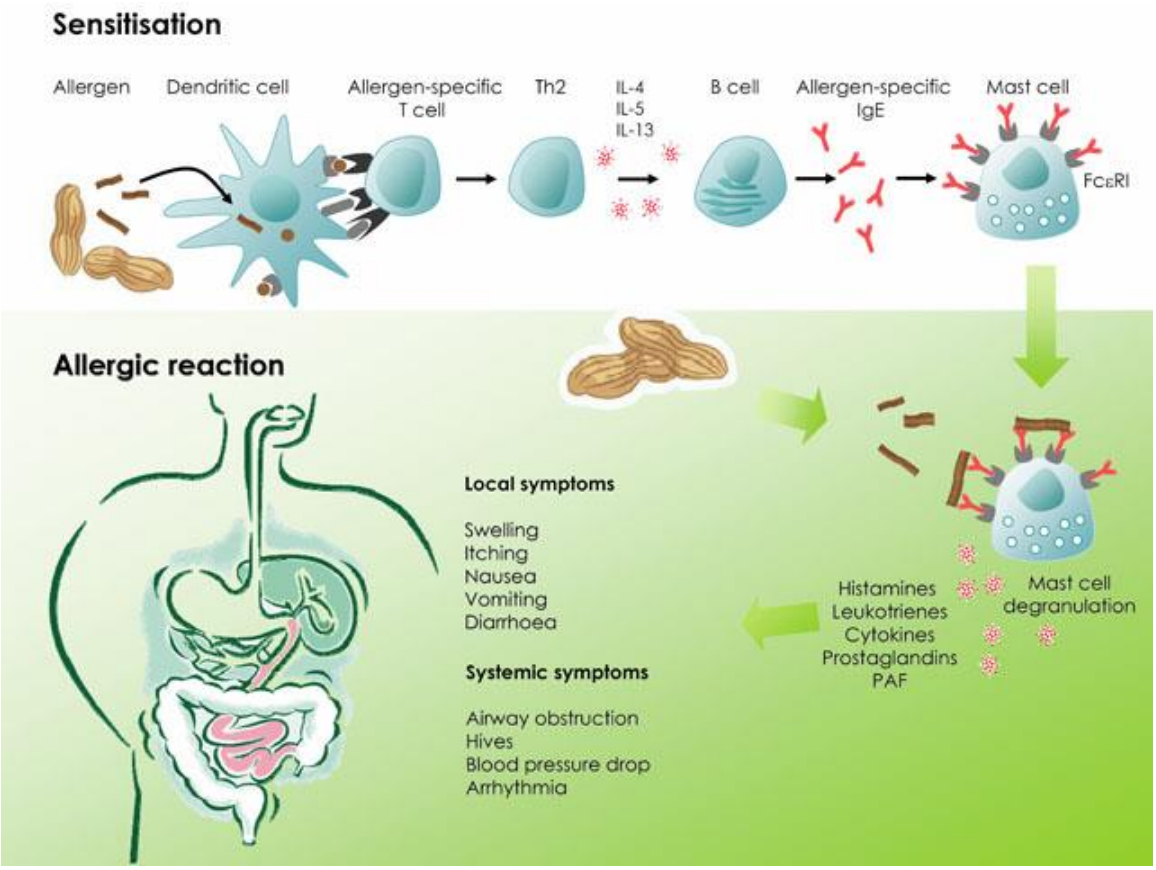

Figure 1.2 Sensitization and allergic reaction[37].

demonstrated in food allergy models requirement for IL-13 were recently shown to effect on vascular and epithelial cell permeability and smooth muscle contractility, the critical factors in food allergy induction[38]. A separate study reveal the complement is activated by aqueous extracts of peanuts and tree nuts but not milk or eggs in both mouse and human plasma[39]. In addition, peanut extract would exacerbate the severity of IgE-mediated anaphylaxis in mice through complement activation mechanism, indicating the complement by components from peanuts contributes to anaphylaxis induction significantly [39].

1.2.1.2 Peanut allergens

Allergens are defined by the binding ability to plasma IgE from allergic individuals and the activation ability of mast cells and/or basophils that have been sensitized with serum rich in IgE[28]. Chung and Champagne reported that roasted peanuts have higher binding ability to IgE than raw peanuts, suggesting their potency in allergy induction[40]. Based on these criteria, 11 peanut allergens have been determined (Table 1).

Among the 11 allergens, Arah 2 and 6 are considered to be more potent in peanut allergy development than others although the other allergen also bind IgE from majority of peanut allergic subjects[41]. To determine the epitopes of Arah 2, some studies have demonstrated that most IgE from peanut-allergic patients' serum binds to the conformational epitopes generated by native polypeptide folding of the allergen, rather than the linear epitopes generated by consecutive amino acids independent of polypeptides folding. However, it is still unclear how this finding explain the previous evidence that the patients with high concentration of serum IgE specific for linear epitopes of peanut allergen usually had the worst allergic symptom of allergy[42].

Some studies aimed at modifying peanut allergenicity by decreasing the levels of Arah 2 and 6. These studies suggested the feasibility of the approach, yet the hypothesis that peanuts

Table 1 Characteristics of Peanut Allergens [43]

Allergen Molecular	Molecular Mass	Characteristics
Ara h 1	63 k-Da	Member of vicilin family of seed storage proteins, a 7S globulin
Ara h 2	17–19 k-Da	Member of conglutin family of seed storage proteins, a 2S albumin
Ara h 3	14–45 k-Da	processed from 64 k-Da protein Member of glycinin family of seed storage proteins; heteromultimeric protein formed from differently proteoltically processed products of the same gene, an 11S globulin
Ara h 4	37 k-Da	Isoform of Ara h 3 Ara h 5 15 k-Da Member of profilin family of G-actin-binding proteins
Ara h 6	15 k-Da	Member of conglutin family of seed storage proteins, a 2S albumin
Ara h 7	17 k-Da	Member of conglutin family of seed storage proteins, a 2S albumin
Ara h 8	16 k-Da	Homologous to major birch pollen allergen, Bet v 1 and other pathogenesis-related proteins
Ara h 9	9.8 k-Da	Lipid transfer protein
Ara h 10	16 k-Da	Oleosin seed storage protein
Ara h 11	14 k-Da	Oleosin seed storage protein

depleted of Arah 2 and 6 have less allergenicity than conventional peanuts was not tested[44]. One concern about this approach is that populations with different geographical distributions or ethnics are prominently sensitive to different allergens. For example, one study demonstrated that Arah 9, an allergen that is relative insignificant to induce allergy in U.S., is a very potent allergen for peanut-allergic patients from Mediterranean area than Arah 1, 2 and 3[45]. Secondly, like mentioned in 1.2.1.1, since peanut allergy is comprised of sensitization and anaphylaxis phases, although Arah 2 and 6 have the highest affinities to IgE in peanut-allergic patients' serum (for the anaphylaxis phase), other allergens play important roles in sensitization phase. With the regard, crude peanut extract is much more allergenic than any of the purified major allergens in animal studies [46].

It is worth noting that despite the less potency of binding IgE from allergic patients, other allergens such as Arah 5, 8 and 9 are types of panallergens, which are responsible for allergic cross-reactivities across a broad range of unrelated plants. The cross-reactivities are always associated with birch and grass pollen or involved in "nsLTP-syndrome)[47].

1.2.1.3 Current therapies for peanut allergy

Currently, the only therapy for peanut allergy is to strictly avoid peanuts and their related products. Allergens from peanut would cause severe anaphylaxis reactions even the patients only had very mild symptoms before [48]. Therefore, besides strict avoidance, the main managements of peanut allergy focus on are to closely observe the early symptoms due to accidental ingestions and to self-administer epinephrine [49]. However, one study reported that these approaches negatively affect patients' life qualities [50]. Hence, therapeutic approaches that modify the immune responses to peanut allergens are needed to protect patients from accidental peanut exposure. Such novel immunotherapeutics are generally classified into allergen-specific and allergen-nonspecific.

Allergen-specific immunotherapy is based on induction of immune tolerance to peanut in IgE-mediated allergic patients by approach of administering patients with progressively higher doses of potent allergen over a relative long period such as weeks or months to induce clinical desensitization. 'Clinical desensitization' refers to the threshold dose of the potent allergen required to induce allergic response. Immune tolerance is defined as to induce a permanent non response to the offending allergen associated with the ability of the allergen ingestion and no more ongoing therapy[51]. Approaches of administering offending allergens including subcutaneous injections, and oral, sublingual or epicutaneous applications. *Subcutaneous immunotherapy (SIT)* is to applicate food allergen through subcutaneous injection. This therapy is usually associated with high risk of severe allergic reactions. Preliminary studies on this therapy showed a reduced skin-prick test (SPT) reactivity and increased tolerance to ingested peanut in the patients with oral challenges[52]. However, systemic reactions occurred in the following therapy and the authors concluded that modified peanut extracts were needed for such treatments [52]. *Oral Immunotherapy treatments (OIT)* have made progress toward safer and more efficient treatment for peanut allergy. Some clinical studies suggest that OIT is effective on clinical desensitization and oral tolerance induction among more than 50% children by raising threshold dose (at least 10 peanuts) of allergic reaction, which showed the clinical efficacy for desensitization during accidental peanuts ingestion[53, 54]. However, other aspects like mechanisms of action, dosing regimens and short-phase and long-phase effects still need to be established[55, 56]. *Sublingual Immunotherapy (SLIT)* is effective for tolerance induction on respiratory allergies [57]. Peanut EPIT is prolonged and repeated administration low dose of peanut allergens through epicutaneous injection. Similar to subcutaneous immunotherapy, preclinical studies showed that EPIT also induce desensitization and tolerance to the subsequent oral peanut challenge [58-60]. In a recent study in France, it was shown that EPIT was to reach a

cumulative threshold dose of peanut allergens 10 times higher than the beginning of the study after 18 months treatment, and 40% of the patients were desensitized to the threshold doses[61]. Therefore, it is indicated that EPIT is safe and effective in desensitizing allergic children to peanut. *Immunotherapy with hypoallergenic mutants combined with bacteria Adjuvant* were shown safe and effective in mice but it is not applicable in humans due to the adverse reactions[51].

Allergen non-specific therapies include silencing allergen-encoding foods in transgenic plants, anti-IgE therapy and DNA vaccines. Since the techniques of these therapies are either not mature enough or have some unsafe factors, most of them need more researches to establish[51].

An efficient therapy might be a good combination of both allergen-specific and allergen-non-specific applications. Therefore, the current most safe and efficient therapy still rests in prevention.

1.2.2 Dietary Fatty Acids

1.2.2.1 Category of Dietary Triglycerides

Dietary Triglycerides (TGs) are indispensable components in our daily diets, they are the main constituents of vegetable oil and animal fats. A TG is composed of three fatty acids (FAs) esterified to a glycerol molecule in one of the three distinct bonding positions. The combination of differences in FA types as well as the positions where they attached to glycerol molecule give rise to the huge heterogeneity of TGs one can ever think of. FAs are the major and functional unites of TGs and make up approximately 90% of total TG mass for most dietary oils we consumed. Three FAs of TGs are generally linear hydrocarbon chains consists with even number of carbons atoms ranging from 4 to 26. FAs that have less than 8 carbons are recognized as short-chain FAs (SCFA), 8 to 14 carbons as medium-chain FAs (MCFA) and long-chain FAs (LCFA)

for those that have 16 or more carbons. The amount of all these FAs varies depend on the source of oil but they all exist in human diets.

In addition to differences in the length of carbons chain, depending on whether or not a FA contains double bonds, FAs can be categorized into two types: saturated FAs and unsaturated FAs, unsaturated FAs can be further sub-categorized into monounsaturated FAs (MUFAs, contains a single double bond) and polyunsaturated FAs (PUFAs, contains two or more double bonds). Two systems have be used to identifying the position of double bonds along the hydrocarbon chain entail carbon counting from either end of the FA molecule. The less commonly used "Δ" system starts the carbon counting from the carboxyl end of the fatty acyl chain, whereas the more commonly used "ω or n" system identification of the position of the first carbon of a double bond relative to the methyl end. For example a ω-3 (n-3) FA, indicates that the first double bond is positioned between the third and fourth carbon atoms from the methyl end. In order for a FA to have a single double bond, it must be at least 12 carbon atoms in length. For PUFAs, each subsequent double bond almost invariably occurs there carbon atoms further along the chain. Thus the number of double bonds within a given FA is restricted depending on its length. And so dietary FAs can contain multiple double bonds up to 6.

Our body have the machinery to insert double bonds at the n-9 position or higher during FAs de novo synthesis but not at any position closer to the methyl end. Thus, FAs with double bonds at the n-3 and n-6 positions can't be constructed by ourselves but they are essential to normal growth in youth and those FAs are considered essential. Essential fatty acids (EFAs) therefor must be obtained from plants and other organisms that possess the machinery for their synthesis. For instance, dietary sources like walnuts, salmon and soybeans are rich in n-3 FAs, poultry meat, eggs and avocado are rich in n-6 FAs. Regardless of their essentiality, whether or not consume EFAs have positive health effects on against chronic diseases such as

cardiovascular diseases (CVDs) and cancer are still under debate. Indeed, some research suggested that excessive levels of certain n-6 FAs relative to n-3 FAs may increase the risk of developing a number of diseases,[62] thus an appropriate ratio of n-6 to n-3 FAs need to be considered when consuming those EFAs, preferentially 1:1 vs. average 15:1 in typical western diet.[63]

In nature, double bonds in USFAs are present in the *cis* configuration, in which the carbon chain extend at the same side of the double bond and thus creates a kink at the site of double bond resulted in a bent hydrocarbon chain; however, trans FAs do present in some food which are a result of hydrogenation of vegetable oil, a process to increase the viscosity of oil and acquire more desirable physical properties, higher melting temperature for example.[64] As appose to cis configuration, carbon chain in *trans* fat extend from the opposite side of the double bond and result in a straighter molecular much like saturated FAs. Although *trans* fat is edible and can be metabolized, intake of *trans* fat perturbs the ability of our body to metabolize essential fatty acids result in changes in fatty acid composition in aorta, moreover, consumption of *trans* fat are associated with increased LDL, decreased HDL and thereby increases the risk of coronary heart disease.[65] On 16 June 2015, the FDA finalized its determination that *trans* fats are not generally recognized as safe since its predetermination in 2013, and set a three year time limit for their removal from all processed foods.

1.2.2.2 TG metabolism

The digestion of dietary lipids begins in the oral cavity via the action of an enzyme known as lingual lipase. With the process of salivation and mastication, lingual lipase released form the serous glands hydrolysis of free FAs from TGs. This enzyme cleaves at the sn-3 position and preferentially hydrolyzing shorter-chain FAs in foods. Continuous digestion happens in

stomach, where gastric lipase promotes further hydrolysis of SCFAs from TGs. However the major site for dietary lipids digestion is in intestine. Intestinal digestion of TGs requires bile salts (BSs) and pancreatic lipase. With the help of colipase, also a pancreatic protein, pancreatic lipase adheres to the surface of TG droplet and acts to hydrolyze ester bonds at sn-1 and sn-3 position of the glycerol moiety. FAs at the sn-2 position however are resistant to hydrolysis by lipase. Hydrolysis products, monoglycerides (MGs) and free FAs are then incorporated in to BSs containing micelle with the help of phospholipids (PLs), cholesterol (CH) and colipase for their transport and subsequent cellular uptake.

The absorption of lipid hydrolysis products occur in large part through passive diffusion. First stage of absorption featured with the penetration of micelles across the unstirred water layer adjacent to the brush border surface of intestine mucosal cells. Due to its smaller size and the amphipathic nature, micelles but not larger lipid droplet are readily to approach and enter the water layer upon formation and continue to shuttle hydrolysis products through in a concentration-dependent fashion. This process primarily count on the lower cellular concentration of lipid hydrolysis products in the enterocyte, this is achieved by the rapid re-esterification of intracellular digestion products to form TGs by enzymes of the endoplasmic reticulum (ER). Also the intestinal FA binding proteins (FABPs) are involved to assist FAs absorption. Besides the process described above, FAs with chain lengths less than 12 carbon atoms are also readily absorbed passively by the gastric mucosal boundary and are taken up by the portal vein. [66] The efficiency of fat absorption is likely independent of the amount consumed but may influenced by the qualitative nature of dietary fat. In general, efficiency increases with the degree of FA unsaturation but decreases as FA chain length increases. In addition, the position or order of FAs attached to TGs also matters. [67]

After absorption, short- and medium- chain fatty acids directly enter the blood via intestine capillaries and travel through the portal vein just as other nutrients. But long-chain fatty acids are absorption into fatty walls of the intestine villi and reassembled again into triglycerides, which coated with cholesterol and apolipoprotein are processed into chylomicrons. The chylomicrons are released into lymphatic circulation and transported to thoracic duct up to a location near the heart. The thoracic duct empties the chylomicrons into the bloodstream via left subclavian vein, where chylomicrons can transport triglycerides to tissues for energy metabolism or storage.

1.2.2.3 Disease related to dyslipidemia

It is conceivable that any defect happened along the process of lipid digestion absorption or transportation might cause disorders in lipid metabolism in our body. For example celiac sprue, a common disorder of small intestine that results in lipid malabsorption is characterized by lesions of the small intestinal mucosa associated with gluten toxicity.[68] Deficiency of pancreatic enzymes necessary for lipid digestion resulted in accumulation of large amount of undigested fat in stools is another common disorder which can be treated by prescribing a low-fat diet or by supplementation of pancreatic enzymes with meals.[69] Bile salt deficiency as consequences of liver disease or gallstone disease results in poor micellar solubilization of lipid digestion products and thus insufficient delivery of lipid molecules to the small intestinal epithelial cells and accumulation of mainly lipid digestion products in the stool. Since dietary fat is the exogenous source of body lipids, malabsorption of fat can lead to hypolipidemia, a form of dyslipidemia that is defined by abnormally lowered levels of any or all lipids and/or lipoproteins in the blood. Hypolipidemia is relatively uncommon and might be asymptomatic in general, however, studies reported close association of this type of dyslipidemia with disorders such as anemia, hyperthyroidism and inflammation.[70] In contrast

to hypolipidemia, hyperlipidemia is one of the most common form of dyslipidemia involves abnormally elevated levels of any or all lipids and /or lipoproteins in the blood. Hyperlipidemia can be divided into primary and secondary subtypes.[71] Primary hyperlipidemia is mainly due to genetic causes, for instance, deficiency in lipoprotein lipase, enzymes that hydrolyzis TGs in lipoproteins, resulting in elevated chylomicrons, the particles that transfer fatty acids from the digestive tract to liver. Mutations affect low density lipoprotein receptor (LDLr) or apolipoprotein B (ApoB, primary apolipoprotein of lipid reach particles such as chylomicrons) result in decreased clearance and elevated blood lipid. Secondary hyperlipidemia or acquired hyperlipidemia are usually consequences of underlying conditions such as diabetes, using of drug, hypothyroidism and so on. This type of hyperlipidemia often mimic the primary form and may have very similar consequences. The fact that hyperlipidemia is such a big health concern is that it is very common in population and it is a strong risk factor for cardiovascular diseases due to its influences on atherosclerosis. However hyperlipidemia as a CVD risk factor is modifiable, thus dietary modification is often the initial approach for treatment of certain types of hyperlipidemia.

1.2.2.4 Pharmacutical effect of TG on intestinal epithelial cells

The intestine is the critical site for dietary lipid digestion and absorption. Hydrolyzed lipid products were uptake by enterocytes through passive diffusion or facilitated by transporters. Enterocytes have the capability of eliminate free fatty acid toxicity in cytosol by quickly synthesize TGs and for its secretion later in the form of chylomicron. However, to what extent this process is controlled have not been fully explored. It is believed that intestine is not just an absorption organ, it is also able to modulate lipoprotein production in response to various stimuli and signaling pathways to control the amount of lipids that enter the body.[72] Recent studies suggested intestine as an insulin sensitive organ, and dysregulation of insulin

signaling in intestine resulted in enhance lipogenesis and lipoprotein synthesis, which might be key mechanism for atherogenic dyslipidemia in patients with metabolic syndrome.[73] Beside as a digestive organ, the intestine also harbors an ecosystem along its length, it is the first barrier for bacteria and antigen infiltration and important party of host defense of our body. The excess amount of dietary lipids present in the intestine lumen can very well be served as an external stimuli that influence the physiology and functionality of the intestine. Perfusion of emulsified lipids but not casein or glucose resulted in dose-dependent disruption of the epithelial monolayer, the damage can be reversed acutely after stimuli withdraw followed by saline perfusion.[74] This suggest that the intestinal epithelium is constantly injured and restitutes during the normal digestion and absorption of a meal. However this balanced process can sometime be disrupted and lead to unfavorable consequences. For instance, excessive amount of fat in complex high fat (HF) diet induces low grade endotoxiemia in parallel with increased intestinal goblet cell number in experimental mice model.[75] In colonic adenoma rat model, chronic HF feeding promote formation of colonic adenoma through inflammation associated with increased circulating TNF-α.[76] Interestingly, the types of TGs present in diet also matters. Diet reach in MCT have been indicated to suppress intestinal mucosa atrophy in septic rats,[77] decrease gut injuries and mortality in rats exposed to LPS.[78] MCT can also blunt ethanol effect to increase gut permeability in experimental mice model.[79] Besides the matter of chain length, diet reach in saturated fat is linked to various chronic disease, including inflammatory bow diseases as well as CVDs, which may attributed to the ability of saturated fat to induce ER stress and macrophage activation result in foam cell formation and tissue inflammation.[80]

1.2.2.5 Dietary fatty acids and allergy

Currently, most of the studies on the relationship between dietary fatty acids and allergy focus on the effect of polyunsaturated fatty acids. Fish oil supplementation during

pregnancy and lactation have demonstrated the benefits of omega-3 PUFAs to the offspring which is associated with immunologic changes[81-83]. These studies indicated the clinical effects of early fish oil provision including reduced sensitization to food allergens and reduced severity and prevalence of atopic dermatitis in the first year. In Australia, a study on around 700 infants proved that high-dose (900mg/day) omega-3 PUFAs intake during pregnancy did not reduce the IgE associated food allergy in the first 12 months of life, although omega-3 PUFAs supplementation lowered egg sensitization[84]. Other studies showed that fish oil supplementation during infancy or childhood resulted in higher omega-3 PUFAs in infants and children and may be associated with immunologic changes in the blood[85-87]. However, the clinical implication of these studies are still not clear.

Western diet is consisted of relative balanced ratio of omega-3 and omega-6 PUFAs that was predominantly rich in omega-6 PUFAs, which may suggest a possible cause of high prevalence of allergic diseases in the developed countries[88]. Predisposition to allergy disease is due to insufficiently balanced types of T help cell, type 1 and type 2 (Th1 and Th2) during fetal life[89]. High levels of dietary omega-6 PUFAs has been shown to promote Th2 differentiation of immune system during ontogeny and development[88]. In contrast, omega-3 PUFAs may shift T helper cell balance by inhibiting production of IL-13, a cytokine related to the induction of allergic disease through inducing IgE synthesis in B cells and Th2 differentiation in T cells[90]. Therefore, it is very likely that diets high in omega-3 PUFAs may regulate development of IgE mediated allergy diseases and the related immune responses[84].

There are very few studies focus on effects of other types of FA on allergy disease such as saturated FAs, long-chain vs. medium chain FAs or trans-FAs. Since the other types of fatty acids comprised the majorities of dietary fats or very hard to avoid, it may be meaningful and more relevant to determine the roles of other fatty acids on food allergy.

1.3 Reverse Cholesterol Transport

1.3.1 Prevalence, pathogenesis and therapeutic strategies of atherosclerosis and the related heart disease

In 2011, the mortality of CVD in the U.S. was 229.6 per 100,000 people, indicating more than 2150 Americans die of CVD each day and 1 death every 40 seconds on average[91]. Among these people, about 155,000 Americans who died of CVD were less than 65 years old. The majority of the lethal CVDs are associated with and caused by atherosclerosis[91](Figure 3), a disease of vascular intima, in which the whole vascular system is involved from aorta to coronary arteries and is characterized by intimal plaque[92].

Atherosclerosis is processed from lesion formation and luminal narrowing of arteries, which give rise to other diseases like cerebrovascular disease coronary artery disease (CAD). The plaque rupture and thrombosis are the signs of acute coronary syndrome (ACS), myocardial infection or stroke. The pathology is characterized by a chronic inflammatory process of a certain site of arterial wall such as branch points, which affect the normal blood flow[93]. The inflammation initiates from endothelial structural alteration and dysfunction which allow accumulation of low density lipoprotein (LDL) in endothelial cells. High levels of apolipoprotein B100 (apoB100)-containing LDL-derived cholesterol is positively correlated with atherosclerosis and cardiovascular disease[94]. Oxidized LDL (oxLDL) and lipids induce the adhesion molecules expression and chemokines secretions in endothelial cells, which initiate intimal immune cells infiltration together with deposition of platelet-derived chemokines[95]. Early lesions comprised of T cells and monocyte-derived macrophage-like lipid loading foam cells. On the site, apoptotic cells, debris and cholesterol crystals accumulation form the necrotic core. Fibroatheromatous plaques are comprised of collagen and smooth muscle cells (SMCs), which will be replaced by macrophages in the thinning inflamed area and induce rupture. The middle area are rich in

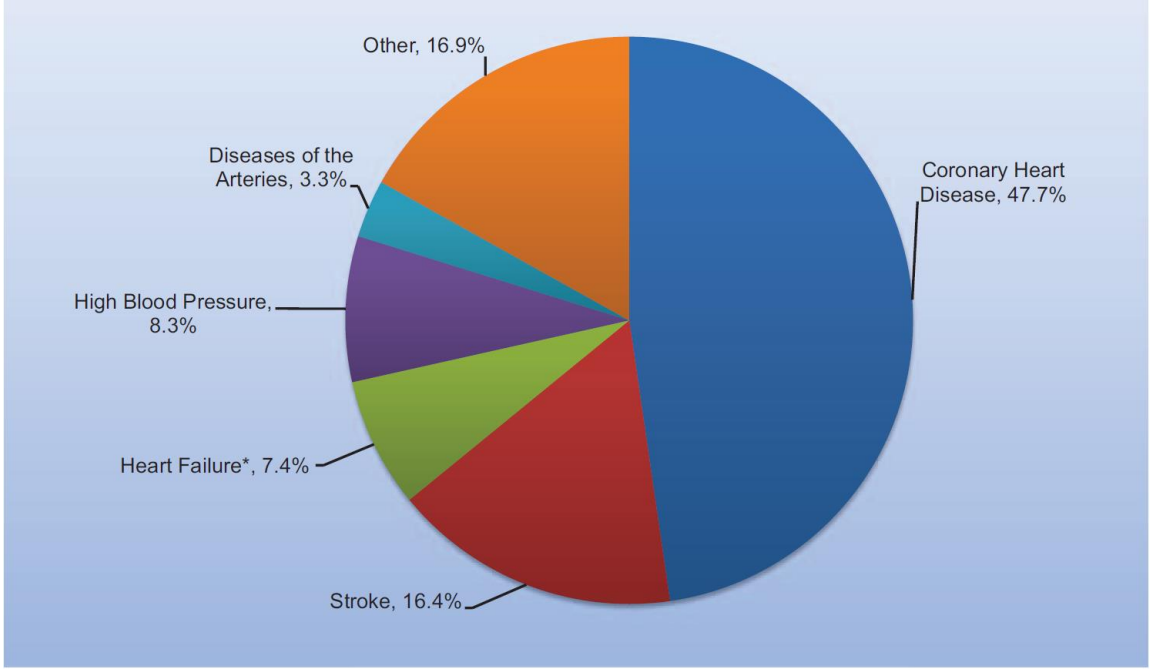

Figure 1.3. Percentage breakdown of deaths attributable to cardiovascular disease (United States: 2011). [91]

infiltrated T cells and mast cells that lead to adventitial inflammation of advanced plaques by producing proinflammaory mediators and enzymes[96].

The healthcare systems are heavily burdened by the high incidence of atherosclerosis-related cardiovascular disease. Current available therapeutics on patients largely focus on hypertension, hyperlipidemia alleviation and enhancing reverse cholesterol transport (RCT) from lipid-laden macrophages[95]. To achieve this purpose, the most established approach includes statin treatment, which yields a pronounced risk reduction due to the effect on LDL-derived cholesterol lowering. Also, statins exerts anti-inflammatory functions, improve endothelial functions and plaque lipids reductions, which limits and stabilize atherosclerotic plaques[97]. High dose of atorvastatin decreased high-sensitivity C-reactive protein (hs-CRP) levels in serum and regression of atheromas by lowering LDLc aggressively[98]. Aggressive statin therapy also decreased the primary endpoints such as myocardial infarction, benefiting the patients with LDLc lowering and CRP both[94]. However, according to the most recent statistics report of Management of Atherosclerotic Cardiovascular Disease Risk, the CVD-associated mortality and morbidity were not reduced prominently with LDLc lowering with statin therapy[99]. Besides LDLc lowering, HDLc elevating therapy had become the promising therapeutic approach due to the role of more relevant predictor of HDLc than LDLc for CVD in a great amount population studies[100] and their significant roles in RCT by using Niacin or CETP inhibitor. Cholesteryl ester transfer protein (CETP) is a protein in plasma that exchange of cholesteryl ester in HDL for triglyceride in larger density lipoproteins like LDL or VLDL. Therefore, CETP is always considered potential atherogenic[101-103] and CETP inhibitor treatment was found to reduce atherosclerosis prominently in animal study[104]. However, besides aldosterone-related adverse effects[105], many clinical trials of niacin[106] and cholesteryl ester transfer protein (CETP) inhibitor Dalcetrapic[107] have shown the independence of high HDLc levels to the risk of

CVD in human beings. The 2013 American College of Cardiology ad American Heart Association cholesterol guidelines made no specific recommendation for targeting HDL-C in therapy of prevention or treatment of atherosclerosis associated CVD due to the lack of convincing data[108]. It was showed that artificial HDL-like apolipoprotein A1 complexes (apoA1-Milano) is promising in CVD regression[109] by their abilities in binding oxidized lipids and anti-inflammatory functions[110]. Likewise, these emerging therapeutic strategies ongoing focus on the mimic the structure of natural HDL or replicate their functions such as RCT, vasodilation, anti-inflammation, and inhibition of platelet aggregation although they still confront with translational challenges[111].

Although many studies and clinical trials failed to confirm the protective role and the positive correlation of HDLc to atherosclerosis associated CVD, it is still well accepted that enhancing process of RCT can prevent or may regress the disease.

1.3.2 Reverse Cholesterol Transport (RCT)

Cholesterol can leave the body as neutral sterols (original cholesterol and its metabolized products by intestinal bacteria) or bile acids. RCT is to classically define the process of cholesterol metabolism from cholesterol efflux from peripheral tissues or macrophages to final excretion into feces either as neutral sterol or bile acids[112-114].

Hepatobiliary RCT has been considered the main pathway for cholesterol elimination for decades. Liver plays a significant role in cholesterol metabolism and cholesterol homeostasis. In the hepatocytes, cholesterol from source of diet or synthesis within liver or intestine is processed into apoB-containing lipoproteins and secreted back to the circulation basolaterally as the supply cholesterol to peripheral tissues[113]. Under certain circumstances, the chemically modified lipoproteins are taken up by macrophages which then result in foam cell formation[115] which are atherogenic as mentioned above. Cholesterol can be effluxed from

macrophages as free cholesterol via either ATP binding cassette transporter A1 (ABCA1) with lipidated apoA-I or ABCG1 with more mature HDL as acceptors[114, 116]. Scavenger receptor class B type 1(SR-BI) and aqueous diffusion may be responsible for other cholesterol efflux capacity[114, 116]. After uptake by HDL, cholesterol is esterified by lecithin-cholesterol acyltransferase (LCAT) thus sparing some space on the surface of HDL for additional free cholesterol[117]. Cholesterol is transported back to the liver through plasma compartments. Hepatocytes uptake HDL selectively by SR-B1 or via a not fully-understand pathway[112]. However, some studies showed that mice with extremely low circulating levels of HDL (apoA-I or ABCA1 deficient mice) have normal biliary and fecal cholesterol excretion, indicating that the levels of circulating HDL cholesterol contribute little to the amount of cholesterol leaving body via bile or feces[118-121]. After uptake by hepatocytes, cholesterol is de-esterified and secreted into bile via ABCG5/G8[22], a heterodimer transporter expressed in liver and intestine in the form of free cholesterol, or through ABCB11, a bile acids transporter in the form of bile acids[122]. Recent studies showed that in mice with altered expression of Niemann-Pick C2 (NPC2, a protein involved in cholesterol intracellular trafficking) indicated that NPC2 might positively correlated with biliary cholesterol secretion[123]. This result was confirmed by the relationship between NPC2 protein levels and human biliary cholesterol concentration. Secreted NPC2 protein can stimulate ABCG5/G8 mediated cholesterol transport which can be explained that by binding to cholesterol, NPC2 can accelerate the transfer of cholesterol to micelles through ABCG5/G8[123]. In humans, rabbits, hamsters and many other species with expression of CETP, cholesterol ester can be transferred to larger size lipoproteins from HDL. Hence cholesterol will be either go back to forward cholesterol transport pathway or to the liver through apoB-containing lipoprotein receptors, indicating that LDL receptor might also be

involved in RCT[124]. However, the role of apoB-containing lipoprotein pathway in humans is far unclear. Finally, bile is secreted into intestinal lumen for fecal loss or reabsorption.

Collectively, the studies in the past decades provide enough evidences that RCT needs to redefinition[125]. Elimination of cholesterol may not only be through hepatobiliary pathways, but via an alternative pathway, Transintestinal Cholesterol Excretion (TICE).

1.3.3 Transintestinal Cholesterol Excretion (TICE)

1.3.3.1 Definition of TICE

Part of dietary and synthesized cholesterol is absorbed and transported back to circulation and the remaining will be excreted as neutral sterol or bile acids. Like mentioned above, ABCG5/G8 transporters mediate the process of cholesterol efflux to bile and hepatobiliary cholesterol would be dramatically eliminated when this transporter is absent[126]. Hence, inhibition of ABCG5/G8 should lead to a drastic lowering of fecal neutral sterol loss. Surprisingly, ABCG5/G8 double KO mice with diminished hepatobiliary cholesterol transport did not show the expected low levels of fecal neutral sterols[22]. Similar result was observed in ABCB4$^{-/-}$ mice. ABCB4 is a phospholipid transporter which located at the canalicular membrane of the hepatocytes. Biliary phospholipid is almost undetectable in ABCB4-/- mice and biliary cholesterol is absent in these mice as well due to lack of efficient cholesterol acceptor[127]. Interestingly, certain amount of intravenously injected radiolabeled cholesterol was recovered in feces of ABCB4-/- mice[128]. Niemann-Pick C1-like 1 (NPC1L1) is the protein expressed mainly in intestine which is required for cholesterol absorption. Hepatic NPC1L1 localizes to the hepatocytes canalicular membrane and allows retention of biliary cholesterol so that mice overexpress NPC1L1 have abrogated biliary cholesterol. However, the fecal neutral sterol levels were very well maintained compared to WT mice[129]. These findings demonstrated that hepatobiliary cholesterol excretion pathway is be the only route for cholesterol elimination.

With disturbed biliary cholesterol secretion, there must be an alternative direct pathway for cholesterol excretion- Transintestinal Cholesterol Excretion (TICE).

The existence of TICE has been proposed about one century ago. As early as 1927, Sperry reported a surprising finding that dogs with bile fistula excreted much more cholesterol into feces than control dogs (Sperry WM. Lipid excretion IV. A study of the relationship of the bile to the fecal lipids with special reference to certain problems of sterol metabolism. J Biol Chem 1927; 1:351–378.). This result was confirmed about half a century late by Pertsemlidis et al. with the similar dog model[130]. Similarly, nondietary originated sterol present in the feces of patients with biliary obstruction[131]. However, these findings did not draw much attention because these models had incomplete enterohepatic cycle of biliary components. In particular, absence of bile acids would affect cholesterol absorption and consequently intestinal cholesterol synthesis. This is probably why these observations have been unnoticed for a long time in the literature. In conclusion, TICE can be defined as a process of cholesterol efflux directly from blood to intestinal lumen for elimination bypassing hepatobiliary pathways.

1.3.3.2 Cholesterol origin of TICE

TICE was directed measured in intestinal perfusion studies in which bile was diverted and bile duct was ligated. Van der Velde et al. proved that TICE occurs primarily and quantitatively most at the proximal part of intestine although the rest part of intestinal also excrete cholesterol to some extent.

It is worth noting that intestinal cell cholesterol synthesis does not account for the secreted cholesterol and TICE route plays an even more important role than hepatobiliary route[132, 133]. Van der Veen et al. demonstrated this by quantification of cholesterol efflux from bile and plasma to feces by using isotope methodology in vivo[133]. The increased fecal

neutral sterol loss of these animals could not be explained by increased fecal loss of de novo synthesized cholesterol but could be directly from blood via intestinal mucosa[133]. However, which lipoproteins, if any, contribute to this route is currently unknown.

1.3.3.2 Transport of cholesterol from blood to intestine

TICE is a specific process for cholesterol elimination from the body and may involve transport protein activity from both basolateral and apical surface of enterocytes.

SR-B1 is known very well as an acceptor for cholesterol uptake from lipoproteins and HDL, in particular, appears to be the more effective ligand[134, 135]. SR-B1 localizes both basolateral and apical membranes in Caco-2 cells, suggesting a potential involvement in intestinal lipoprotein uptake[136]. Mice fed with Western-diet or a high fat diet have upregulated TICE rates, in which intestinal SR-B1 mRNA levels and protein levels are positively correlated with TICE[137]. Unexpectedly, SR-B1 deficient mice have significantly increased TICE compared to controls[137]. In addition, mice overexpressed SR-B1 specifically in small intestine have increased TICE compared to WT mice when treated with ezetimibe to block cholesterol absorption. However, these mice have similar fecal neutral sterol levels to their WT littermates when treated with ezetimibe[138]. When crossed with NPC1L1LiverTg mice, which have increased TICE, these double transgenic mice had similar biliary cholesterol concentration, cholesterol absorption and fecal neutral sterol levels[138]. From these findings, it can be concluded that SR-B1 overexpression is not positively correlated with increased TICE. Furthermore, TICE may not be stimulated by increasing SR-B1 in the gut. Thus, the role of HDL as cholesterol donor for intestine remains questionable.

Another potential efficient lipoprotein receptor is LDLr due to the fact that LDL may also involve in RCT[124]. In a recent study, Le May's group found that in the mice that LDLr expression was conditionally deficient by continues injection of PCSK9, TICE was accordingly

decreased. Similarly, when LDLr expression was conditionally increased by PCSK9 deficiency, reduced TICE was detected consistently. Surprisingly, they also detected an increased TICE in LDLr-/- mice[139]. Moreover, LXR activation was shown to increase TICE significantly, yet the intestinal LDLr protein levels were nearly abrogated due to the E3 ubiquitin ligase Idol action[128, 133]. These results indicate that the role of LDLr and probably LDL in stimulating TICE may need further studies.

As to the lipoprotein donor for TICE, it had been previously reported that whole body ABCA1-/- mice which have abrogated circulating HDL levels maintain normal biliary and fecal cholesterol levels[120, 121]. Recently, Vrins and colleagues also showed that ABCA1-/- mice have unchanged TICE compared with WT littermates[140]. Current data suggest that raising HDL may not be a potential therapeutic target to increase TICE. In contrast, apoB-containing lipoproteins seem to enhance TICE. It has been reported that mice with liver specific knockdown of acyl-CoA:cholesterol acyltransferase (ACAT2) have normal biliary cholesterol secretion but increased fecal neutral sterol excretion, indicating a potential contribution to TICE[141]. In the same study, this liver specific ACAT2 knockdown mice accumulated a large amount of cholesterol originating from nascent VLDL in the small intestine compared to control mice[141]. In a follow-up study, ACAT2 liver specific knockdown mice fed with cholesterol containing diet had rapidly increased fecal neutral sterol levels accompanied with accordingly increased circulating apoB lipoproteins, but had no change on biliary cholesterol levels[142]. Microsomal triglyceride transfer protein (MTP) is required for apoB lipoprotein secretion. Hepatic knockdown of MTP in mice would decrease hepatic triglyceride secretion yet the biliary cholesterol levels was not affected[143]. However, fecal neutral sterol levels was significantly decrease in MTP and NPC1L1 liver specific knockout mice[143]. In conclusion, these results indicate that VLDL secretion is necessary for TICE.

Currently many questions related to the intestinal receptors and cholesterol donors involved in TICE remain unanswered. Defining the TICE lipoprotein donor and intestinal receptor systems has potential therapeutic significance.

1.3.3.3 Transport of cholesterol from enterocyte to intestinal lumen

In the process of cholesterol transport from enterocyte to intestinal lumen, cholesterol efflux protein ABCG5/G8 are thought to be the good candidate mediating cholesterol efflux. Transgenic mice overexpressing ABCG5/G8 had significantly increased fecal neutral sterol levels[23]. However, this increased excretion was inhibited by Mdr2 deficiency in the transgenic mice, suggesting that biliary cholesterol secretion contribute mostly in their fecal neutral sterol loss[144]. Moreover, ABCG5 and/or ABCG8 deficiency leads to mild[22, 145] or no[21] decreased fecal neutral sterol levels. Besides, both mRNA and protein levels of ABCG5/8 were not relevant to TICE rate in the same intestinal segments. In addition, with intestinal perfusion tool, TICE was directly measured in ABCG8-/- mice and found no differences compared to control mice[132]. Wang and colleagues recently highlighted the possible role of intestinal ABCG5/G8 in TICE. In the study, to determine the role of hepatic and intestinal ABCG5/G8 in RCT, liver (L-G5G8-/-), intestinal (I-G5G8-/-) and whole body (G5G8-/-) knockout mice were injected with 3H-cholesterol and they found that the percentage of 3H-cholesterol was in found highest in the WT and I-G5G8-/- mice, then the L-G5G8-/-, G5G8-/- had the least. Compared to WT mice, I-G5G8-/- mice had decreased fecal 3H-cholesterol excretion which was explained by reduced TICE[146]. Collectively, ABCG5/G8 are very likely to contribute to TICE particularly intestinal expressions. But their roles seem to strongly depend on the conditions and need more elucidation.

ABCB1 is a multidrug transporter, locating at apical surface of enterocytes, which can pump cholesterol as a cholesterol floppase[147]. Mice with deficiency of ABCB1 for both

isoforms spontaneously develop hepatic steatosis, obesity, diabetes mellitus and increase HDL-C. Le May and colleagues reported that the mice with deficiency of ABCB1 had about 26.5% decreased TICE compared to WT mice, indicating a contributive role of ABCB1 in TICE[139].

1.3.3.4 Regulation of TICE

Although the mechanisms for TICE have not been clear yet, it is found that TICE can be stimulated or inhibited by some factors.

Van der Velde and the colleagues found that TICE increased two-fold in mice receiving Western type or a high fat diet. This results were positively correlated with the increased fecal neutral sterol levels in these mice as well. However, high cholesterol diet did not upregulate TICE as the other two types of diets. The authors concluded from these results that TICE rate is not dependent on cholesterol content in the diet but only fat content[137]. The increased fecal neutral sterol levels in mice when fed with high fat diet were demonstrated by de Vogel-van der Bosch at el, whereas all known cholesterol transporters expressions were decreased in these mice[148]. According to the authors, this results could not be explained by increasing TICE rather than decreasing cholesterol absorption. Connor and colleagues recruited male subjects with characteristic serum cholesterol concentrations without known metabolic disorders to investigate the effect of dietary fat on fecal neutral sterol levels. They found that poly-unsaturated fats affect RCT by increasing fecal neutral sterol levels two-fold higher than cholesterol calculated to leave plasma[149]. This results were confirmed later by Nestel[150] 38 and Oh and Monaco39. Since polyunsaturated fatty acids are natural ligands for peroxisome proliferator-activated receptors (PPARs), their findings may explain the study that PPARδ activation in mice led to increased TICE[151].

Recently, two studies reported if ezetimibe, a clinically approved drug used to treat hypercholesterolemia through blocking NPC1L1 to decrease cholesterol absorption, increases

RCT through TICE pathway. Uto-Kondo et al. and colleagues found that Ezetimibe increased fecal macrophage-derived 3H-neutral sterol levels in bile duct-ligated hamsters[152]. However, this increase is not as significant as in sham-operated hamster. The authors concluded that ezetimibe can stimulate TICE and the effect will be maximized by the existence of hepatobiliary cholesterol secretion120. Overall, it can be concluded that fat content in the diet can increase TICE. Furthermore, blocking cholesterol absorption by ezetimibe can promote TICE but is likely to require hepatobiliary cholesterol secretion pathway to maximize the effects.

Plosch and colleagues showed that liver X receptor (LXR) activation led to increase fecal neutral sterol excretion, which could not only be attributable to hepatobiliary cholesterol secretion. This result suggested a potential contribution in intestine to total cholesterol elimination[153]. This result was demonstrated by Kruit and colleagues who showed that LXR activation induced increased fecal neutral sterol loss was independent of biliary cholesterol secretion[128]. With a kinetic approach to measure cholesterol efflux rates, Van der Veen et al. reported that LXR activation stimulated TICE as well[133]. Collectively, these findings suggest that LXR is involved in TICE.

It was reported that the intracellular vesicle transport proteins, Rab9 and LIMPII were upregulated when TICE was enhanced[151]. However, to determine their role in TICE, more studies with intestinal specific knockout mice for these proteins are required.

In conclusion, there are many factors that can significantly stimulate TICE, suggesting a promising approach to consider TICE acceleration as a potential effective therapy particularly in patients with deficient biliary cholesterol excretion.

1.3.3.5 TICE in humans

Early in 1959, fecal sterols from nondietary cholesterol origin was found in feces of patients with biliary obstruction[131], which may be the first evidence suggesting the existence

of TICE in human. However, it remains unclear if TICE presents in normal healthy conditions and whether it can be stimulated. In 1967. Simmonds et al. performed intestinal perfusions in humans and detected significant cholesterol secretion in intestine yet the amount of secreted cholesterol was not quantified[154]. Based on the average total estimated cholesterol secreted from body per day (1g)[155, 156], average daily cholesterol intake amount (400 mg)[157] biliary cholesterol secretion amount (1000mg)[158] and cholesterol absorption rate (50%)[155], Van der Velde and colleagues calculated the possible amount of cholesterol secreted from intestine in human per day[159] which is about 300mg/day. Given 70kg body weight, this amount is about one-third of the biliary cholesterol secretion amount. However, studies on quantification of this amount need to be performed in humans.

If this data can be confirmed, stimulation of TICE in human may suggest a new therapeutic approach to reduce hypercholesterolemia especially in the patients with deficient biliary cholesterol secretion. TICE has never been tested to predict plasma cholesterol. The measurement of cholesterol kinetics in humans by stable isotype studies may provide new view of regulation of plasma cholesterol, which will also provide foundation of new therapeutic strategies to treat hypercholesterolemia and atherosclerosis associated CVD in humans.

CHAPTER II. Dietary medium-chain triglycerides promote oral allergic sensitization and orally induced anaphylaxis to peanut protein in mice.

2.1 Introduction

Peanut allergy affects about 2% of the Western population, and its prevalence is rising [160-163]. The condition is rarely outgrown and there is no cure. To stem the rise of food allergies, it is important to unravel mechanisms that lead to allergic sensitization. Peanuts and many allergenic foods contain significant amounts of triglycerides ("fat") or are most likely ingested with a fat-rich meal, especially in Western societies, which are also more affected by food allergies. However, little is known about the effect of dietary fat in allergic sensitization or immune responses to dietary proteins.

Recent work has demonstrated that the intestinal epithelium plays a key role in immune responses to dietary antigens. Intestinal epithelial cells control access of luminal antigenic material to the lamina propria and beyond, and it has been suggested that increased intestinal permeability could be a risk factor for allergic sensitization [164, 165]. On the other hand, properly controlled intestinal absorption of small amounts of dietary antigen may protect against food allergies by promoting oral tolerance [166]. However, the mechanisms involved in soluble antigen absorption are poorly understood.

Fatty acids, released in significant amounts from dietary triglycerides in the upper gastro-intestinal tract, have potent detergent properties and may induce transient mucosal damage and gut leakiness [167] which could enable translocation of dietary antigen. The type of dietary fat might determine antigen absorption. We recently observed that dietary long-chain triglycerides (LCT), which contain fatty acids that have more than 12 C-atoms and are absorbed via mesenteric lymph as part of chylomicron particles, promoted the absorption of the dietary

antigen ovalbumin (OVA) into lymph and blood [168]. In contrast, dietary medium-chain triglycerides (MCT; fatty acids with 12 or fewer C-atoms), which are absorbed via portal blood, promoted less antigen absorption [168]. This would suggest that MCT should differ from LCT in their effects on allergic sensitization and anaphylaxis.

Fatty acids of different chain length may also differ in pharmacological properties, especially in their effects on the intestinal epithelium with which they interact in large numbers. Intestinal epithelia not only control antigen absorption, but also secrete factors that significantly affect nearby immune cells. For example, the intestinal epithelial cytokine TSLP (Thymic Stromal Lymphopoietin) promotes the induction of Th2- responses through multiple mechanisms [169]. Interestingly, TSLP has been implicated in allergic diseases, including experimental food allergy [170]. Other Th2-biasing cytokines, such as IL-25 (IL-17E) and IL-33, are also expressed in the intestinal epithelium, and support Th2-mediated expulsion of parasitic worms[171, 172]. Fatty acids are known to affect intestinal epithelial cytokine expression [173, 174], although it is unclear how epithelial cytokines relevant to food allergy are regulated.

Based on these putative immune-modulating effects of dietary fat, we evaluated how dietary fats affect oral sensitization in naïve mice and immune responses to oral antigen challenges in sensitized mice. We decided to compare LCT with MCT, based on their different effects on OVA absorption. As model system for oral sensitization we slightly modified a recently reported, adjuvant-free model[175]. This model and a classical systemic sensitization model were also used to test the effect of triglycerides during oral antigen challenges. To acutely block chylomicron formation during LCT feeding, a small amount of the chylomicron secretion inhibitor PL81 was added in some experiments[168, 176, 177].

Interestingly, when we replaced LCT in peanut butter with MCT or gavaged OVA with MCT, we observed marked allergic sensitization, which was associated with a significant induction of intestinal epithelial Th2 cytokine expression in the jejunum, a reduction of antigen absorption into blood, and an increase in antigen absorption into Peyer's patches. LCT did not induce sensitization unless PL81 was added. Moreover, our data suggest that chylomicron formation during antigen ingestion may promote oral tolerance and may protect against allergic sensitization.

2.2 Methods

2.2.1 Animals

Experiments with peanut protein were performed with male C3H/HeJ mice. Experiments with OVA used male C3H/HeJ mice, female BALB/c mice and DO11.10 mice (BALB/c background) of both genders, and female Sprague-Dawley (SD) rats. Approximately 70% of CD4 T-cells of the transgenic DO11.10 strain express a T-cell receptor for an OVA peptide (residues 323-339) [178]. The mice, ordered at 5 weeks of age from The Jackson Laboratory, were housed three per cage in a room of a specific pathogen-free animal facility with a 12h light / dark cycle, and were used at 6 weeks of age. Rats were purchased from Taconic. Unless indicated otherwise, the animals received standard rodent diets and filtered tap water ad libitum. All animals were handled in strict accordance with good animal practice as defined by the relevant national and local animal welfare bodies, and all animal work was approved by the Institutional Animal Care and Use Committee of the University of Kentucky (Animal Welfare Assurance Number of the University of Kentucky A3336-01; U.K. IACUC protocol 2008-0306).

2.2.2 Gavage suspensions and diets

Gavages: Jif-brand peanut butter or grade V OVA (Sigma Aldrich Corp.) were used for gavage studies. Peanut butter was diluted with two volumes of MCT oil (distilled coconut oil, Nestle Nutrition Corp.) and passed through a sieve with a 70 µm pore size to prevent clogging of the feeding tube. The filtrate was centrifuged (4 minutes; 16,800xg) and the supernatant replaced with triglycerides or water to reconstitute the initial volume of peanut butter. Triglycerides consisted of MCT oil or LCT (food grade peanut oil; "Hollywood" brand). In some cases, PL81 was added at 3% by volume to LCT to block chylomicron secretion into lymph [168, 176, 177]. We found this quantity to completely block chylomicron formation without toxic side effects [168, 177].

Diets: All diets were from Harlan Teklad Corp. Their standard rodent diet, with casein as protein source and soybean oil as fat source, was considered the LCT diet. The MCT diet differed only in fat source (partially hydrogenated coconut oil instead of soybean oil). In OVA studies, 10% of casein was replaced with egg-white solids (Deb-El brand). In peanut feeding studies, 10% of casein was replaced with defatted peanut flour (Byrd Mill brand) and soybean oil was replaced with peanut oil.

2.2.3 Allergic sensitization

By acute feeding: Mice were fasted for 4h, then gavaged once with 0.3 ml of antigen suspensions in various vehicles (~80 mg peanut protein or 60 mg OVA). An 18G 38mm polypropylene feeding tube with rounded tip (Harvard Apparatus Corp.) was used to avoid injury. Mice were returned to standard diets and blood was drawn 18 to 21 days later for antibody measurements. In some instances (as indicated), mice received a second oral sensitization one week after the first. *By chronic feeding*: Mice were fed MCT- or LCT- based peanut or control diets for 4 weeks, with weekly blood sampling. *By systemic sensitization*: Mice received two

intraperitoneal (I.P.) injections with 10 µg OVA in 0.2 ml alum (Accurate Chemical and Scientific Corp.), with one week between injection. One week later, blood was tested for anti-OVA IgE. Rats were sensitized by a single subcutaneous injection with 0.1 mg OVA and 1 mg alum in saline and their blood was obtained two weeks later after euthanasia. Serum was also obtained from non-sensitized animals.

2.2.4 ELISA for antigen-specific IgE and IgG

To detect antigen-specific IgE and IgG, we developed an ELISA, which involved coating 96 well BD Falcon ELISA plates with 20 µg peanut butter protein or 20 µg OVA in carbonate buffer (pH 9.6). Washed plates were treated with blocking solution ("NAP blocker", GBiosciences Corp.), and mouse serum, diluted 1:500 in blocking solution for IgG measurements and 1:10 for IgE measurements, was added for 1 h at room temperature. Unbound antibody was washed with Tris-Buffered Saline containing Tween 20 (TBST). Alkaline-phosphatase conjugated rabbit anti-mouse IgG (1:5000; A1902, Sigma-Aldrich.) or goat anti-mouse IgE (1:800; SouthernBiotech Corp.) were added in blocking solution, and incubated for 60 min or longer at room temperature in complete darkness (in case of IgE sometimes several hours). After washing unbound antibody, AP substrate (femto-ELISA-AP Substrate; GBiosciences) was added and the absorption at 450 nm (A450) was read after addition of 50 µl stop solution (3 M NaOH).

2.2.5 In vitro splenocyte stimulation assays

Spleens were aseptically removed, gently minced, and passed through a 70 µm mesh into culture medium (Dulbecco's Modified Eagle's Medium:F12 (1:1; Lonza Corp.) with 10% heat-inactivated fetal calf serum (Gibco Corp.) and antibiotics (Gibco)). Cell viability was assessed with Trypan Blue staining. Splenocytes were seeded in 96-well plates at 1 x 10^6 live cells/cm^2 and kept in an incubator maintaining 100% humidity, 37°C, and 5% atmospheric CO2. Cells were

pulsed with homogenized peanut butter (50 µg protein / ml) or 1 µg / ml OVA peptide 323-339 (Invivogen Corp) or vehicle (control), and medium obtained 4 days after the pulse was analyzed for cytokine content (ELISA kits from eBiosciences).

2.2.6 In vitro basophil stimulation assays

RBL-2H3 cells, a rat leukemia line with basophil and mast-cell characteristics, were obtained from American Type Culture Collection and grown till confluency in 96 well tissue culture plates in Eagle's Medium with 10% heat-inactivated serum and antibiotics. They were exposed to serum from OVA-sensitized rats (1:1 dilution) or naïve rats for two hours, washed twice with PBS containing 0.1% BSA, then incubated with 0.1 ml of serial dilutions of OVA in PBS/BSA, for 1h. Included with the OVA was an emulsion with chylomicron-like composition and physiological properties [Robinson, 1979 #886] (Intralipid 20%; Baxter Healthcare) at 1:1000 or 1:500 dilution. One hour after the incubation, 30 µl medium was mixed with 30 µl 4-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma Aldrich; 1 mg / ml in citrate buffer, pH 5) and the coloring reaction was stopped after 1h with carbonate buffer (pH 10.5). p-Nitrophenol was subsequently quantified by measuring the optical density at 405 nm. OD405 values of OVA-free solutions were subtracted to correct for light absorption by emulsion particles. The emulsions were non-toxic at the indicated dilutions as reflected by lack of stimulation of spontaneous hexaminidase release and the cells' appearance after incubation was identical to controls (not shown).

2.2.7 In vivo antigen challenges

For systemic challenges, mice received 30 mg peanut butter protein I.P. in 0.3 ml sterile PBS[175]. Body temperature was measured telemetrically immediately before and every 5 minutes after the challenge, using subcutaneously placed micro transponders (Bio Medic Data Systems Corp.), and mast cell histamine release was estimated by measuring mouse mast-cell

protease 1 (mmcp-1) serum levels 90 minutes after the challenge [175] (eBioscience ELISA). Signs of anaphylaxis were estimated with a semi-quantitative scoring, with “0” being assigned to mice with no symptoms, “1” to mice which were stationary but moved when provoked, “2” to mice which remained immobile even when provoked, and “3” to mice lying on their side.

In some experiments, mice were injected ip 16h before the challenge with rat-monoclonal blocking antibodies against IgE (clone EM-95; 0.1 mg/mouse) or-FcγRII/III (clone 2.4G2; 0.5 mg/mouse), to determine whether anaphylaxis was IgE- or IgG- dependent. These antibodies have been described elsewhere [179, 180].

For oral challenges with peanut protein, 80 mg peanut butter protein was resuspended in 0.3 ml vehicle. For oral challenges with OVA, 50 mg OVA was suspended in 0.15 ml water and mixed with 0.15 ml triglycerides.

2.2.8 Effect of triglycerides on epithelial Th2 cytokine expression

To study acute effects of dietary fat on intestinal epithelial cytokine expression, fasted mice were gavaged with 0.3 ml MCT or LCT (with or without PL81) and jejuna were obtained 5 h later. To study chronic effects, mice were fed 3 wks with MCT or LCT diets and intestines were obtained from non-fasted mice in the morning. Epithelia were isolated by treating washed sections with 1 mM dithiothreitol and 30 mM Ethylenediaminetetraacetic acid as described elsewhere [181]. Epithelial RNA was isolated with an EZRNA kit (Omega-Biotech Corp.) and reverse-transcribed into cDNA with a Q-script kit (Quansys Corp.). The cDNA was analyzed for abundance of TSLP, IL-25 and IL-33 mRNA relative to GAPDH with primer pairs ACG GAT GGG GCT AAC TTA CAA / AGT CCT CGA TTT GCT CGA ACT (TSLP), ACA GGG ACT TGA ATC GGG TC / TGG TAA AGT GGG ACG GAG TTG (IL-25), ATT TCC CCG GCA AAG TTC AG / AAC GGA GTC TCA

TGC AGT AGA (IL-33) and CCA GGT TGT CTC CTG CGA CTT / CCT GTT GCT GTA GCC GTA TTC A (GAPDH) using a Bio-Rad CFX-96 realtime quantitative polymerase chain reaction machine.

2.2.9 Effect of triglycerides on Th2 responses

To test the effect of MCT on Th2 responses to dietary antigen, DO11.10 mice were fed egg protein-containing MCT or LCT diets (or corresponding egg-free diets) for one week and spleen cells were challenged *ex vivo* with OVA peptide or not. Cytokines in the culture supernatants were quantified by ELISA (eBioscience).

2.2.10 Effect of triglycerides on antigen absorption

Peanut butter protein was labeled with ^{125}I according to a slightly modified iodine monochloride procedure [182]. Prior to protein labeling, the peanut butter was delipidated with hexane - isopropanol (2:1), resuspended in phosphate-buffered saline (PBS), dialyzed against PBS, and concentrated with a 10 kDa ultra filter. Fasted C3H/HeJ mice were gavaged with 80 mg peanut butter protein spiked with radiolabeled protein, suspended in 0.3 ml triglycerides. Plasma ^{125}I levels 30 minutes after gavage were measured in a gamma counter. Absorption was expressed as percentage of gavaged material. Absorption of OVA was studied using DQ-OVA (Invitrogen), which only emits fluorescence when degraded in lysosomes. For this, fasted BALB/c mice received gavages of 1 mg DQ-OVA in water, MCT, LCT, or LCT + PL81, and were then deprived of food for at least another hour. The next day, single cell suspensions from mesenteric lymph nodes (MLN), Peyer's patches and spleen were stained with Alexa 647 anti-CD11c (Biolegend Corp.) and analyzed by flow cytometry (FACScalibur, Becton Dickinson corp.).

2.2.11 Statistics

Results were analyzed with Graphpad Prism version 5 and are displayed as average ± S.E.M. ANOVAs were followed by between-group post-hoc analyses (Newman-Keuls). Anaphylaxis scores were compared with Mann–Whitney U tests. Temperature data were analyzed by comparing maximum temperature drop or area under the curve. Columns in graphs that do not share letter labels differ significantly from each other ($P<0.05$). All figures show representative results of at least two repeats per experiment.

2.3 Results

2.3.1 MCT and LCT differentially affect antigen absorption and dissemination

MCT were previously found to decrease absorption of dietary OVA into blood compared to LCT [168]. To test whether this also applies to peanut protein, radiolabeled peanut protein was fed to fasted mice together with MCT, LCT, or LCT + PL81, and blood was collected 30 min later. As shown in Fig 2.1A, gavage with MCT resulted in significantly reduced antigen absorption compared with LCT. However, addition of PL81 to LCT (which trapped chylomicrons within jejunal epithelial cells; Fig 2.1B) reduced absorption to levels seen with MCT (which does not cause chylomicron release).

To further test the effect of postprandial chylomicron formation on antigen absorption, we measured DQ-OVA uptake by antigen presenting cells one day after DQ-OVA gavage in the presence of different triglycerides. Surprisingly little signal was found in the MLN of either group (<1% positive cells positive), with slightly stronger signal in the spleen (approximately 3%). However, there were no significant differences between groups for any of these sites (not shown). In contrast, a pronounced difference was observed in the percentage of DQ-OVA positive cells in the Peyer's patches among groups, with significantly more DQ-OVA-positive cells after gavage with MCT and LCT + PL81 than after gavage with water or LCT (Fig 2.1C). Thus, prevention or inhibition of chylomicron formation suppressed antigen absorption into the circulation while enhancing antigen delivery to Peyer's patches.

2.3.2 Dietary MCT promote allergic sensitization

Because MCT and LCT differed in their effects on antigen absorption, we next evaluated their effects on immune responses to dietary antigens. Strikingly, antigen-naïve male C3H/HeJ mice gavaged once with peanut protein (80 mg) in MCT produced significantly more IgE and IgG

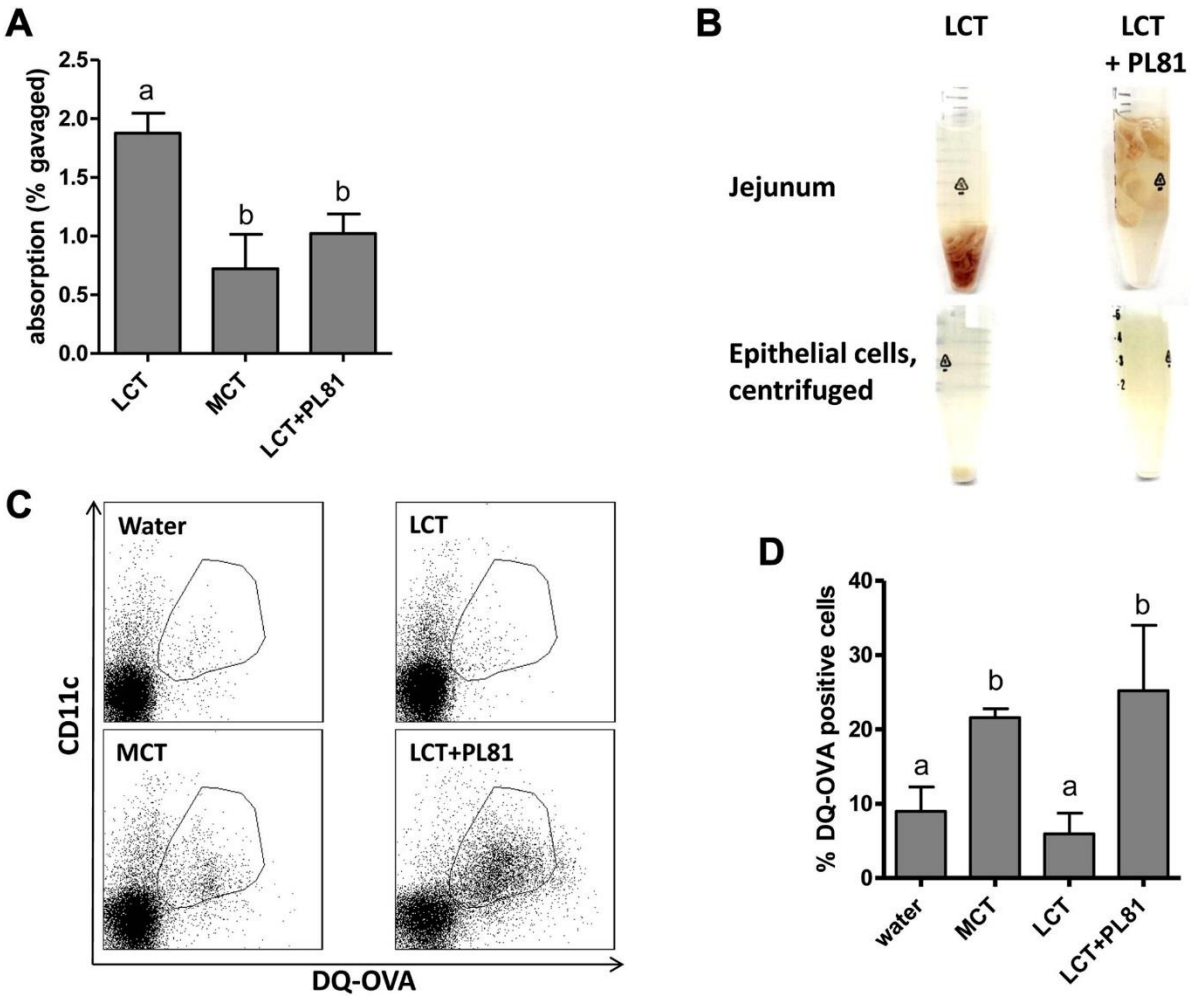

Figure 2.1 Effects of triglycerides on antigen absorption. A, Plasma iodine 125 levels 30 minutes after gavage of radiolabeled peanut protein with indicated vehicles (n = 6). B, Features of mice intestines and epithelial cells in PBS 30 minutes after gavage with indicated vehicles. PL81 blocks LCT absorption, as reflected by increased buoyancy because of lipid entrapment. Tissue was obtained 5 hours after gavage. C and D, CD11c+ and DQ-OVA+ cells in Peyer patches 16 hours after DQ-OVA gavage in indicated vehicles (n = 4).

against peanut protein than mice gavaged with the same amount of protein in LCT or water (Fig 2.2A, B). Usage of another LCT, soy oil, yielded the same results (Fig 2.2C). The protective effect of LCT was abrogated by adding PL81 (Fig 2.2A, B). In addition, splenocytes from mice gavaged with peanut protein in MCT produced more IL-13 when stimulated *in vitro* (Fig 2.2D). The failure to induce sensitization with LCT may involve induction of oral tolerance by LCT, since a single gavage of peanut protein in LCT prevented subsequent sensitization by antigen in MCT (Fig 2.2E). Interestingly, the pro-allergenic effect of MCT was not limited to peanut protein, since a single gavage of OVA (60 mg) in MCT or LCT + PL81 also resulted in increased IgE anti-OVA antibody production compared to gavage in water or LCT (Fig 2.2F).

Collectively, these data suggest that dietary MCT promotes allergic sensitization to food antigens and that chylomicrons may prevent sensitization.

2.3.3 Dietary MCT promote intestinal epithelial Th2 cytokine expression and Th2 bias

Recent reports indicated that intestinal epithelial cells can contribute to allergic sensitization by releasing cytokines that promote Th2 responses [170]. To determine whether the sensitizing effect of MCT was associated with increased TSLP expression, we measured intestinal epithelial TSLP mRNA in jejunal epithelia 5h after gavage with MCT or LCT. As shown in Fig 2.3A, mice gavaged with MCT had significantly higher TSLP mRNA in jejunal epithelial cells than mice gavaged with LCT, unless PL81 was added (Fig 2.3B).

We next tested whether chronic MCT feeding also stimulates intestinal epithelial TSLP mRNA expression. Indeed, C3H/HeJ mice fed three weeks with the MCT diet had significantly higher levels of TSLP mRNA in their duodenal and jejunal epithelia than control mice (Fig 2.4A). TSLP, which is more highly expressed in the distal gastrointestinal tract [183], was not upregulated by MCT in sites distal from fat absorption. There was also a stimulatory effect of MCT on IL-25 and

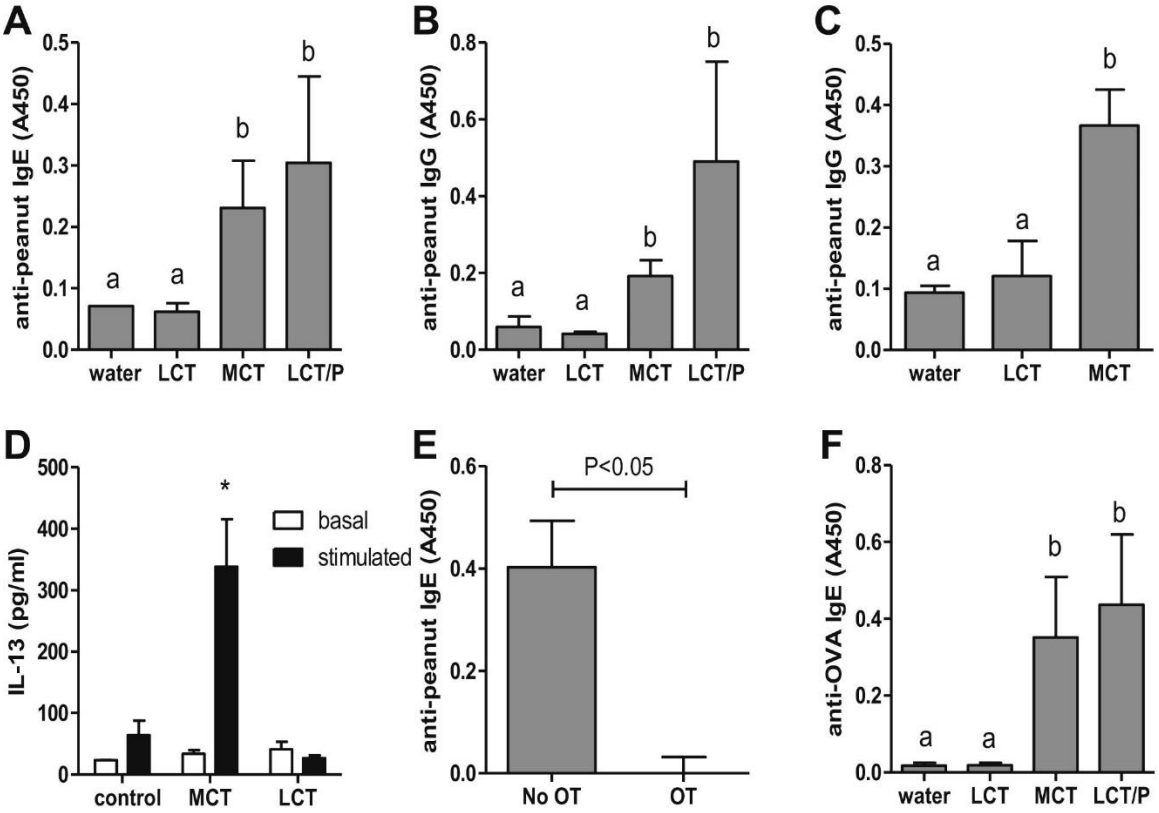

Figure 2.2. MCT causes allergic sensitization. A-C, Anti-peanut antibodies 18 days after gavage with peanut protein (80 mg) in indicated vehicles (n = 6). The LCT was peanut oil in Fig 2, A and B, and soybean oil in Fig 2, C. D, Splenocyte IL-13 release (n = 4). ∗P < .05. E, Gavage of peanut protein in the LCT (OT) 1 week before gavage with the MCT induces oral tolerance (n = 6). F, Anti-OVA IgE levels 18 days after gavage with OVA (60 mg) in indicated vehicles (n = 6).

OT Oral tolerance

LCT/P LCT plus PL-81

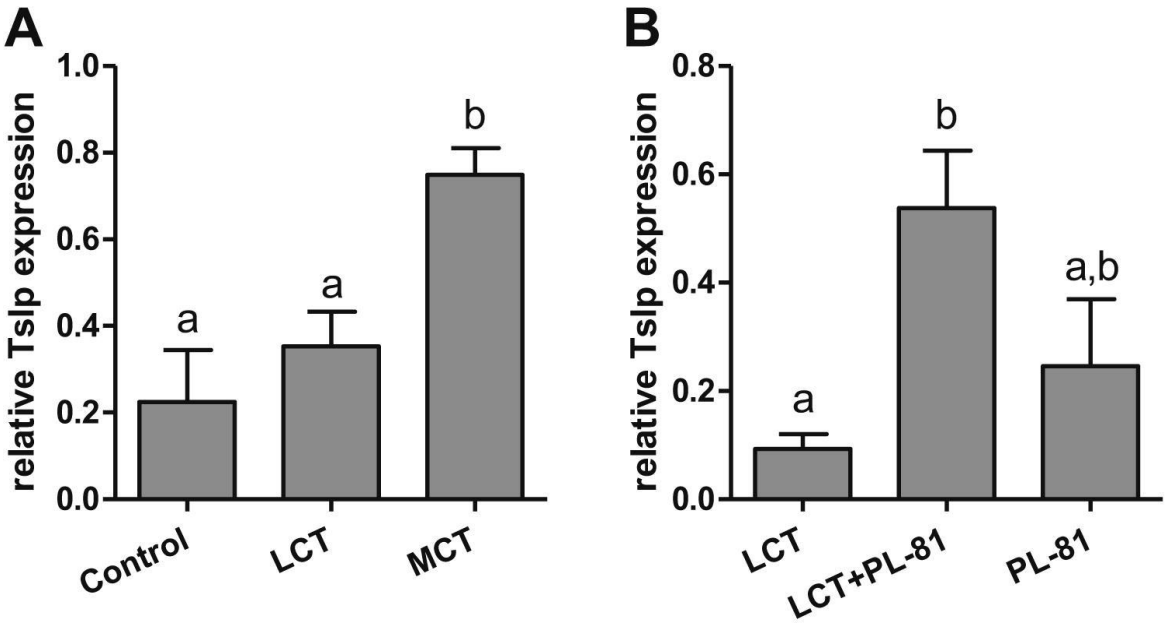

Figure 2.3. Tslp mRNA in epithelial cells of the jejunum 5 hours after gavage of 0.2 mL of the MCT, the LCT, or saline (A) or 0.2 mL of the LCT, the LCT plus PL81, or saline plus PL81 (B; n = 6).

IL-33 mRNA expression (Fig 2.4B). However, TSLP protein levels were below the level of detection by ELISA (not shown).

The sustained effect of MCT on epithelial cytokine expression prompted us to test whether addition of peanut protein to MCT diets would cause sensitization during sustained dietary exposre. Indeed, C3H/HeJ mice receiving peanut protein in the context of MCT had detectable amounts of anti-peanut IgE in their serum after 4 weeks (Fig 2.4C).Thus, dietary MCT promote allergic sensitization, which partially involves upregulation of expression of Th2-biasing cytokines in the epithelium of the jejunum. To investigate whether dietary MCT causes a general Th2 bias, we fed DO11.10 mice with MCT- or LCT- diets with or without egg-white protein for 1 week and then stimulated splenocytes *ex vivo* with OVA peptide. In the absence of OVA peptide, none of the splenocyte preparations produced detectable IL-4 or IFN-γ (Fig 2.5). In contrast, stimulation with OVA peptide resulted in significant production of these cytokines, due to the large numbers of OVA-reactive CD4 T-cells in DO11.10 mice. However, splenocytes from mice fed MCT diets with OVA produced more IL-4 and less IFN-γ than the LCT/OVA diet, suggesting that dietary MCT had indeed promoted Th2 responses to the antigen.

2.3.4 IgG-dependent anaphylaxis upon systemic antigen challenge

We next tested whether sensitized mice would develop anaphylaxis upon antigen re-exposure. Mice (C3H/HeJ) were gavaged once with peanut protein in MCT, LCT, or LCT plus PL81, then challenged I.P. 18 days later with 30 mg peanut protein in 0.2 ml PBS. As shown in Figure 6A and B, mice sensitized with peanut protein in MCT or LCT+PL81 released much more mmcp-1 into the bloodstream and showed a greater drop in body temperature than mice sensitized by gavage with peanut protein in LCT. Semi-quantitative clinical scoring of anaphylaxis (based on mouse mobility) in a separate experiment yielded negligible scores in mice sensitized with

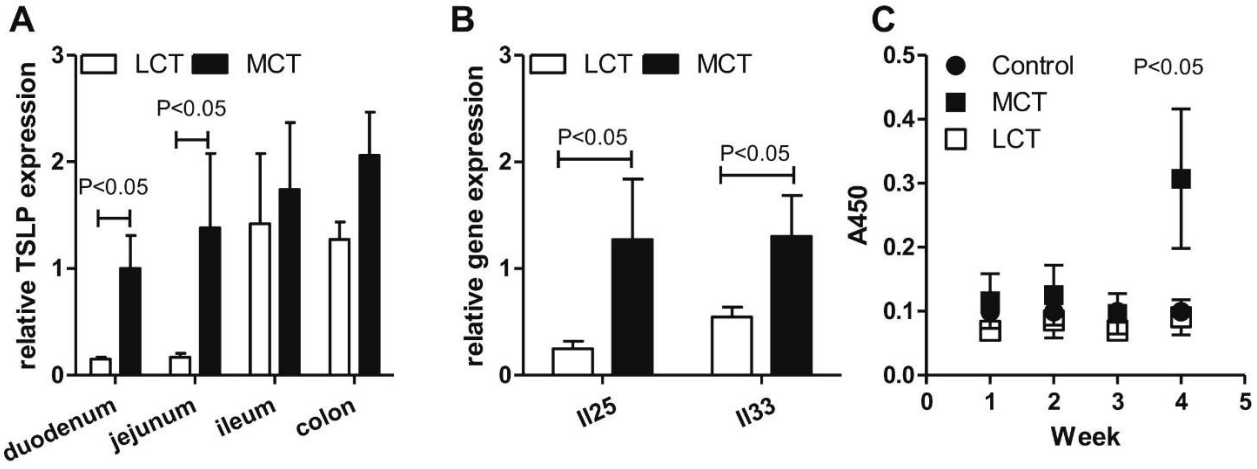

Figure 2.4. Effect of MCT feeding on intestinal epithelial TH2 cytokine expression. A, TSLP mRNA in epithelia from indicated sections 3 weeks after feeding (n = 5). **B,** IL-25 and IL-33 mRNA in jejunal epithelial cells 3 weeks after feeding (n = 5). **C,** IgE against peanut protein during 4 weeks of feeding with diets enriched with peanut flour (n = 6).

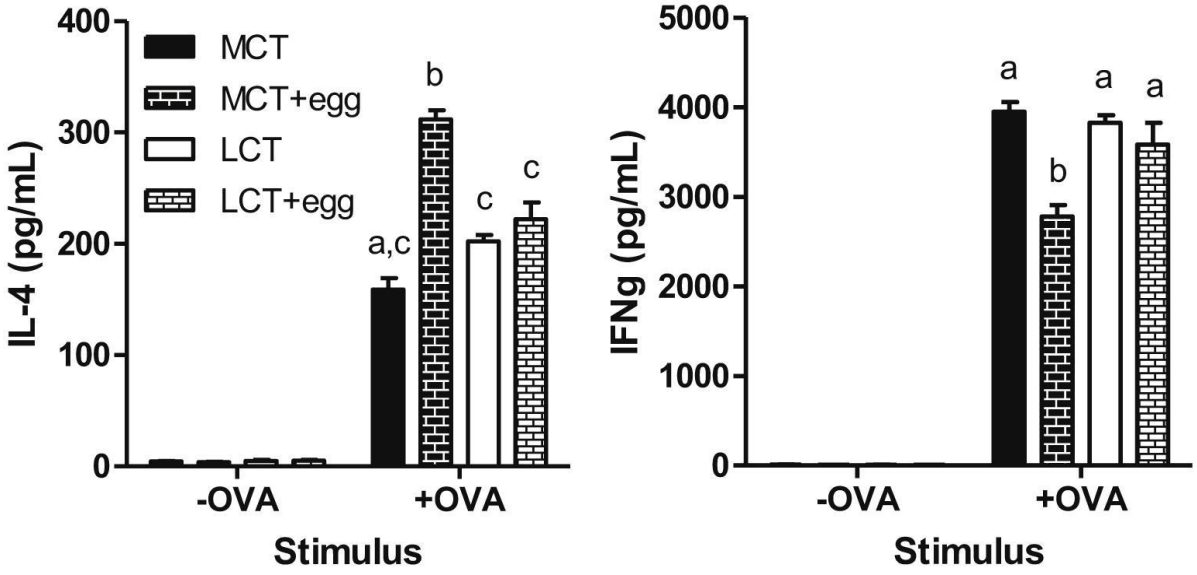

Figure 2.5. Promotion of TH2 responses by dietary MCTs. DO11.10 mice were fed MCT- or LCT-based diets containing egg white (*MCT+egg* and *LCT+egg*) or without egg white (*MCT* and *LCT*; n = 5 per group). One week later, splenocytes were stimulated *ex vivo* with OVA peptide, and indicated cytokines were measured by using an ELISA.

peanut protein in water or LCT, while mice sensitized with MCT or LCT plus PL81 had significantly increased scores upon challenge (Fig 2.6C). However, diarrhea or significant stool softening was not evident in any group. To determine whether anaphylaxis was IgE- or IgG-dependent, mice sensitized by gavage of peanut protein in MCT were treated 16h before the systemic challenges with rat monoclonal antibodies that block IgE- (EM-95) or IgG- mediated pathways of anaphylaxis (2.4G2), or both [184]. As shown in Fig 2.6D, anaphylaxis was prevented when the mice were pre-treated with anti-FcγRII/III, but not anti-IgE, suggesting that anaphylaxis was IgG-dependent. The mild drop in body temperature in naïve mice may relate to complement activation by peanut protein [185].

2.3.5 IgE- dependent anaphylaxis upon oral antigen challenge

Next, we tested whether mice sensitized by gavage of peanut protein in MCT develop anaphylaxis when re-exposed to antigen via the oral route. To this end, mice were gavaged with peanut protein in MCT on day 1 and 8 (double sensitization), and injected 40 days later with IgE-blocking antibodies or vehicle. One day later, the mice received a third gavage with peanut protein in MCT, and body temperature was monitored. As shown in Fig 2.7A, oral antigen challenged mice showed a significant drop in body-temperature, provided that no blocking antibody had been injected. In contrast, mice pre-treated with blocking antibodies against IgE did not respond to the oral antigen challenge, suggesting that orally-induced anaphylaxis was IgE- dependent. It should be emphasized that sensitization in this experiment was enhanced by performining two gavages with MCT, separated by one week. In contrast, gavage with peanut protein in LCT prior to gavage with MCT led to tolerance (Figure 2.2D).

Orally induced anaphylaxis is exacerbated when chylomicron formation is inhibited

To test how MCT might affect responses to oral antigen challenge, mice sensitized by two gavages with peanut protein in MCT were orally challenged 40 days later with peanut protein in water, LCT (peanut oil), MCT, or LCT + PL81 (Fig 2.7B). In agreement with the experiment in Fig 2.7A, mice challenged with peanut protein in MCT showed a significant drop in body temperature (Fig 2.7B). On the other hand, mice challenged with peanut protein in water or LCT showed no significant response. However, when PL81 was added to LCT, the response was similar to that with MCT (Fig 2.7B, C). Chylomicron formation may have protected against anaphylaxis, most likely by inhibiting the ability of absorbed antigen to gain access to mast cells or basophils. In vitro studies indeed revealed that basophils decorated with anti OVA IgE responded significantly less when OVA was added in the presence of chylomicron-like particles (Fig 2.7D). Moreover, in an OVA-based model of food allergy it was observed that allergic responses were significantly more severe when challenges occurred with MCT or LCT + PL81 than with LCT alone (Fig 2.7E). In particular, the response of mice challenged with MCT was very robust.

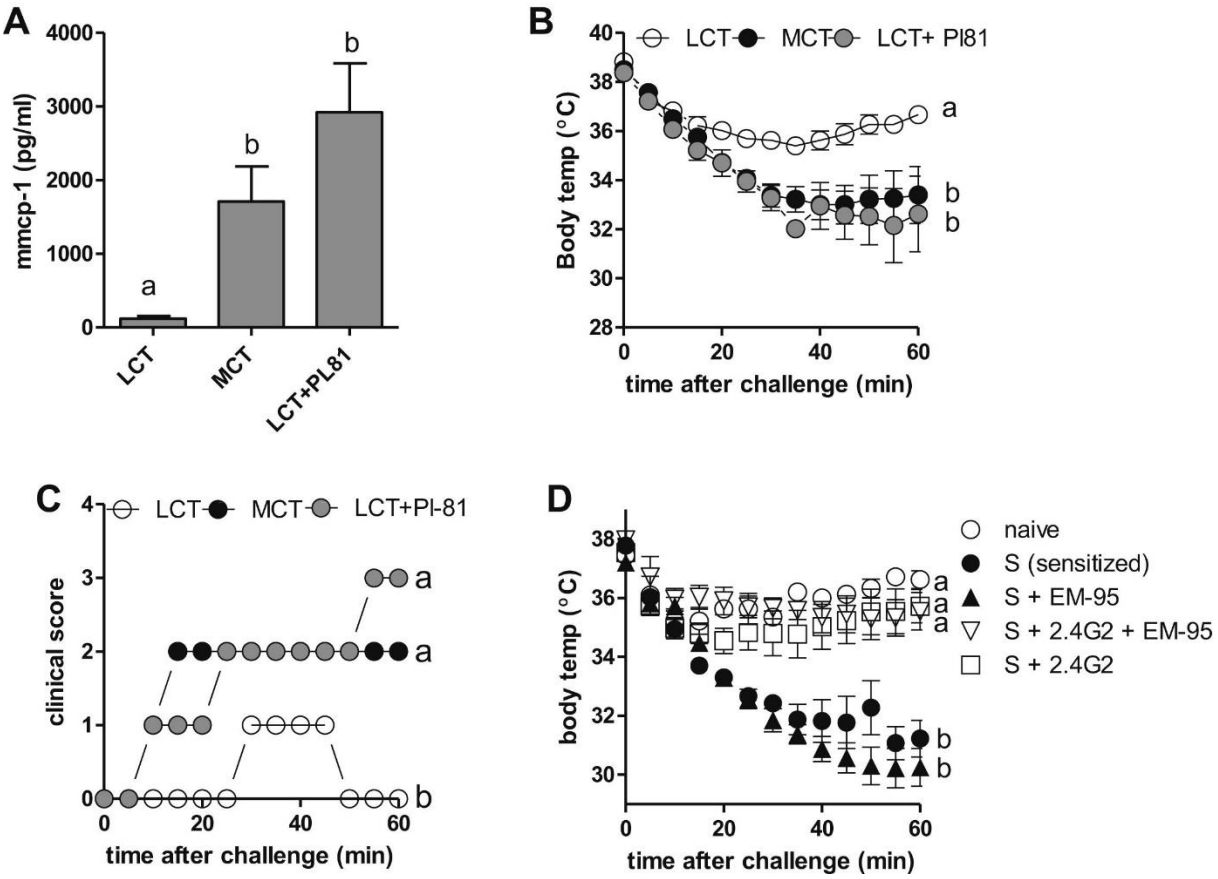

Figure 2.6. IgG-dependent anaphylaxis upon systemic challenge. Mice were gavaged once with peanut butter protein in indicated vehicles and then challenged by means of intraperitoneal injection 18 days later (n = 6). **A-C,** Parameters included plasma mouse mast cell protease 1 *(mmcp-1)* 90 minutes after the challenge (Fig 6, *A*), body temperature (Fig 6, *B*), and clinical score (Fig 6, *C*). **D,** Pretreatment with blocking antibodies against IgG receptors (2.4G2) but not against IgE (EM-95) blunted anaphylaxis.

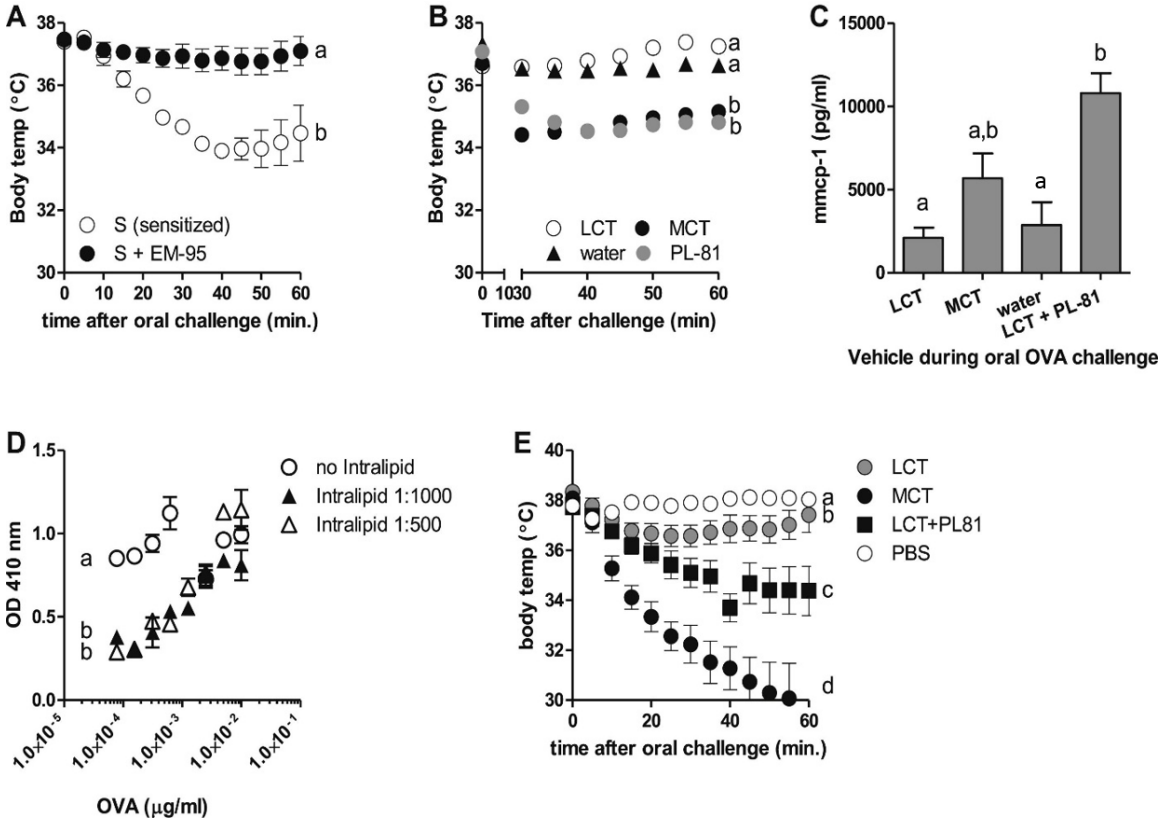

Figure 2.7. IgE-dependent orally induced anaphylaxis is aggravated by MCTs. A, Body temperature of mice (n = 6-7) challenged with peanut protein in the MCT with or without pretreatment with IgE blocking antibody. Sensitization occurred by means of gavage on days 1 and 8, and challenges were performed on day 48. **B,** Mice (n = 6) were sensitized as in Fig 7, *A*, but the oral challenge was performed by using gavage of peanut protein in different vehicles. **C,** Plasma mouse mast cell protease 1 *(mmcp-1)* levels in mice in Fig 7, *B*. **D,** Inhibition of basophil activation by including Intralipid (2 different dilutions) with OVA added to sensitized basophils (n = 4 per group). **E,** BALB/c mice (n = 9 per group) were sensitized intraperitoneally with OVA in alum and then challenged with 60 mg of OVA by means of gavage in the indicated vehicle. All experiments were repeated at least twice with similar outcome, except those shown in Fig 7, *B* and *C*, which were performed once.

2.4 Discussion

In addition to confirming and expanding our previous observation that more antigen is absorbed when ingested with LCT than with MCT[168], we have made six novel and important observations: 1) antigen delivery to Peyer's patches is significantly enhanced by MCT; 2) both acute and chronic MCT feeding promote allergic sensitization to concomitantly ingested antigens, as shown by increased antibody production and anaphylaxis following antigen re-exposure; 3) ingestion of antigen with MCT promotes the expression of the Th2-biasing cytokines TSLP, IL-25 and IL-33 by upper gastrointestinal tract epithelial cells; 4) MCT-based diets induce a Th2 bias in the host (probably a result of point 3); 5) MCT promote the ability of ingested antigen to induce anaphylaxis in sensitized mice; and 6) almost all MCT effects are mimicked by mixing LCT with an inhibitor of epithelial cell chylomicron secretion, suggesting that chylomicons inhibit antigen access to mast cells, basophils and dendritic cells that present antigen in a stimulatory manner. Each of these points and its relevance is discussed below.

Antigen absorption: We previously demonstrated that chylomicron formation promotes intestinal antigen absorption into the bloodstream [168]. We now additionally demonstrate that failure to secrete chylomicrons causes retention of LCT (and presumably, associated antigens) within the intestinal mucosa and lamina propria, where they could more readily interact with relevant immune cells. A similar mechanism may also explain how feeding MCT instead of LCT increases antigen within Peyer's patches. The role of Peyer's patches in food allergy is unclear, although they are reported to be associated with oral tolerance [186, 187]. We found, however, that DQ-OVA was present in a large fraction of Peyer's patch dendritic cells, when gavaged with MCT or LCT plus PL81. In contrast, surprisingly few DQ-OVA positive cells were found in the MLN, even when DQ-OVA was gavaged with LCT. The apparent conflict between the latter observation and our previous finding of increased OVA in the MLN of OVA/LCT-gavaged mice may be

explained by the possibility that chylomicrons traffic through the MLN but prevent the uptake or processing of associated antigens by MLN dendritic cells.

Sensitization by MCT-rich diets and the role of chylomicrons: Our findings demonstrate that MCT-based diets promote allergic sensitization to simultaneously ingested antigens in an acute and a chronic feeding model. MCT-containing oils are regularly prescribed to patients with fat malabsorption or intestinal inflammation, and are added to some infant formulas and commercially available peanut butter (Eckhardt, Li, unpublished observations). The amounts of MCT required to promote peanut allergy remains to be determined, however. MCT, unlike LCT, do not form chylomicrons, which might protect from allergic sensitization. Chylomicron induction of macrophage cytokine production [188, 189], for example, may prevent Th2-biasing phenotypes in antigen-presenting cells (APC) and may, as suggested by our data, promote oral tolerance (Fig 2.3C). This is perhaps because of the high chylomicron content of retinol [190], which promotes oral tolerance by stimulating regulatory T-cell development [191-193].

Effect of MCT on Th2 cytokine expression in epithelial cells: The first cells to interact with dietary fatty acids are intestinal epithelial cells. These play a role in food allergies because of their barrier function, and of their active participation in immune responses to microbial and dietary antigens. One novel epithelial cytokine, TSLP, was recently shown to be important for the induction of experimental food allergy [170]. Interestingly, our studies showed that mice fed MCT via acute gavage or via dietary enrichment increase intestinal-epithelial expression of three Th2-biasing cytokines: TSLP, IL-25 and IL-33. This effect is greatest in the jejunum, which is the principal site of fat absorption. This may explain why MCT feeding also promoted antigen-driven IL-4 production and decreased IFN-γ production by isolated splenocytes.

Chylomicrons and anaphylaxis: Besides their protective role in the sensitization phase of peanut and OVA allergy, chylomicrons were also protective in the effector phase of food allergy. Both in the peanut protein model and in a classical OVA model, gavage of the antigens with LCT did not cause anaphylaxis, unless PL81 was added. In contrast, gavage of antigens with MCT caused clinically significant anaphylaxis. This suggests that chylomicrons prevent the access of ingested antigens to mast cell- and basophil-associated IgE, and our in vitro basophil activation tests support this hypothesis in that the presence of chylomicrons greatly reduced the effect of OVA on basophils (Fig 2.7D) to an extent that would more than compensate for the increase in antigen absorption that is associated with chylomicron formation. We used Intralipid as proxy for chylomicrons, which are similar in size and lipid content but lack Apolipoprotein B48. However, they do acquire other apolipoproteins from serum [Robinson, 1979 #886] and show similar metabolic clearance rates [Bryan, 1976 #1361]. Nevertheless, we cannot exclude that other factors present on chylomicrons in vivo may differently affect mast cells or basophils. Moreover, whereas the inhibitory effect is significant, we do not know whether the effect is due to OVA sequestration by chylomicrons [Wang, 2009 #1171] or by an inhibitory effect on the basophils themselves. Our findings nevertheless suggest that postprandial lipid transport via the lamina propria in the upper GI tract, where most mast cells reside, or through the bloodstream, could have important effects on the effector phases of food allergies.

Ingestion of antigen with water, rather than a fat, also failed to induce anaphylaxis. We currently cannot distinguish the possibility that antigen ingested with water is poorly absorbed from the possibilities that antigen ingested with water becomes associated with chylomicrons or is otherwise protected from access to mast cell and basophil IgE. Regardless of the mechanism involved, our finding is still potentially relevant for optimization of oral desensitization, as currently attempted in small clinical trials [194]. Feeding antigen with LCT might prevent

anaphylaxis during oral treatment while boosting its effectiveness by promoting oral tolerance (Fig 2.2D).

Concluding remarks: Our study suggests that dietary MCT may have a previously unappreciated effect on immune responses to dietary antigens, both on sensitization and anaphylaxis, by affecting antigen absorption and by promoting a Th2 bias. Although it is premature to vilify MCT, they nevertheless could be a novel dietary risk factor for allergies. In this respect, it is important to note that considerable MCT is present in breast- and infant formula. On the other hand, the Th2-biasing properties of MCT could be exploited to treat or prevent "Th1/Th17 diseases", such as Crohn's disease and diabetes. Interestingly, most of the effects of MCT could be mimicked by adding an inhibitor of chylomicron formation to LCT, which suggests that postprandial chylomicron formation plays an important role in immune responses to dietary antigens. This intriguing observation suggests that subtle genetic defects in the production, secretion, transport and clearance of chylomicrons may be a risk factor for food allergy development.

CHAPTER III. Dietary palmitic acid as a potential natural adjuvant in peanut allergy

3.1 Introduction

The prevalence of food allergies is rising, including allergies to peanuts, which is one of the more persistent and dangerous food allergies and results in numerous emergency room visits and approximately 200 deaths per year in the United States[28]. Peanut allergy is also increasing in countries with traditionally high peanut consumption, with dynamics that make it highly likely that environmental factors may contribute[195].

Several environmental factors may increase the risk for food- and other allergies, such as altered hygiene, lifestyle, and pollution. However, Western and westernizing societies have also undergone significant changes in their alimentation in a rather small time period, and it could be that some unsuspected dietary components increase the risk for allergic sensitization. Certain dietary components may affect the intestinal mucosal immune system and predispose it to develop inappropriate Th2 responses to dietary antigens. In a recent study at the role of one of the most common food components, fatty acids, we observed that mice can be made spontaneously allergic to peanut protein when force-fed a single dose of peanut butter protein in the presence of saturated medium-chain triglycerides (MCT) or when fed peanut protein for several weeks in the context of an MCT-rich diet[196]. This was associated with an effect of MCT on Th2 cytokine expression in the intestinal mucosa, including the cytokines Thymic Stromal Lymphopoietin (TSLP), IL-25, and IL-33. Other oils, such as soybean oil and peanut oil, did not appear to induce peanut allergy, unless an inhibitor of chylomicron formation was added. Since MCT, in contrast to the other oils, does not require chylomicron formation for its absorption by virtue of the short size of its fatty acids, we argued that postprandial chylomicron formation

protects from allergic sensitization, partially by affecting lymphatic absorption of dietary antigens[168, 196].

However, MCT also differs from soybean and peanut oil in the degree of saturation of its fatty acids. While refined MCT is virtually completely saturated, soybean and peanut oils are mainly unsaturated. We therefore wanted to test whether a mainly saturated oil with similar fatty-acid chain lengths as soybean- or peanut oil, and which is absorbed in a chylomicron-dependent manner, would also spontaneously cause allergic sensitization to peanut protein. As a saturated fatty acids component in commercial vegetable oil, palmitic acid is predominantly esterified to the sn-1,3 positions of the triglyceride. It is reported that such palmitic-acid increases intestinal erosion and mucosal damage by failing to induce immunosuppressive regulatory T cells responses[197]. Our results show that palm oil significantly promotes spontaneous allergic sensitization to concomitantly ingested allergens, much like MCT. Therefore, chylomicron formation and fatty acyl saturation likely are independent risk factors for allergic sensitization. The physiological relevance of our findings are discussed.

3.2 Materials and Methods

3.2.1 Animals

Male 5 week old C3H/HEJ mice were ordered from The Jackson Laboratory and were housed three per cage in a room of a specific pathogen-free animal facility with a 12h light / dark cycle. The mice were allowed to acclimatize for 1 week prior to experimentation. Unless indicated otherwise, the mice received standard rodent diet and filtered tap water ad libitum. All animals were handled in strict accordance with good animal practice as defined by the relevant national and local animal welfare bodies, and all animal work was approved by the Institutional Animal

Care and Use Committee of the University of Kentucky (Animal Welfare Assurance Number of the University of Kentucky A3336-01; U.K. IACUC protocol 2008-0306).

3.2.2 Peanut extract preparation

Jif-brand peanut butter ("To Go; Creamy") was diluted with two volumes of MCT oil (Nestlé Nutrition) to reduce viscosity and then passed through a sieve with a 70 µm pore size to remove particles that would clog the gavage needle. The filtrate was then centrifuged (4 minutes; 16,800xg) and the oily supernatant was completely removed and replaced with the respective experimentally used oils. The final volume of the suspensions equaled the initial volume of peanut butter. Triglycerides consisted of food grade palm- (Spectrum, PA105), peanut (Hollywood brand) or soybean (Kroger) oil.

3.2.3 Effect of palm oil on acute allergic sensitization to peanut protein

To determine whether palm oil promotes allergic sensitization to peanut protein, antigen naïve and fasted (4h) mice received a single 0.3 ml bolus of peanut butter protein in palm- or other oils via intragastric gavage with an 18G 38mm polypropylene feeding tube with rounded tip (Harvard Apparatus). The amount of protein per gavage was estimated to be around 80 mg using a Bicinchoninic acid (BCA) assay (Pierce). Mice were returned to their cages and kept for three more weeks on standard rodent diet before a blood sample was taken for anti-peanut IgE measurements.

3.2.4 Effect of palmitate on jejunal epithelial TSLP mRNA expression

Fasted mice were gavaged with 0.3 ml palm oil or peanut oil. After 5h, the mice were euthanatized and their jejuna were isolated and opened in cold PBS. Mucosal scrapings were collected and snap frozen for RNA isolation using the EZRNA kit from Omega-Biotech. RNA was

reverse-transcribed into cDNA with a Q-script kit from Quansys. The resulting cDNA was analyzed for abundance of TSLP relative to villin (which is restricted to the epithelium) with primer pairs ACG GAT GGG GCT AAC TTA CAA / AGT CCT CGA TTT GCT CGA ACT (TSLP) and TAA GGC TGC ACT CAA GCT GTA CCA/AGT CCT CAT GCT TGA GCA GGT CTT (villin; a marker chosen to normalize expression) using a Bio-Rad CFX-96 realtime PCR machine and SYBR Green-based PCR reagent.

3.2.5 Effect fatty acids on epithelial TSLP expression in vitro

Effects of fatty acids on TSLP expression *in vitro* were tested using CMT93 cells. This murine rectal epithelial line was obtained from American Type Culture Collection and cultured in an incubator at 37°C, 100% humidity, and 5% atmospheric CO_2 in HEPES- and bicarbonate-buffered DMEM/Ham's F12 medium supplemented with10% fetal calf serum (FCS) and antibiotics. Culture media and reagents were from Hyclone or Gibco. Cells were seeded in 12-well tissue culture plates, and when a confluent monolayer was obtained (usually within three days), the cells were washed with serum-free medium and incubated overnight with sodium salts of fatty acids (0.75 mM of either palmitic-, oleic-, or linoleic acid; highest purity available from Sigma) solubilized with 0.5% fatty-acid-free bovine serum albumin (Sigma) in serum-free medium. After overnight incubation, medium was aspirated and RNA isolated and analyzed for TSLP expression as with jejunal mucosa, except that TSLP mRNA was normalized for GAPDH mRNA (primer pair CCA GGT TGT CTC CTG CGA CTT / CCT GTT GCT GTA GCC GTA TTC A) instead of villin. None of the treatments significantly affected cell viability as assessed with Trypan-Blue staining.

3.2.6 Effect of palmitate on spontaneous allergic sensitization to peanut protein

To study the effect of dietary triglycerides on allergic sensitization during longer-term feeding, male C3H/HeJ mice were fed for up to 6 weeks with custom diets in which 10% of the protein

source consisted of partially defatted peanut flour (the other 90% consisted of casein). The diets were prepared by Harlan Laboratories, Inc., using 12% dark-roasted peanut flour (Byrd Mill brand). As source of fat (20% of calories) served either palm oil (Harlan diet TD.110685) or peanut oil (Harlan diet TD.110684).

3.2.7 Systemically allergen challenge

Three weeks after gavage with peanut protein or 4 weeks after chronic feeding of peanut protein in the various diets, mice received 30 mg of peanut butter protein intraperitoneally in 0.3mL of sterile PBS [198]. Body temperature was measured telemetrically immediately before and every 5 minutes after the challenge, using subcutaneous transponders (Bio Medic Data Systems Inc.) and mast cell degranulation was estimated by measuring serum levels of mouse mast-cell protease 1 (mmcp-1) 90 minutes after the challenge by ELISA (eBioscience). Signs of anaphylaxis were estimated with a semi-quantitative scoring, with “0” being assigned to mice with no symptoms, “1” to mice which were stationary but moved when provoked, “2” to mice which remained immobile even when provoked, and “3” to mice which lied down.

3.2.8 ELISA

To detect antigen-specific IgE, we used a custom-developed ELISA as described before[196]. In short, 96 well BD Falcon ELISA plates were coated with 20 µg/100ul peanut butter protein in carbonate buffer (pH 9.6) at 4 ˚C overnight. Washed plates were then treated with blocking solution (NAP blocker, GBiosciences) for 1 h at room temperature. Mouse serum, diluted 1:10 in “NAP” blocking solution (GBiosciences), was added for 1 h at room temperature. Unbound ligand was washed with Tris-Buffered Saline containing Tween 20 (TBST). Alkaline-phosphatase conjugated goat anti-mouse IgE (SouthernBiotech) was added at 1:800 dilution in blocking solution and incubated for 60 min or longer at room temperature in complete darkness After

washing unbound antibody, AP substrate (femto-ELISA-AP Substrate; GBiosciences) was added and the absorption at 450 nm (A450) was read after addition of 50 µl stop solution (3 M NaOH).

3.2.9 Statistics

We used Graphpad Prism version 5 to analyze the results and the data were displayed as average ± S.E.M. One way ANOVAs were used for intergroup analysis following by between-groups post-hoc analyses (Newman-Keuls). Temperature data were analyzed by comparing maximum temperature drop or area under the curve. Statistical significance was assumed at $P<0.05$. All figures show representative results of at least two repeats per experiment.

3.3 Results

3.3.1 Gavage of peanut butter protein in palm oil induces allergic sensitization

To test whether palm oil can promote oral allergic sensitization to peanut protein in a single gavage model similar as seen previously with MCT oil[196], we gavage fed male C3H/Hej mice with a single dose of 80 mg peanut protein in 0.3 ml of palm oil, peanut oil, or a 1:1 mixture of palm oil and peanut oil. Serum anti-peanut IgE was measured three weeks later. As shown in Figure 1A, mice that were gavaged with peanut protein in palm oil alone or palm oil mixed with peanut oil had significantly higher anti-peanut protein IgE than the mice gavaged with peanut protein in peanut oil only. Using soybean oil instead of peanut oil also did not result in significant allergic sensitization (Figure 3.1D). To test the anaphylaxis level of mice sensitized by gavage with peanut protein mixed with different oil, they were challenged intraperitoneally with 30 mg peanut protein in 0.3 ml PBS to determine anaphylaxis. As shown in figure 3.1B and C, mice sensitized with peanut protein in palm oil alone or mixed 1:1 with peanut oil released more mmcp-1 into the bloodstream and showed a stronger drop in body temperature than mice sensitized by gavage with peanut protein in peanut oil only and water. While mice responding with a temperature drop and mmcp1 release showed clinical signs of anaphylaxis, such as immobility and hunched postures, diarrhea or significant stool softening was not evident as seen previously in experiments with MCT[196] (not shown). Similarly, Figure 3.1E and F showed the similar protective role of soybean oil as peanut oil in peanut protein sensitization and anaphylaxis.

3.3.2 Feeding peanut flour in palm-oil based diets induces spontaneous allergic sensitization

We previously reported spontaneous allergic sensitization in mice fed peanut-protein containing diets in which the fat source consisted of MCT (refined coconut oil). We tested whether palm-oil

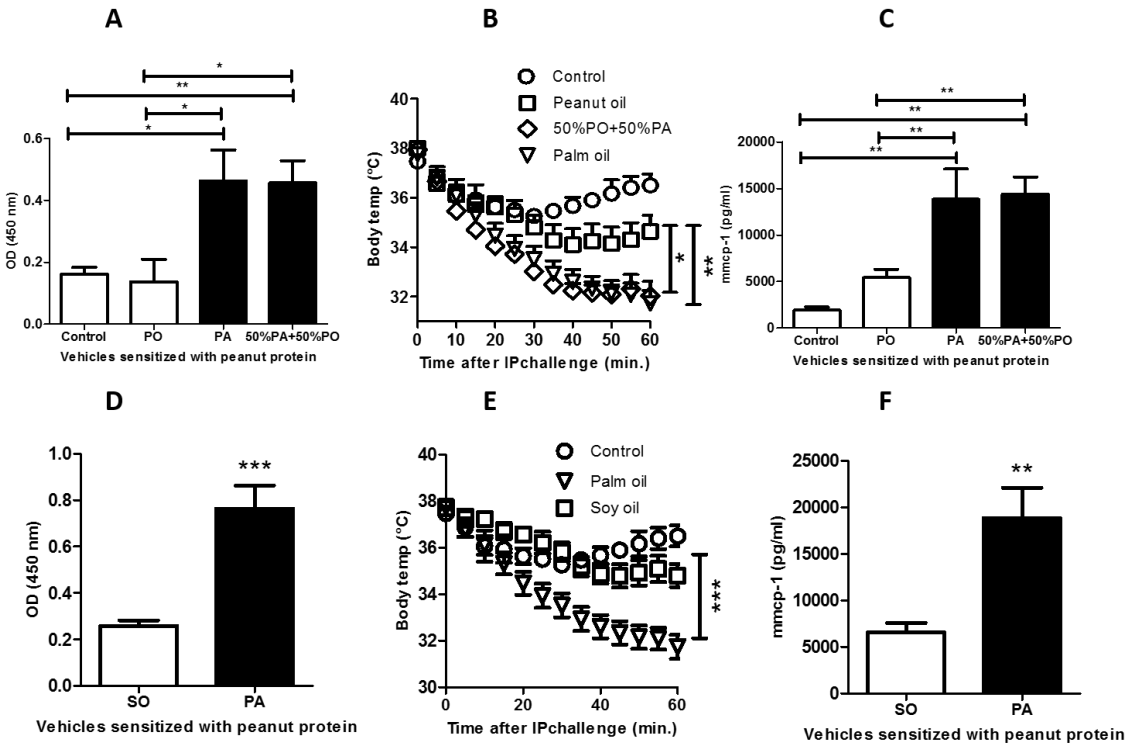

Figure 3.1. Palm oil causes allergic sensitization and anaphylaxis. (A)&(D) Anti-peanut IgE 18 days after gavage with peanut protein (80mg) in indicated vehicles (n=6). All the values are after control subtraction. (B)&(E) Body temperature drop after challenge by means of intraperitoneal injection 18 days after sensitization (30mg peanut protein in 0.3ml PBS). (C)&(F) Plasma mouse mast cell protease 1(mmcp-1) 90 minutes after the challenge. *P<0.05, **P<0.01, ***P<0.001. All the data are shown as mean±SEM.

PA-Palmitic acid

PO-Peanut oil

based diets would have similar effects, and observed indeed that mice fed with peanut flour in palm-oil based diets but not peanut-oil based diets developed anti-peanut protein IgE within 3-4 weeks (Figure 3.2A). Consistent with this, we observed that the mice in the palm oil group, when challenged intraperitoneally with peanut protein in MCT as per our previous protocol[196], showed signs of anaphylaxis that were also reflected by increased serum mmcp1 levels (Figure 3.2B).

3.3.3 Palm oil induces jejunal TSLP expression

Oral sensitization with dietary antigens may involve stimulation of intestinal epithelial expression of cytokines that promote Th2 responses, such as TSLP. To test whether the observed allergic sensitization with palm oil involved intestinal mucosal TSLP expression, we gavaged mice with palm oil or peanut oil (0.3 mL each) and isolated the jejuna 5h later. TSLP expression in mucosal scrapings was measured by RT-PCR and appeared to be significantly increased by palm oil compared with peanut oil or water. We next investigated whether palmitic acid, the major fatty acids derived from palm oil, is able to stimulate TSLP expression *in vitro*. To this end, CMT93 cells were incubated with PBS, palmitic, oleic-, or linoleic acid (as sodium salts; the major fatty acids in palm oil, peanut oil, and soybean oil, respectively), and TSLP mRNA was quantified by realtime PCR the day after. As shown in Figure 3.3B, palmitic acid significantly increased TSLP expression compared to the other fatty acids Thus, it appears as if allergic sensitization through palm oil gavage is associated with upregulation of TSLP expression in the jejunum.

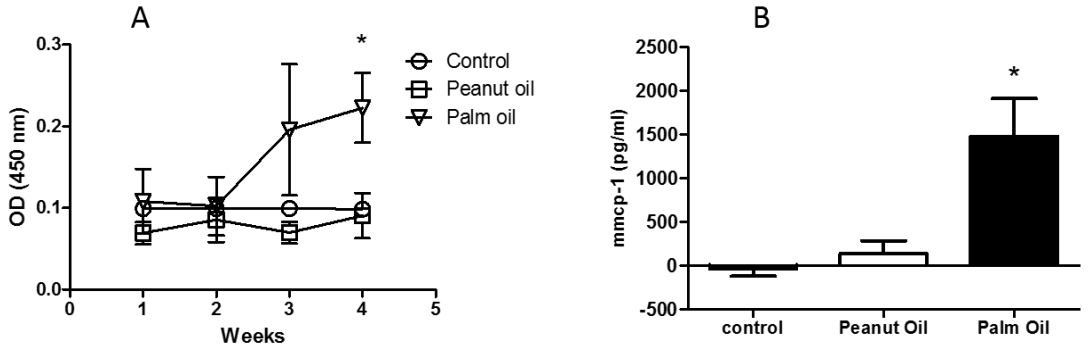

Figure 3.2. Effects of palm oil feeding on peanut sensitization and anaphylaxis. (A) Anti-peanut IgE during 4 weeks of feeding with diets enriched with peanut flour in which oil was replaced as indicated. (B) Plasma mouse mast cell protease 1(mmcp-1) 90 minutes after intraperitoneally challenge (30mg peanut protein in 0.3ml PBS). *P<0.05, **P<0.01, ***P<0.001. All the data are shown as mean±SEM.

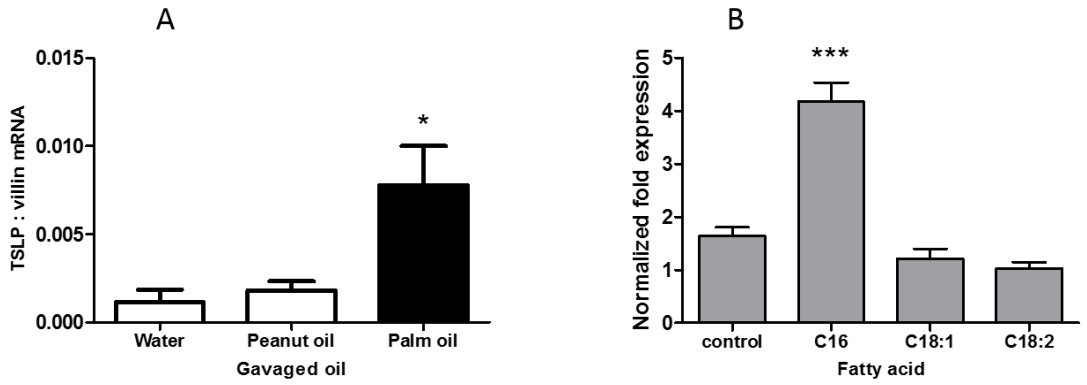

Figure 3.3. Effects of palm oil intestinal epithelial TH2 cytokine expression in vivo and in vitro. (A) TSLP mRNA in epithelia from jejunum 4 hours after indicated vehicle gavage (n=6). (B) TSLP mRNA fold change in CMT93 cells 12hrs after incubating with 0.75mM of either palmitic-, oleic- or linoleic acid solubilized with 0.5% fatty-acid free bovine serum albumin in serum-free medium (n=3). *P<0.05, **P<0.01, ***P<0.001. All the data are shown as mean±SEM.

3.4 Discussion

The main finding in this study is that acute or chronic palm oil feeding promoted allergic sensitization to concomitantly ingested antigens, as evidenced by increased antibody production and anaphylaxis following antigen re-exposure. This may be explained by our second finding that palmitate oil can induce the expression of novel Th2-biasing cytokines in the epithelium of the upper gastrointestinal tract. In the following, we will discuss each of these findings and address their potential relevance.

Not only for cooking, palm oil existed broadly in the products around us such as body lotion, detergent, butter and other food products. Based on the evidence that Angiopoietin-like protein 4 (Angptl4), an important modulator of LPL activity, can protect against severe proinflammatory effects characterized by severe mesenteric lymphadenitis of saturated fat by inhibiting fatty acid uptake into macrophages in mesenteric lymph nodes[199], we believe saturated fatty acids have inflammatory effect on different tissues as refer to their pharmacological properties. Furthermore, Lu and his colleagues found that the palmitic-acid esterified to the sn-1,3 positions of the glycerol backbone, the main form of palmitic acid in vegetable oil, leads to intestinal erosions and morphological damage in Muc2-/- mice by failing to induce immunosuppressive regulatory T cells [197]. We previously demonstrated that acute or chronic MCT (medium chain triglycerides, which contains C8 and C10 mostly) feeding promoted allergic sensitization to concomitantly ingested antigens, as evidenced by increased antibody production and anaphylaxis following antigen re-exposure. The results were significantly different if we used LCT (long chain triglycerides, which contains mainly oleic and linoleic-acid.)[196]. MCT fatty acids that are from coconut oil are notably known as “saturated fatty acids”, this most likely gives us a hint of the different pharmacological properties on food allergy both in sensitization and anaphylaxis phases of saturated fatty acids and unsaturated ones. Interestingly, the palm oil we

used had much similar effects on promoting peanut protein allergy (Figure 3.1 and 3.2) as MCT that we reported.

We then liked to investigate whether saturated fatty acids differ from unsaturated fatty acids in terms of immune responses to dietary antigen. The first cell barriers to interact with dietary fatty acids are the intestinal epithelial cells. It is now becoming increasingly clear that intestinal epithelial cells not only play a role in food allergies because of their barrier function, but also because of their active participation in immune responses to microbial and dietary antigens. TSLP, one novel epithelial cytokine was recently proved to be important for the induction of experimental food allergy[200]. Interestingly, our study showed that mice gavaged with palm oil had a significant increase in intestinal-epithelial expression of TSLP (Figure 3.3). The effect was biggest in the jejunum, which is the principal site of fat absorption. Then we got the same result in vitro when we used CMT 93 cells and different free fatty acids (Figure 3.3).

In conclusion, our study suggests that dietary saturated fatty acids (Palm oil) may have a previously unappreciated effect on immune responses to dietary antigens, both on sensitization and anaphylaxis, by promoting a Th2 bias. Thus we have reasons to suspect the risk role of saturated fatty acids on some Th2 responses induced disease such as food allergy, pollen allergy, eczema and etc since Palm oil exists in a lot of daily necessities including baby products. This finding may explain why food allergy has regional properties. It also inspired us to expand our research targets to other saturated fatty acids to further prove our findings and to study TSLP involved JAK/STAT pathway.

CHAPTER IV. Materials and Methods of Chapter V and VI

4.1 Animals and diets

C57BL/6J (Stock #000664) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed individually ventilated cages with free access to food and water and maintained in a temperature-controlled room under negative pressure with a 14h/10h light:dark cycle. Mice homozygous for the ABCG5 and ABCG8 mutant alleles (G5G8 KO) and their WT littermates were obtained from trio breeding of male and female hemizygous mice as described[201]. G5G8 KO mice and their WT littermates were weaned between 18 and 21 days onto a pellet plant sterol free diet (PSF diet, Research Diet, D10040301) to prevent STSL[201]. Plasma levels of phytosterols were barely detectable compared with G5G8 KO mice on chow diet[202].

To induce STSL, G5G8 KO mice and their WT littermates were transferred to PSF diet after weaning for two weeks followed by standard chow diet for 10 weeks until the plasma levels of phytosterol reach steady statues (Figure 4.4A).

Mice carrying the human CETP minigene (B6.CBA-Tg(CETP)5203Tall/J) were obtained from The Jackson Laboratory (Stock number: 003904). Mice were housed individually ventilated cages with free access to water and food. The colony was maintained in a temperature-controlled room under negative pressure with a 14h/10h light/dark cycle. Male hemizygous and female WT (C57BL/J) mice were bred by trio. Male CETP transgenic (Tg) and their WT littermates were weaned between 18 and 21 days onto standard rodent chow diet.

To induce expression of transgene, mice analyzed between 10-12 weeks of age were transferred to high fat, high cholesterol Western diet (41% kcal fat, 1.5% w/w cholesterol, Research Diets Inc., New Brunswick, NJ. Product number: D12079B) for two weeks.

4.2 Plasma, bile and intestinal perfusate analysis

Biliary and intestinal perfusate total cholesterol content were measured by commercial colorimetric-enzymatic assays (Wako Chemicals, Richmond, VA). Plasma phytosterol concentration was measured by GC-MS, using modifications of previously published methods[203]. Plasma CETP activity was determined by using commercial CETP activity assay kit (Roar CETP Activity Assay Kit, Cat. No. RB-CETP). Bile flow was determined gravimetrically assuming a density of 1 mg/mL.

4.3 Intestine perfusion procedures

Mice were anesthetized with 2mg urethane/g body weight intraperitoneally and placed in a temperature-controlled moist chamber (HUGO SACHS ELEKTRONIK HARVARD APPARATUS GmbH D79232, Germany) with steady temperature of 37°C to maintain body temperature. A longitudinal cut was made from the lower abdominal incision site along the midline to the sternum. Two hemostatic forceps were used to separate the skin and expose the peritoneum. A sterile cotton- tipped applicator wetted in sterile PBS was used to push aside the liver lobes and intestine to expose the common bile duct and gall bladder. The bile duct was ligated with a 3cm length silk suture and the gall bladder cannulated with a 10cm length of PE-tubing. The tubing was secured to the gall bladder with a 3cm length silk suture. Diverted bile was collected every 10 or 15 minutes for 90 minutes and stored on ice. The tail vein was cannulated with a Tail Vein Catheter (Braintree Scientific, INC. No. MTV-01) attached to a syringe pump containing 20mM

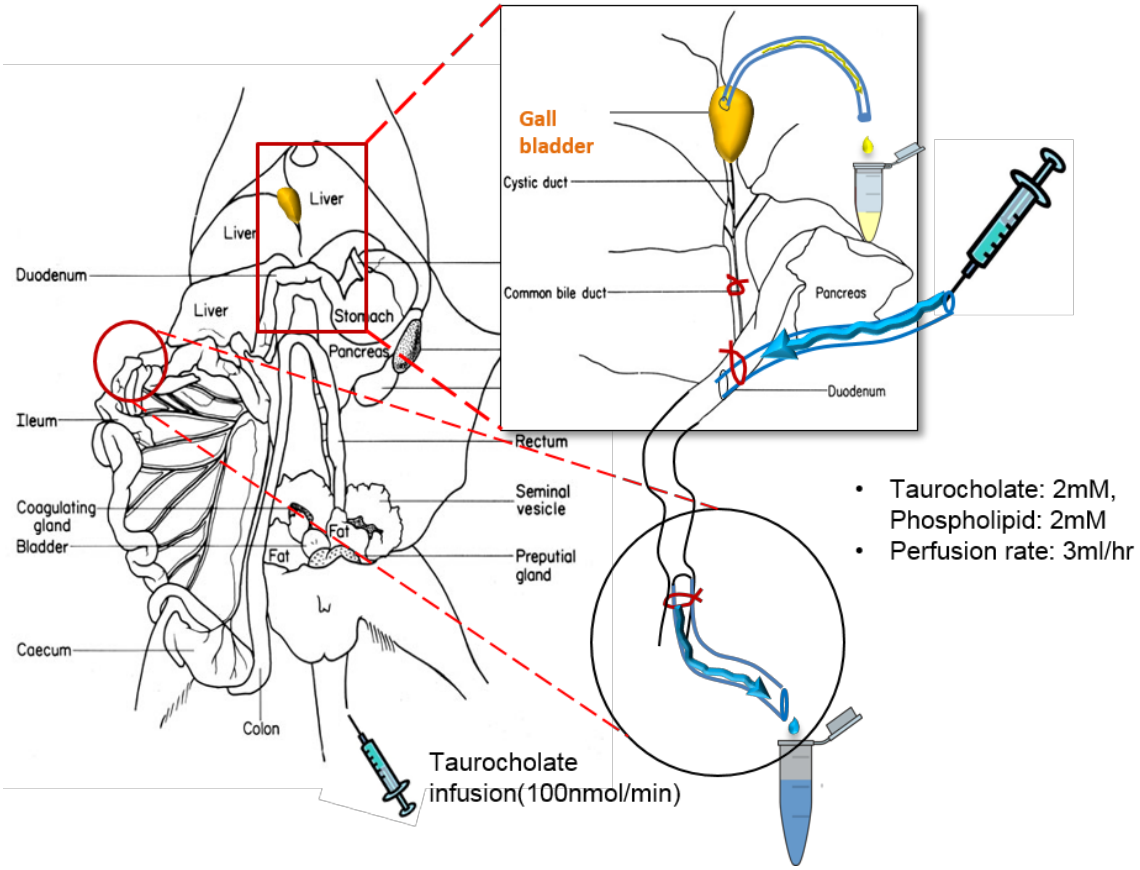

Figure 4.1. Perfusion assay for measurement of biliary and intestinal cholesterol secretion rates simultaneously.

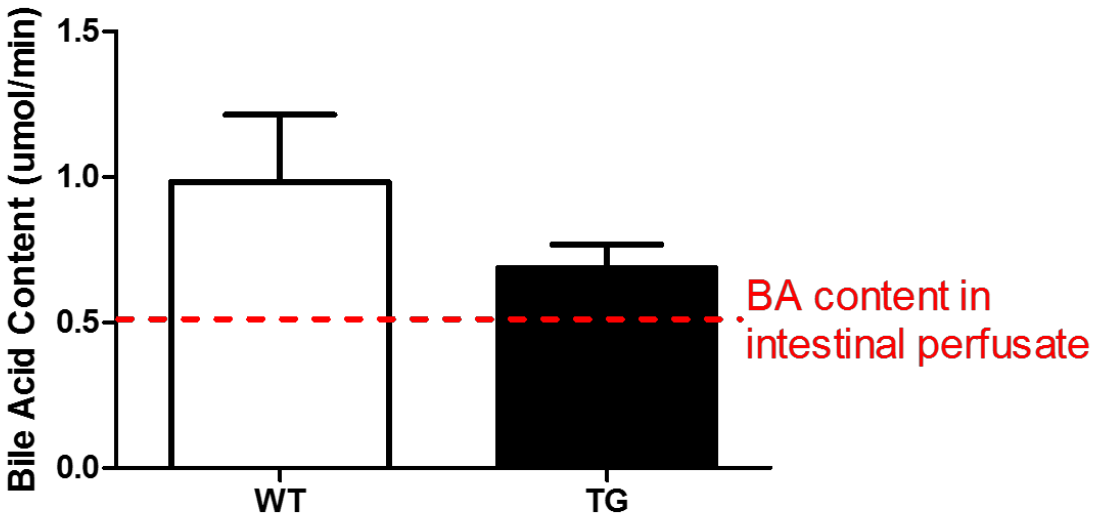

Figure 4.2. Bile acid content in mice bile and intestinal perfusate. The red dash line indicates the bile acids content in intestinal perfusate. Data were determined by two-tailed t-test.

tauracholate. Taurocholate was infused at a rate of 100nmol/min to maintain biliary lipid secretion. (Figure 4.1)

Perfusion of the proximal (first 10 cm) small intestine where highest TICE rate was observed [132] were performed simultaneously. An inflow catheter attached to a peristaltic pump was inserted in the small intestine just below the fundus of stomach and an outflow catheter was fitted 10 cm distal to the inflow catheter for perfusate collection. Both catheters were fixed with intestine with 3cm length silk sutures. The proximal intestine was flushed with 5 mL PBS (37°C) to remove the lumenal contents and fill with perfusate (37°C, see fluid composition below). Perfusions were performed at a fixed flow rate (3 mL/h) over the 90 minute period. Perfusate fractions were collected every 10 or 15 minutes and stored on ice simultaneous to bile collection. At the end of the perfusion period, blood was collected by cardiac puncture and serum was obtained by means of centrifugation (12,000rpm, 2.5 min). (Figure 4.1)

The rate of bile acid transit is 0.5 µmol/min and is similar to the rate of hepatic bile acid output (Figure 4.2) and allows for a comparison of the relative rates of biliary vs. intestinal cholesterol secretion.

To examine HDL-mediated cholesterol delivery to bile vs. intestine, mice were injected with 0.6µCi/mouse [^{3}H]-cholesteryl oleate HDL retro-orbitally 30 minutes before the perfusion procedure.

4.4 Perfusion fluid composition

Perfusions were carried out with a modified Krebs solution (119.95 mmol/L NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4 · 7H2O, 15 mM HEPES, 1.3 mM CaCl2 · 2H2O and 10 mM L-glutamine; final pH 7.4) supplemented with 10 mM bile salt and 2mM phospholipid (10 mM taurocholate [TC]: 2mM phosphatidylcholine [PC]). Mixtures were made as follows:

taurocholate (Sigma) was dissolved in methanol and egg yolk L-α-phosphatidylcholine (Sigma) was dissolved in chloroform. Two preparations were mixed in a volume ratio of 1:1 and solvents were evaporated under a mild stream of nitrogen at 45°C. The residue after evaporation was lyophilized overnight. Lyophilized samples were sealed and stored -80°C until the day of the intestine perfusions. Before the start of the intestine perfusions, the films were dissolved in perfusion buffer (room temperature).

4.5 Mouse estrogen replacement treatment

Ovariectomy was performed on WT female mice (C57BL/6, 8weeks age) and 2 weeks later these mice were assigned to groups to receive estradiol(E2, 0, 36, 200 or 600ug/ml, n=5 mice/group) via silastic tubing capsules for one month. A group of intact female mice (n=5) without drug treatment were included for control comparison. All of the surgery work was done by Dr. Lisa Cassis group, University of Kentucky.

4.6 Immunoblot and Quantitative and Real-time PCR

The preparations of proteins, SDS-PAGE, and immunoblotting were conducted as previously described[201]. STAT-60 (Tel-Test, Inc.) was used in total RNA extraction from liver and intestine and reversely transcripted into cDNA with iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA). The relative abundance was determined by RT-PCT using SYBR Green detector on Applied Biosystem 7900HT fast-Real Time PCR System (Carlsbad, CA)[201].

4.7 Isolation and labeling of lipoproteins

Mouse HDL and human HDL (ρ = 1.063–1.21 g/ml) was isolated from fresh plasma from healthy human volunteers or male WT and CETP transgenic mice at age of 12 weeks by density gradient ultracentrifugation as described. All isolated fractions were dialyzed against 150 mM NaCl and

0.01% EDTA (saline-EDTA), and stored under nitrogen gas at 4°C. Protein concentrations were determined by the method of Lowry et al. HDL was labeled with ^{3}H-Cholesteryl oleate as described previously[204]. Briefly, ^{3}H-Cholesteryl oleate in acetone (15 µCi/mg protein) was added dropwise with swirling to 2 ml lipoprotein deficient serum (LPDS) in a glass tube at room temperature. The acetone was then evaporated under nitrogen gas for 45 min. 5 mg HDL in 2 ml saline-EDTA was added to the labelled LPDS and was incubated at 4°C for 2 h The density of the labelled lipoprotein was adjusted by solid KBr to 1.21 g/ml , then the labeled lipoprotein was recovered by ultracentrifugation for 11.5 h at 55000 rpm, dialyzed against saline-EDTA, and stored under nitrogen gas at 4°C. All procedures were performed under sterile conditions[205].

4.8 Statistical analysis

All Data were presented as mean±S.E.M. and analyzed using GraphPad Prism. Data shown in were analyzed by two-tailed t-test, two-way ANOVA and linear regression. Significance was set at *$p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$.

CHAPTER V. Sexually dimorphic adaptive responses maintain cholesterol elimination in the absence of ABCG5 and ABCG8

5.1 Introduction

ABCG5 and ABCG8 encode a pair of ATP-binding cassette half-transporters that form a complex (G5G8) promoting the elimination of neutral sterols and cholesterol[206] through hepatobiliary secretion. The expression of G5G8 is restricted in the liver and intestine. Mutations in either ACBG5 or ABCG8 result in sitosterolemia (STSL), a monogenic recessive disorder characterized by the accumulation of phytosterols in plasma, tendon, and tuberous xanthomas; elevated low density lipoprotein (LDL) cholesterol; and premature coronary artery disease[207]. Genetic ablation of G5G8 in mice disrupted cholesterol homeostasis[208], caused infertility and lipoatrophy[209], and liver abnormalities and cardiac lesions[210]. Disruption of both ABCG5 and ABCG8 in mice leads to extremely low biliary cholesterol concentrations yet biliary phospholipids and bile acid concentrations are not altered[22]. Similar results are got from mice lacking either ABCG5 or ABCG8[21, 211]. Conversely, overexpression of ABCG5 and ABCG8 in mice leads to 5-fold increase in biliary cholesterol concentrations[23]. Besides, accelerating biliary cholesterol secretion by administration of adenoviral vectors encoding ABCG5 and ABCG8 (AdG5G8) restores glycemic control and reduces plasma triglycerides in obese db/db mice[212]. It is also worth noting that G5G8-independent biliary cholesterol excretion has been found under some circumstances which needs further studies[213, 214].

With the exclusive function in cholesterol elimination, G5G8 plays a significant role in reverse cholesterol transport (RCT), the process consists of delivery of cholesterol from peripheral tissue and macrophages to liver, followed by secretion into bile by G5G8 and its final fecal elimination. This hepatobiliary pathway is considered to be the classic and sole route for cholesterol

secretion from body, playing a critical role in cardiovascular disease (CVD) prevention my mediating the cholesterol transferring to liver for disposal. However, it is found that some animal models which hepatic cholesterol metabolism were genetically altered still maintained certain amount of fecal neutral sterol levels[22, 128, 129, 141, 215, 216]. In ABCB4-/- mice with abrogated biliary phospholipid and cholesterol secretion that intravenously injected free cholesterol partly ended up in the feces as neutral sterol [128], suggesting a direct route from blood to intestinal lumen. This pathway was known as Transintestinal Cholesterol Excretion (TICE) [132], an important pathway for cholesterol elimination in mice. It was further proved that the cholesterol secreted into intestinal lumen originated from blood directly rather than intestine itself. Of importance of G5G8 in classic RCT, it is crucial to determine the role of G5G8 in TICE. It was reported that the G5G8 KO still maintained certain amount of fecal neutral sterols loss[22]. Furthermore, when intestine perfusion was performed on G8 KO mice, no significant effect of lack of ABCG8 on cholesterol secretion from intestine was observed[132]. In another hand, in a mouse model with increased TICE induced by PPARδ agonist, intestinal G5G8 mRNA levels were found no significantly different[151]. Therefore, the role of G5G8 in TICE remains undetermined and needs further exploration.

Like mentioned above, mutations in either ACBG5 or ABCG8 result in sitosterolemia (STSL) marked with elevated circulating phytosterol levels[208]. It was reported that circulating levels of sitosterol and campesterol are correlated with fractional and absolute absorption of dietary cholesterol positively, but are related to whole body cholesterol synthesis inversely. Therefore, accumulation of non-cholesterol sterols may account for the very low rates of cholesterol synthesis in STSL[217]. Yu et al. found there is approximately 50% decline in cholesterols in the livers and plasma of G5G8 KO mice due to low hepatic cholesterol synthesis relative to WT

mice[218]. Therefore, to better understand the role of G5G8 in RCT and TICE, it is necessary to exclude the effect of sitostrolemia.

Among previous studies on cholesterol homeostasis such as biliary cholesterol secretion and transintestinal cholesterol efflux (TICE), male animals were the prevalent subjects to study on and gender differences were frequently neglected in the past. However, the rates of gallstone disease happen in women two to three times higher than men[219, 220]. One possible mechanism suggests that estrogen increases biliary cholesterol secretion causing cholesterol supersaturation of bile[221]. Besides, female mice expressed more ABCG5 and ABCG8 at mRNA levels stimulated by high cholesterol synthesis or dietary intake via LXRs[222] in the absence of cholesterol[223]. Therefore, it is rational to study cholesterol homeostasis on both genders.

We speculated, on the basis of the above findings, that the role of G5G8 in TICE and whole body cholesterol homeostasis is underestimated due to the effects of STSL. It is also important to know the effect of STSL in whole body cholesterol homeostasis. Female and male mice were both used to study the sex-related difference in cholesterol elimination in this manuscript.

5.2 Results

5.2.1 Female mice have higher biliary and lower intestinal total cholesterol secretion rates than male mice

To determine if sex would affect the biliary and intestinal cholesterol secretion rates and the relationship between them, we use both male and female WT mice to perform perfusion assay described in Methods and Materials. Not surprisingly, we found the female mice had higher bile flow rates and bile cholesterol concentration than male mice (figure 5.1A&B). Similarly, we found higher biliary total cholesterol secretion rates in female mice than males in a time window of 90 minutes as expected (figure 5.1C&E). Interestingly, we found that female mice had lower intestinal total cholesterol secretion rates than male mice (figure 5.1D&F). Furthermore, It is worth noting that the sum of total cholesterol secreted from both hepatobiliary and transintestinal pathways remained similar in both genders (figure 5.1G). These data may suggest that female mice may prefer hepatobiliary pathways to eliminate cholesterol rather than transintestinal pathway compared with males. It also confirmed a complimentary relationship between hepatobiliary and transintestinal cholesterol secretion routes to maintain whole body cholesterol elimination that we found previously.

5.2.2 Female mice adapt to G5G8 deficiency by increasing TICE

To determine the impacts of G5G8 in TICE, we compared both biliary and intestinal cholesterol excretion rates in WT and G5G8 KO mice of both sexes. All mice were maintained on a PSF diet beginning at weaning to prevent the development of STSL. We found no differences on bile flow rate between either genotypes or sexes (figure 5.2A). No surprisingly, G5G8 KO mice had lower bile cholesterol concentration and biliary cholesterol excretion rates compared to their WT

littermates (figure 5.2B, C&E) as previously reported[201]. However, this difference is significantly

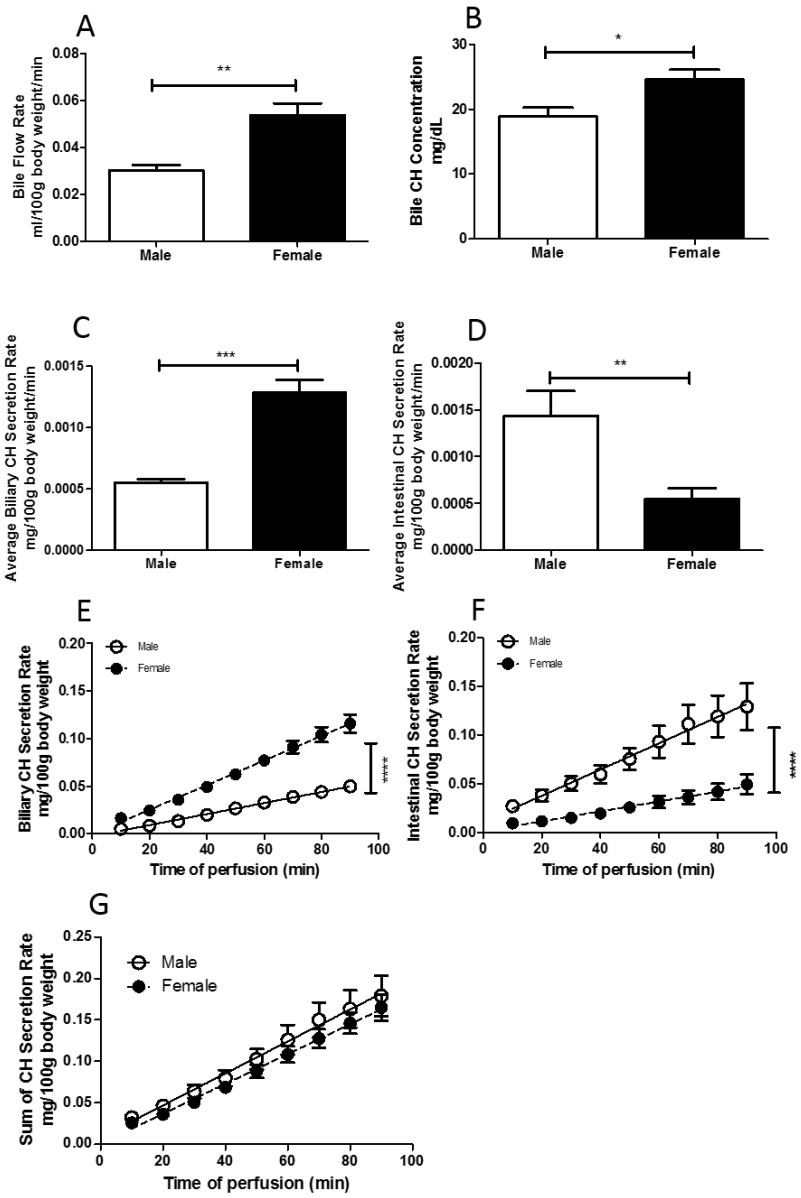

Figure 5.1. Female mice have higher biliary and lower transintestinal cholesterol secretion rates than males. (A) Bile flow rate. (B) Biliary cholesterol concentration. (C-D) average biliary and intestinal cholesterol secretion rates. (E-F) Cumulative biliary and intestinal cholesterol

secretion rates. (G) Sum of cholesterol secretion. ****$p<0.0001$. Data were analyzed by T test (upper) and linear regression (bottom). All data are shown as mean±SEM.

greater in females compared with males (figure 5.2B, C &F). The differences of biliary cholesterol concentration we found in male mice between genotypes were not as prominent as what was reported before[224]. The possible reason might be the effect of plant sterol in the diet that would inhibit cholesterol synthesis[208] that our experimental mice did not have access to yet was ingested by the mouse model used previously. Interestingly, intestinal total cholesterol excretion was increased in female KO mice compared with their WT littermates, indicating a possible G5G8 independent cholesterol elimination. The difference not observed in males (figure 5.2D, G&H). Besides, no differences of the genes implicated in RCT were found different between genotypes (figure 5.3). It is worth noting that NPC2 expressions are high in females than males in both liver and intestines in both genotypes (figure 5.3).

5.2.3 G5G8 KO male mice showed pronounced decrease in biliary cholesterol secretion when fed with PS containing diet (chow diet)

G5G8 KO male mice have about 90% lower biliary cholesterol levels compared to their WT littermates fed with chow diet[201]. We found that G5G8 KO mice had lower bile cholesterol concentration and biliary cholesterol excretion rates compared to their WT littermates yet this difference is significantly greater in females compared with males fed with PSF diet (figure 5.2B, C &F). To check if the plant sterol is the factor that affect the cholesterol secretion in G5G8 KO mice, we transferred male G5G8 KO mice and their WT littermates from PSF diet back to chow diet and monitor the development of STSL. About 10 weeks later, the plasma plant sterol levels of G5G8 KO mice reached a steady state and were more than 120-fold than WT littermates (figure 5.4A). Perfusion assay was performed on these mice and we detected more pronounced difference in biliary cholesterol secretion rates between G5G8 KO and WT mice (figure 5.4B) than what was found in mice fed with PSF diet (figure 5.2F). When maintained on chow diet, accumulation of non-cholesterol sterols may lead to the very low rates of cholesterol synthesis

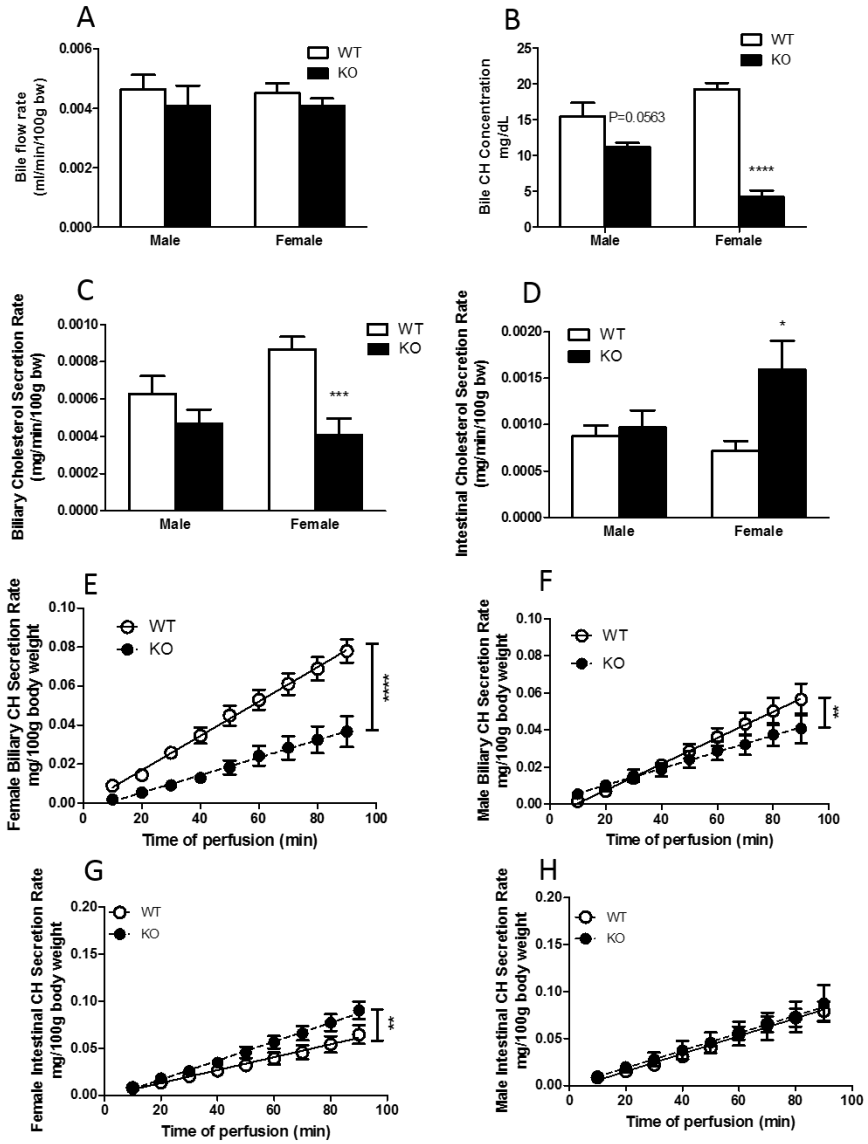

Figure 5.2. Female mice adapt to G5G8 deficiency by increasing TICE. (A) Bile flow rates. (B) Biliary cholesterol concentration. (C-D) average biliary and transintestinal cholesterol secretion rates of G5G8 KO mice and WT mice in both sexes during surgery. (E-F) Cumulative biliary cholesterol secretion of G5G8 KO and WT mice in both sexes during 90 minutes. (G-H) Cumulative intestinal cholesterol secretion of G5G8 KO and WT mice in both sexes during 90 minutes surgery. ****p<0.0001. Data were analyzed by two way ANOVA (upper) and linear regression (middle and bottom). All data are shown as mean±SEM.

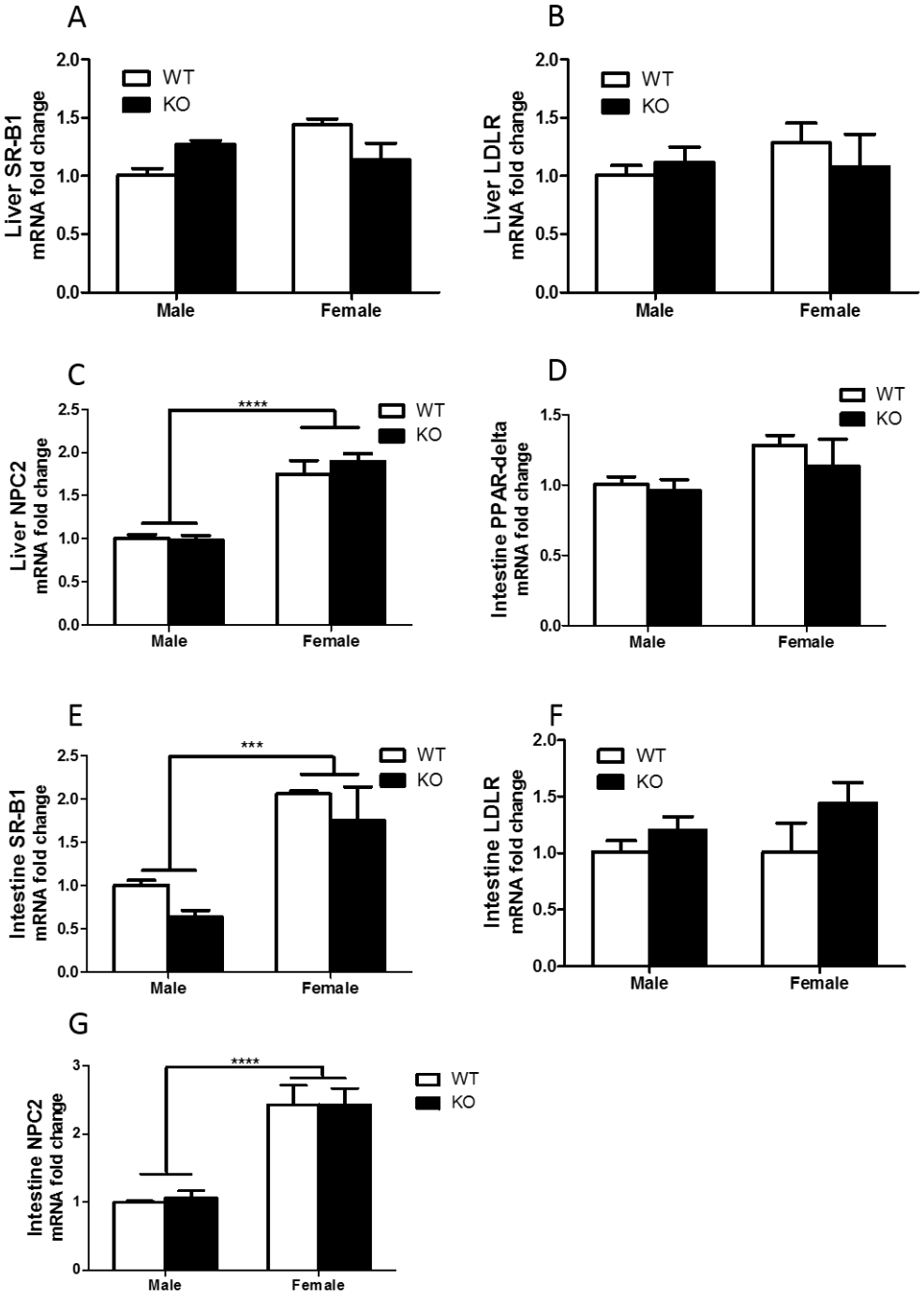

Figure 5.3. Relative mRNA expression in the livers and intestines. mRNA expressions were determined by real-time PCR. n=4~5 per group. Data were analyzed by two-way ANOVA. ***p<0.001, ****p<0.0001

in STSL[225] and that may accounts for the low cholesterol levels in bile of G5G8 KO mice fed with PS containing diet. This result also indicated that the exclusive role of G5G8 in biliary cholesterol secretion in male mice may be overestimated.

5.2.4 Estrogen can manipulate G5G8 expression in female mice

To determine the possible mechanism accounts for the greater dependence on hepatobiliary cholesterol secretion in female mice, we test liver G5G8 protein and mRNA levels of both sexes and female mice underwent estrogen replacement treatments. Ovariectomy was performed on WT female mice (C57BL/6, 8weeks age) and 2 weeks later these mice were assigned to groups to receive estradiol(E2, 0, 36, 200 or 600ug/ml, n=5 mice/group) via silastic tubing capsules for one month. A group of intact female mice (n=5) without drug treatment were included for control comparison. As expected, we found bare G5G8 expression in G5G8 KO mice in both sexes. And we also found that in WT groups, females had much more G5G8 expressions in the livers than males (figure 5.5A), which is consistent with the finding that female tend to secrete more cholesterol into bile than males[221]. Accordingly, we found E2 treatment upregulated liver ABCG5 expressions compared to intact control mice (figure 5.5B) that may explain why females express more G5 than males. Interestingly, the G5G8 mRNA expressions of livers were the opposite of their protein levels (figure 5.5C&D). There might be a post-translational protein manipulation mechanism that needs to be further determined.

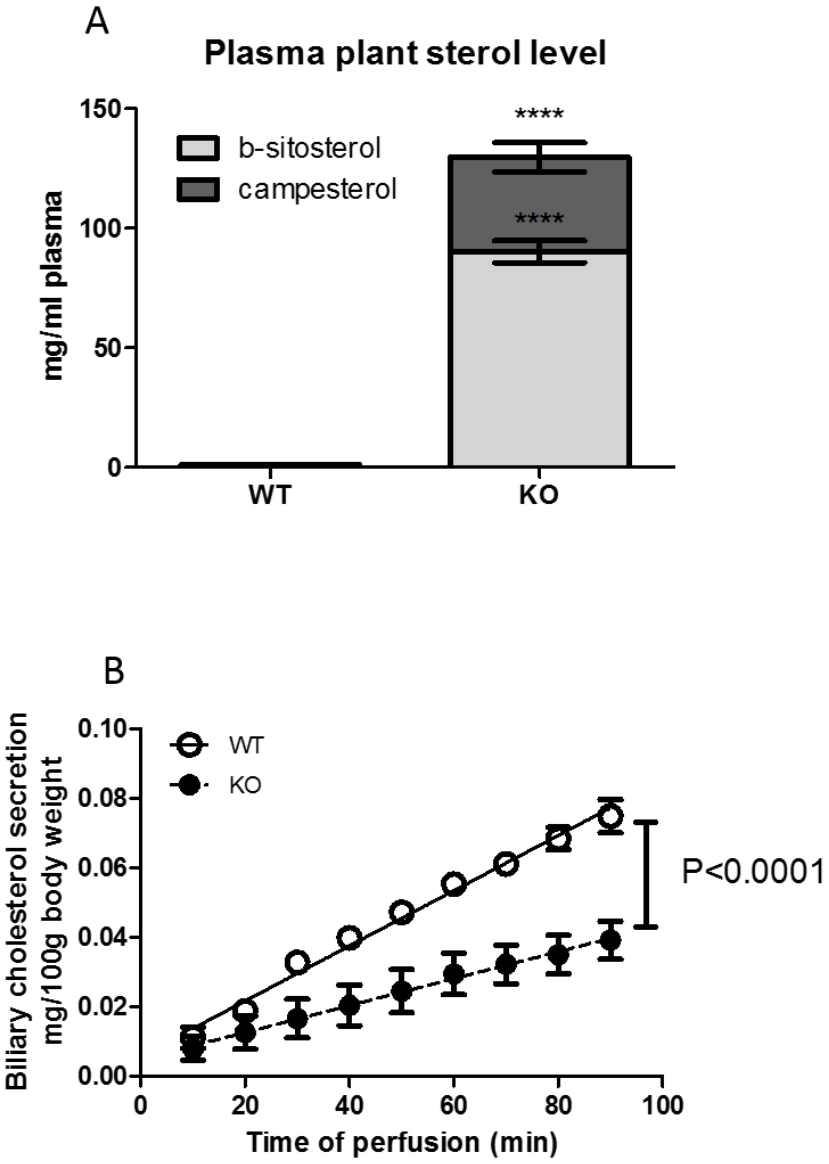

Figure 5.4. Biliary cholesterol secretion rate was recovered in male ABCG5/G8 KO mice when transferred back to chow diet for 10 weeks. (A) plasma plant sterol levels of male WT and G5G8 KO mice three weeks after transferring to chow diet. (B) Cumulative biliary cholesterol secretion of WT and G5G8 KO mice during 90 minutes. ****p<0.0001. Data were analyzed T test (left) and linear regression (right). All data are shown as mean±SEM.

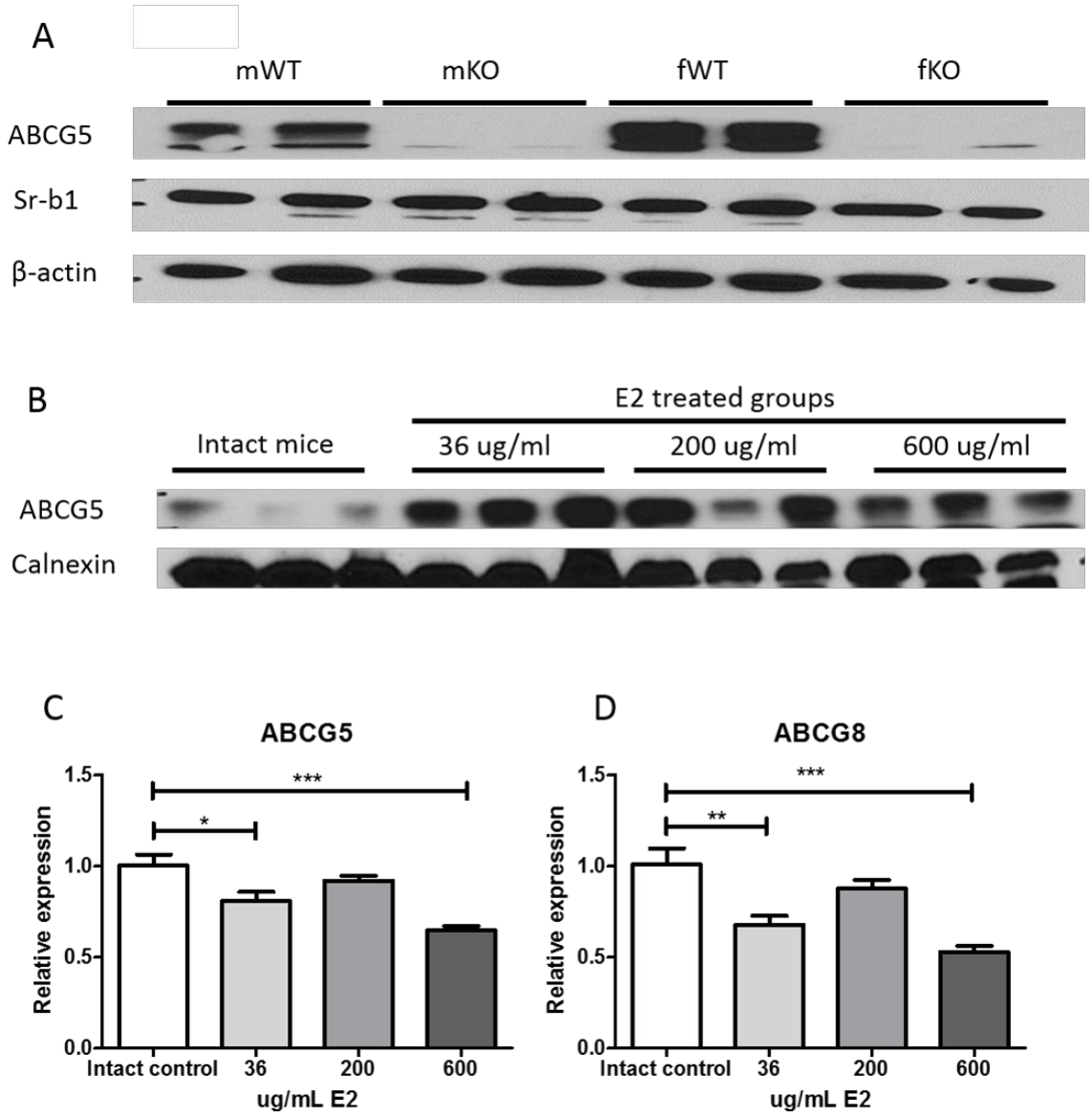

Figure 5.5. Estrogen upregulate ABCG5 expression in mRNA levels in the livers. (A-B) Western blot of ABCG5 and SR-B1 protein levels in livers of ovariectomized female mice treated with different doses of estrogen. (C-D) Relative mRNA expression for livers of ovariectomized female mice treated with different doses of estrogen. Data were analyzed by T test. All data are shown as mean±SEM. *p<0.05, **p<0.01, ***p<0.001.

5.3 Discussion

We made several findings within the present study: 1) Female mice preferentially eliminate cholesterol through the biliary pathway, whereas male mice depend on intestine. 2) Estrogen increases G5G8 that may account for this difference. 3) Female mice adapt to G5G8 deficiency by increasing TICE. 4) In the absence of sitosterolemia, male G5G8 KO mice maintain biliary cholesterol secretion with an unknown mechanism.

TICE is a specific pathway that accounts for up to 70% of daily total body neutral sterol secretion in mice[140]. However, the possible transporters that accounts for cholesterol delivery to intestinal lumen remains unknown. The sterol efflux proteins ABCG5/ABCG8 seem to be good candidates for the mediation of cholesterol secretion from enterocytes to the intestinal lumen. However, many studies disagree on the relevance of G5G8 to TICE[132, 151, 208], making its role unclear.

However, there are three points that are worth noting in the previous studies. Firstly, male mice are the most prevalent subjects in cholesterol homeostasis and TICE studies, and gender differences were frequently neglected in the past. However, the rate of gallstone disease in women is two to three times higher than in men[219, 220], due to the cholesterol supersaturation of bile[221] caused by increased biliary cholesterol secretions. In addition, female mice express much more ABCG5 and ABCG8 at mRNA levels, stimulated by high cholesterol synthesis or dietary intake in the absence of cholesterol[223]. We also found that female mice express more G5 protein in livers than male mice fed with a PSF diet (figure 5.5A). It is rational to research cholesterol homeostasis in both sexes. Secondly, in previous TICE studies, intestinal perfusion assays were widely used to measure the average rate of transintestinal cholesterol secretion. In some of the studies, bile was cannulated for 15 minutes to measure the basal bile flow and the

cholesterol concentration, yet hepatobiliary cholesterol secretion rates were not measured at the same time as TICE[137, 151]. We developed an assay that allowed us to compare the relationship between the rates of hepatobiliary and transintestinal cholesterol secretion and how they cooperate in whole body cholesterol homeostasis. Lastly, mutations in either ACBG5 or ABCG8 result in STSL marked with elevated circulating phytosterol levels[208] and accumulation of non-cholesterol sterols may account for the very low rates of cholesterol synthesis in STSL[217]. Most of the previous studies were based on G5G8 KO mice fed with a chow diet. It is important to understand the role of G5G8 in cholesterol homeostasis exclusive of STSL. In our experiment, we measured and compared both biliary and intestinal cholesterol secretion rates on G5G8 KO mice and their WT littermates in both sexes. These mice were maintained on PSF diet to prevent STSL after weaning. Thus, we can determine the effects of sex and STSL on the cholesterol elimination process.

Not surprisingly, we found that female mice had high biliary cholesterol concentration and secretion rates than male mice (figure 5.1), which is consistent with the phenomenon that females tend to secrete more cholesterol into bile than males[221]. Furthermore, females express higher G5 in the liver than male mice, and estrogen upregulated the G5 expression in female mice livers (figure 5.5A). In addition to estrogen induced biliary cholesterol saturation, these results may also explain why patients of any gender who underwent hormone replacement therapy tend to have increased risk for gallstone formation[221]. But we did not find a consistent expression of G5G8 at mRNA levels (figure 5.5B). There might exist a posttranslational mechanism that accounts for it. However, the female mice had lower total cholesterol secretion rates in the intestine than male mice (figure 5.1D&F), and the sum of the total cholesterol secreted from both hepatobiliary and transintestinal pathways remained similar in both sexes (figure 5.1G). This data suggests that female mice defaults hepatobiliary

pathways to eliminate cholesterol, rather than transintestinal pathways as compared with males. The results also confirmed a complimentary relationship between hepatobiliary and transintestinal cholesterol secretion routes to maintain whole body cholesterol elimination, a finding that posited in another study.

As expected, we found that G5G8 KO mice have lower cholesterol concentration than WT mice in both sexes, a finding which was widely reported (figure 5.2B&C). However, this difference is significantly greater in females compared with males (figure 5.2B, C &F). The differences in male mice between genotypes were not as prominent as what was reported before[224], which means that the G5G8 KO mice maintained a certain level of biliary cholesterol secretion in the absence of plant sterol. These mice were maintained on a PSF diet, thus explaining the plant sterol's effects on cholesterol secretion that our experimental mice did not have access to, unlike the previous models. Based on the mechanism that was mentioned in the second paragraph, it is very likely that blockage of cholesterol synthesis induced by accumulation of plant sterol made the role of G5G8 in RCT overestimated. Furthermore, deficiency in plant sterol may stimulate some possible G5G8 independent pathways in male G5G8 KO mice for biliary cholesterol elimination.

In addition, we found that the total cholesterol excretion in the intestine was increased in female KO mice compared with their WT littermates, indicating another possible G5G8 independent cholesterol elimination pathway. Two possible G5G8 independent cholesterol elimination pathways were reported before. Annemiek Groen at el. reported that the ATP8B1 deficient mice maintained a high cholesterol output as WT mice did independent of G5G8 activity. The possible cause for this phenotype is due to the direct extraction of cholesterol from the canalicular membrane by hydrophobic bile salts caused by an ATP8B1 deficiency[214]. Donna at el. reported a G5G8 independent mechanism for basal biliary cholesterol secretion in

rats during lactation, but a dependence on G5G8 for maximal biliary cholesterol secretion[213]. The possible transporter responsible for increased TICE in female G5G8 KO mice needs to be further explored.

In summary, the present studies demonstrate sexually dimorphic cholesterol elimination in mice. Female mice preferentially eliminate cholesterol through the biliary pathway, whereas males depend on the small intestine with the possible mechanism that estrogen increases G5G8 expression in the liver. Female mice adapt to G5G8 deficiency by increasing TICE by an unknown G5G8 independent cholesterol elimination mechanism. In the absence of STSL, male G5G8 KO mice maintain biliary cholesterol secretion by an unknown mechanism, and the role of G5G8 may be overestimated in mice with STSL.

CHAPTER VI. CETP alters route of total and HDL cholesterol elimination

6.1 Introduction

Cardiovascular disease (CVD) is the leading cause of death globally and account for approximate one-third of all deaths in the United States[226]. Plasma concentration of LDLc is thought to be one of the primary predictors of CVD incidence and lowering LDLc has been the primary therapeutic target for preventing and treating CVD for decades [226]. However, CVD-associated mortality and morbidity were not reduced prominently with LDLc lowering with statin therapy [227], HDLc elevating therapy has become the promising therapeutic target because HDLc was shown to be the even more relevant predictor than LDLc for CVD in a great amount population studies [100]. HDLc's cardioprotective role was explained by many mechanisms, but functioning in facilitating Reverse Cholesterol Transport (RCT) is widely accepted.

The classic RCT pathway starts from the efflux of cholesterol from peripheral tissues and from macrophages in the arterial wall through the cholesterol transporter adenosine triphosphate-binding cassette transporter ABCG1 and ABCA1 [228, 229]. The cholesterol carried by lipoproteins is transported to the liver for uptake by the specific receptors SR-BI on the hepatocyte surface. HDL, although some findings on transgenic mice indicate that LDL was also involved [124], is widely accepted as the main lipoprotein that mediates this process. The cholesterol is transferred to bile through canalicular membrane of hepatocyte after uptake at the basolaterol side by cholesterol transporter ABCG5 and ABCG8[22]. Biliary cholesterol is secreted to intestinal lumen where a certain amount of reabsorption of cholesterol occurred [230]. Remaining of cholesterol is eliminated through feces.

This hepatobiliary pathway is considered to be the classic and only route for cholesterol secretion from body. However, it is found that some animal models which hepatic cholesterol

metabolism were genetically altered still maintained certain amount of fecal neutral sterol levels [22, 128, 129, 141, 215, 216]. It was reported that in ABCB4-/- mice with abrogated biliary phospholipid and cholesterol secretion that intravenously injected free cholesterol partly ended up in the feces as neutral sterol [128], suggesting a direct route from blood to intestinal lumen. This pathway was known as Transintestinal Cholesterol Excretion (TICE) [132], an important pathway for cholesterol elimination in mice. It was further proved that the cholesterol secreted into intestinal lumen originated from blood directly rather than intestine itself. Which lipoproteins are involved in this transport remains controversial. Several receptors mediate hepatic uptake of cholesterol from circulating lipoproteins for cholesterol elimination. Scavenger receptor B1 (SR-B1) preferentially binds to ApoA1 and mediates the uptake of esterified cholesterol mainly from HDL [134]. Unexpectedly, TICE was increased about two-fold in SR-B1-deficient mice [137]. Similarly, ABCA1-/- mice that lack normal HDL content secreted radiolabeled cholesterol from plasma into intestinal lumen at a similar rate as WT mice did and TICE was unaltered between two mice models [140]. Low density lipoprotein receptor (LDLR) plays an important role in the classic hepatobiliary route and clears apolipoprotein (apo)B-containing LDL particles or apoE lipoproteins [231]. However, contrary to liver, Cédric Le May *at el*. found that the acute repression of TICE was dependent on the LDLR yet LDLR-/- mice tended to have increased TICE [139]. Thus the findings on the lipoproteins contributed to TICE remains unclear and needs to be explored.

Cholesteryl ester transfer protein (CETP) is a 74kDa glycoprotein to transfer cholesteryl ester from HDL to larger lipoprotein like LDL or VLDL, and to exchange triglyceride from larger lipoproteins to HDL concomitantly [232]. CETP transgenic mouse strains have shown higher cholesterol levels of VLDL and LDL and lower levels of HDL than normal WT mice [233]. In this study, we investigated the roles of different lipoproteins in TICE by using CETP transgenic mouse

model with varied plasma lipoprotein pools. We also evaluated the role of HDL in TICE by injecting tritium labeled HDL particles to mice of two genotypes. Data indicated that CETP enhanced hepatobiliary cholesterol secretion in mice yet which was not contributed by HDL. In contrast, CETP modified HDL particles favored TICE for cholesterol elimination.

6.2 Results

6.2.1 CETP had no effects on physiological features and plasma lipoprotein profile in mice maintained on Rodent Chow Diet

To define if CETP changed physiological features on mice under basal conditions, male WT and CETP Tg mice were transferred to Rodent Chow Diet after weaning until 12 weeks for experimental use. As expected, we found that the plasma CETP activity was barely detected in WT mice, but was much abundant in transgenic mice and was comparable to what is in human plasma (Figure 6.1B). Interestingly, body weight and plasma cholesterol concentration seemed not affected by this and did not differ between two genotypes (Figure 6.1 A&C). Similarly, FPLC analysis did not show plasma lipoprotein distribution differences between genotypes (Figure 6.1D). CETP genes facilitates the transport of cholesteryl esters and triglycerides between lipoproteins in plasma. The inefficiency of CETP in transgenic mice fed with Rodent Chow Diet may be due to the lacking of sufficient triglycerides pool as substrates for CETP to work with in these mice.

6.2.2 CETP had no effects on either biliary or transintestinal total cholesterol secretion rates in mice maintained on Rodent Chow Diet

To determine if CETP has effects on biliary and tranintestinal cholesterol secretion rates under basal conditions, we performed perfusion assays described in Materials and Methods on male CETP and WT mice at age of 12 weeks to compare biliary and transintestinal cholesterol secretion rates simultaneously. We found that CETP does not affect bile flow rates and bile cholesterol concentrations in mice maintained on Rodent Chow Diet (Figure 6.2A&B). Similarly, we did not detect a difference on biliary and transintestinal cholesterol secretion rates in a

period of 90 minutes between genotypes either (Figure 6.2C-F). Interestingly, compared to the relative

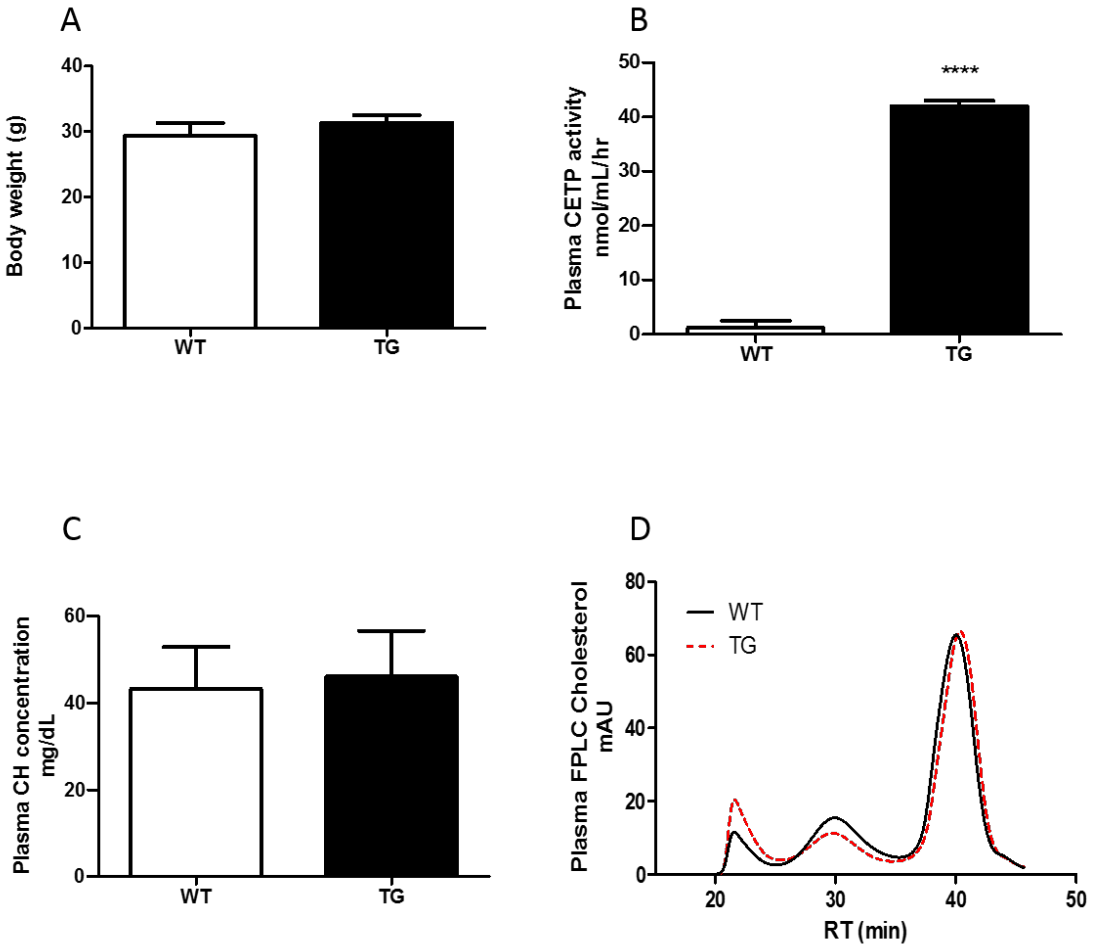

Figure 6.1. CETP had no effects on mice body weights, plasma cholesterol concentration and plasma lipoprotein distribution in mice fed with Rodent Chow diet. (A) Mice body weights, (B) plasma CETP activity, (C) cholesterol concentration and (D) FPLC fractions analysis of 12-week male WT and CETP transgenic (Tg) mice fed with rodent chow diet. n=3~4 per genotype. Data are mean ± S.E.M. Panel (A-C) were determined by two-tailed t-test. ****p<0.0001

low biliary cholesterol secretion rates (Figure 6.2E), both genotypes performed a compensatory increased intestinal cholesterol secretion rates (Figure 6.2F). But when cholesterol secretion rates were compared within genotypes, we found that the both male WT and CETP mice favored intestine to eliminate cholesterol when maintained on chow diet (Figure 6.8A&B). (Female mice were the opposite, unpublished data) Furthermore, the sum of total cholesterol secreted from both hepatobiliary and transintestinal pathways remain similar in both genotypes (Figure 6.8C). These data may suggest a complimentary relationship between hepatobiliary and transintestinal cholesterol secretion routes to maintain whole body cholesterol elimination.

6.2.3 CETP upregulated mice body weights, liver weights and plasma cholesterol levels and altered HDL and LDL cholesterol contents predominantly in response to two-week high fat, high cholesterol Western Diet feeding

It is known that cholesterol upregulates CETP activity and hepatic CETP gene expression in transgenic mice expressing human CETP, and high fat content diet further enhance this effect [234]. To make the physiological features of CETP transgenic mice more prominent, 8 weeks CETP transgenic mice and WT littermates were transferred to high fat, high cholesterol Western Diet for two weeks. As expected, CETP activity was predominantly elevated by 2 weeks Western Diet feeding (Figure 6.3B). Different from mice fed with Rodent Chow Diet, the body weights and plasma cholesterol levels were higher in CETP transgenic groups compared to WT controls accordingly (Figure 6.3A & C). Furthermore, CETP predominantly reduced HDL and elevated LDL content according to mice plasma FPLC analysis (Figure 6.3D).

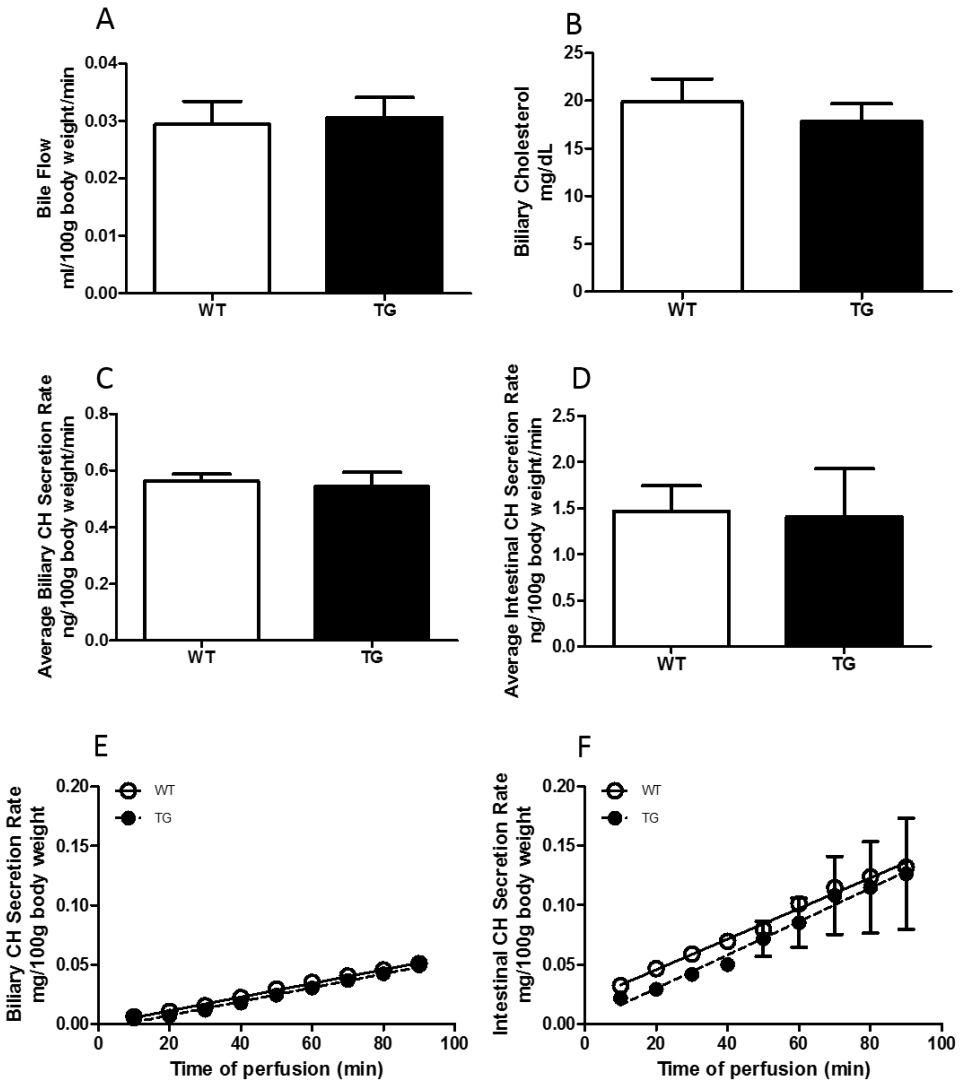

Figure 6.2. CETP had no effects on either biliary or transintestinal total cholesterol secretion rate in mice fed with rodent chow diet. (A) average bile flow rates, (B) Biliary cholesterol concentration, (C) average biliary cholesterol secretion rates and (D) average transintestinal cholesterol secretion rates of male WT and Tg mice at age of 12 weeks in a period of 90 minutes. (E) Cumulative biliary cholesterol secretion rates and (F) Cumulative intestinal cholesterol secretion rates of 12 weeks male WT and Tg mice throughout 90 minutes. n=3 per genotype. Data are mean ± S.E.M. Panel (A-D) were determined by two-tailed t-test. Panel (E&F) were determined by linear regression. P value less than 0.05 is considered to be significant.

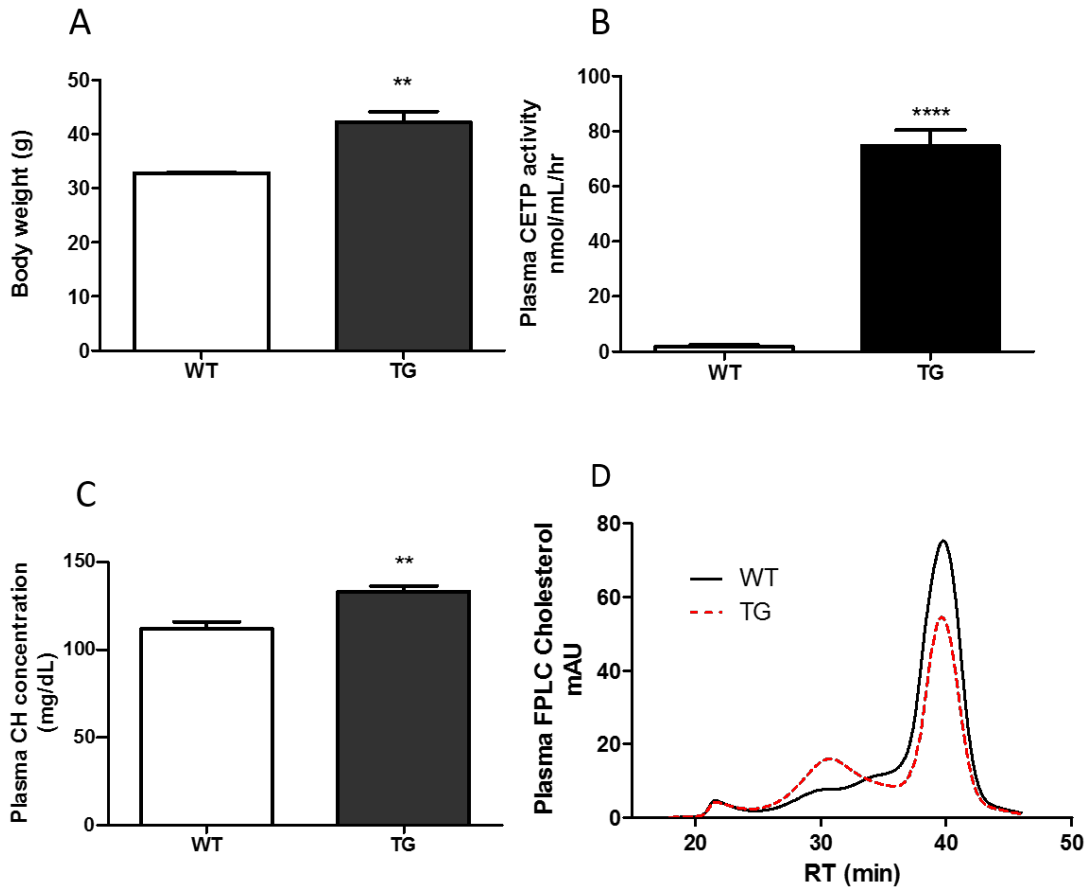

Figure 6.3. Tg mice had higher mice body weights, plasma cholesterol concentration and had altered plasma lipoprotein distribution in response to two weeks western diet feeding. Mice body weights (A), Plasma CETP activity (B), cholesterol concentration (C) and FPLC fractions analysis (D) of 12-week male WT and Tg mice fed with Western Diet for two weeks. n=6 per genotype. Data are means ± S.E.M. Panel (A-C) were determined by two-tailed t-test. *p<0.05, **p<0.01, ****p<0.0001

6.2.4 CETP altered hepatobiliary and transintestinal total cholesterol secretion rates in mice maintained on Western Diet for two weeks

Similar as described above, we performed perfusion assay on 12-week male mice maintained on Western diet for two weeks. Bile total cholesterol concentrations, but not bile flow rates of CETP group were higher compared to WT mice (Figure 6.4A&B), which indicated both increased average and cumulative biliary cholesterol secretion rates in a period of 90 minutes (Figure 6.4C&E). In contrast, CETP mice showed a decreased intestinal total cholesterol secretion rates compared to WT group (Figure 6.4D&F). Similarly, when cholesterol secretion rates were compared within genotypes, we found that when maintained on Western Diet, WT mice did not have differences between biliary and intestinal total cholesterol secretion rates yet CETP mice had much lower intestinal cholesterol secretion rates compared to their biliary pathway (Figure 6.9A&B). Interestingly, when cumulative biliary and intestinal cholesterol secretion rates were added up, there were no differences between two genotypes on sum of cholesterol secretion rates (Figure 6.9C). Combine with the results in mice fed with chow diet, this results may further illustrate that the hepatobiliary and intestinal cholesterol secretion routes are compensatory to each other in cholesterol elimination independent of genotype. Furthermore, CETP may facilitate cholesterol to hepatobiliary route for elimination when stimulated by Western Diet. Most of genes involved in cholesterol homeostasis were not found significantly different between genotypes in both liver and intestine (figure 6.5).

6.2.5 CETP altered hepatobiliary and transintestinal HDL-derived cholesterol secretion rates in mice fed with Western Diet for two weeks

By knowing that CETP would alter hepatobiliary and transintestinal total cholesterol secretion rates when stimulated with Western Diet, we next want to illustrate if this alteration was due to

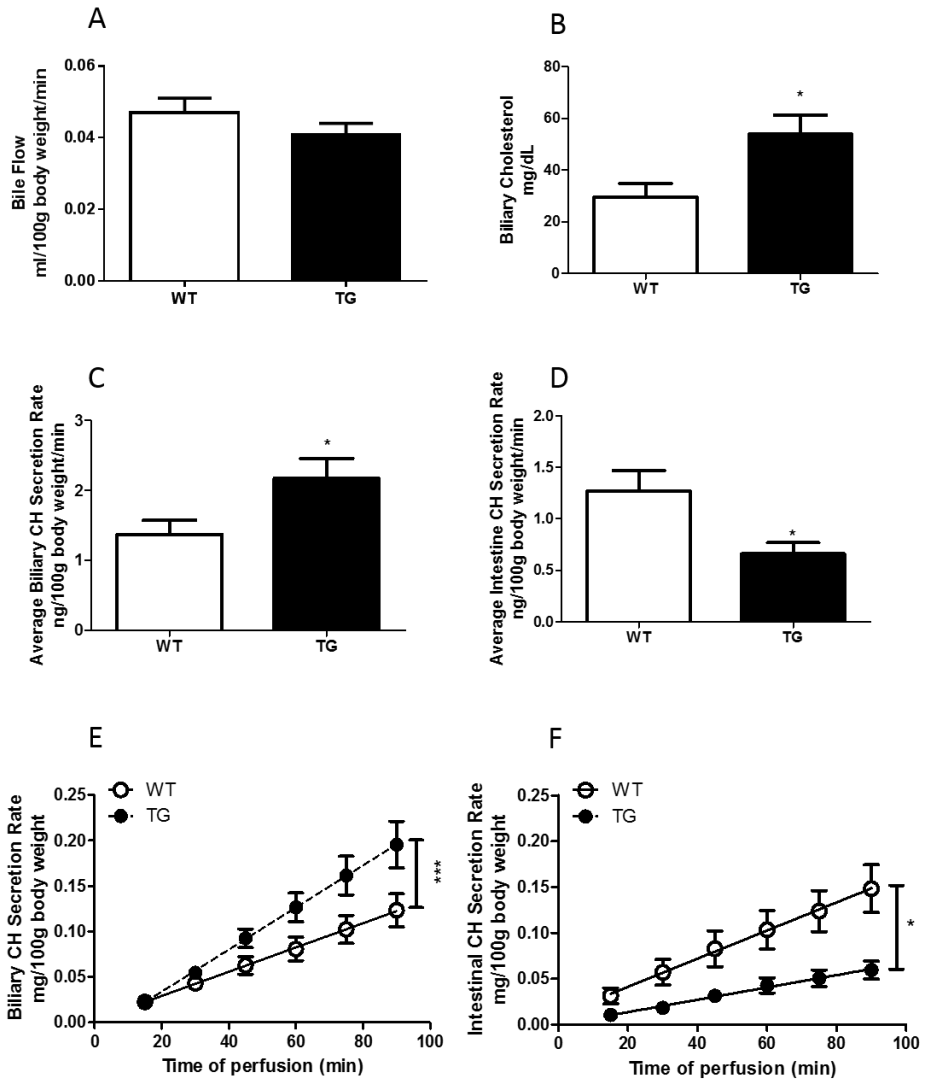

Figure 6.4. CETP upregulated biliary and downregulated intestinal total cholesterol secretion rates in mice fed with western diet for two weeks. Unlike bulk cholesterol secretion, HDL-derived cholesterol were preferentially delivered to the intestine in Tg mice. (A) average bile flow rates, (B) Bile cholesterol concentration, (C) average biliary cholesterol secretion rates and (D) average intestinal cholesterol secretion rates throughout 90 minute. (E) Cumulative biliary cholesterol secretion rates and (F) transintestinal cholesterol secretion rates throughout 90 minutes. n=6 per genotype. Data are means ± S.E.M. Panel (A-D) were determined by two-tailed t-test. Panel (E&F) were determined by linear regression. *p<0.05, **p<0.01, ****p<0.0001.

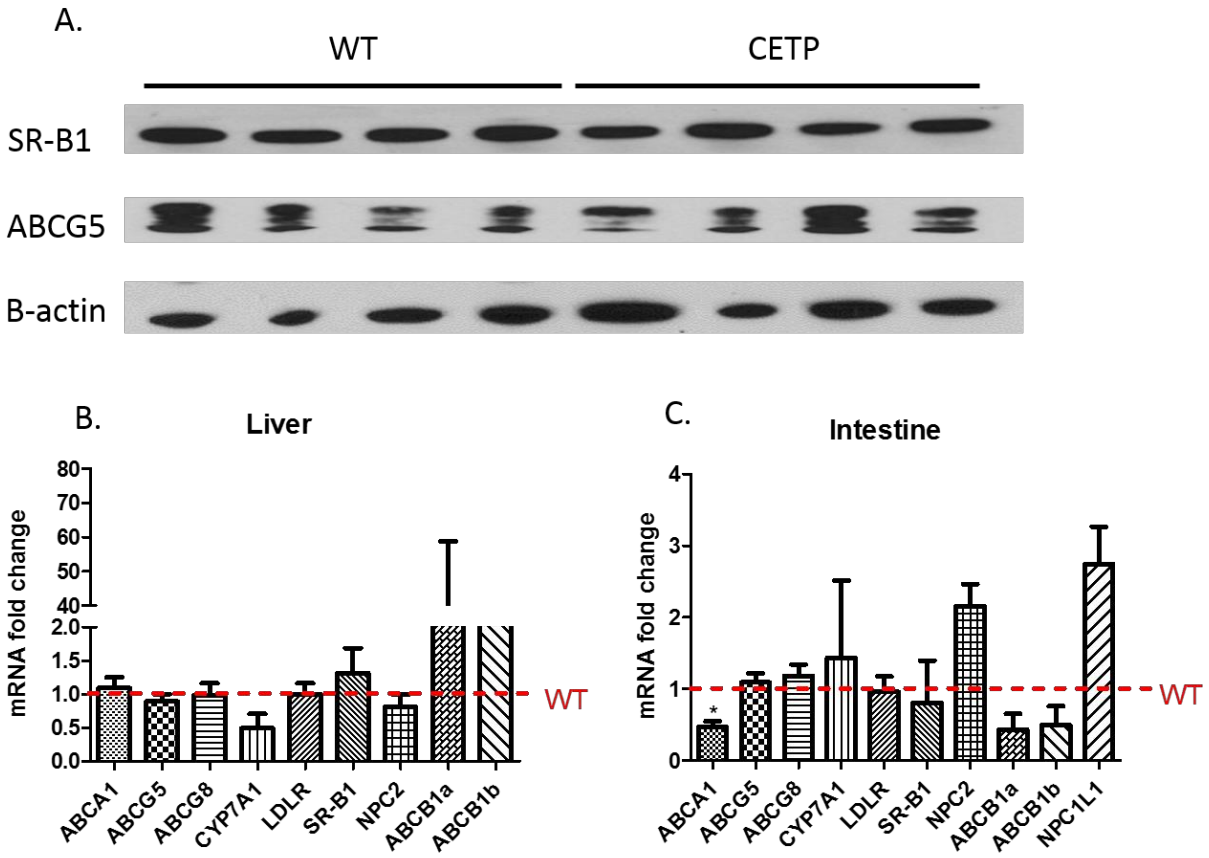

Figure 6.5. Gene expression in both protein levels and mRNA levels in liver and intestines of WT and CETP transgenic mice. (A) Western blot of ABCG5 and SR-B1 protein levels in livers of WT and CETP transgenic mice. (B) Relative mRNA expression of genes for livers of WT and CETP transgenic mice. (C) Relative mRNA expression of genes for intestines of WT and CETP transgenic mice. Data were analyzed by T test. All data are shown as mean±SEM. *$p<0.05$.

the different routes that lipoprotein-derived cholesterol went through in the presence of CETP. In another study, we injected 12 weeks male CETP and WT mice fed with both Rodent Chow and Western Diet with 0.6μCi/mouse [^{3}H]HDL-cholesterol ester retro-orbitally 30 minutes before perfusion assay. Similar to total cholesterol secretion patterns, when maintained on Chow diet, CETP has no effects on HDL-derived cholesterol secretion rates from both routes (Figure 6.6A&B). Interestingly, we found that HDL-C secretion patterns of both biliary and intestinal routes were the opposite to total cholesterol secretion rates when the mice were fed with Western Diet for two weeks. CETP mice showed decreased hepatobiliary and increased intestinal HDL-C secretion rates compared to WT controls (Figure 6.6C&D). These results suggest that under Western Diet feeding condition, HDL-derived cholesteryl esters were preferentially delivered to the intestinal lumen directly rather than bile in CETP transgenic mice for cholesterol elimination. Therefore, the increased bulk biliary cholesterol secretion rates we observed in CETP Tg mice were not mainly contributed by HDL.

6.2.6 CETP-modified HDL favored intestine for cholesterol elimination

It was reported that treatment with CETP may delay the clearance of rat HDL cholesteryl ester from blood and its uptake by the liver[235]. This finding may explain why HDL-derived cholesteryl ester in CETP mice preferentially delivered to the intestinal lumen directly rather than bile for cholesterol elimination. To further explore if CETP-modified HDL altered the pathway for HDL-derived cholesteryl ester elimination, we extracted and labeled HDLs from both 12 weeks WT and CETP male mice maintained on western diet for two weeks with [^{3}H]-cholesteryl ester. 0.6μLCi/mouse labeled WT and CETP HDLs were injected back to two groups of male WT mice retro-orbitally respectively 30 minutes before perfusion assay. We did not detect differences between biliary WT HDL and CETP HDL derived cholesterol secretion rates (Figure 6.7A), which is consistent to what was reported [235]. However, intestinal CETP HDL-C

secretion rates were higher than that of WT HDL-C, which indicated that CETP modified HDL preferentially delivered cholesterol to intestine for cholesterol elimination (Figure 6.7B).

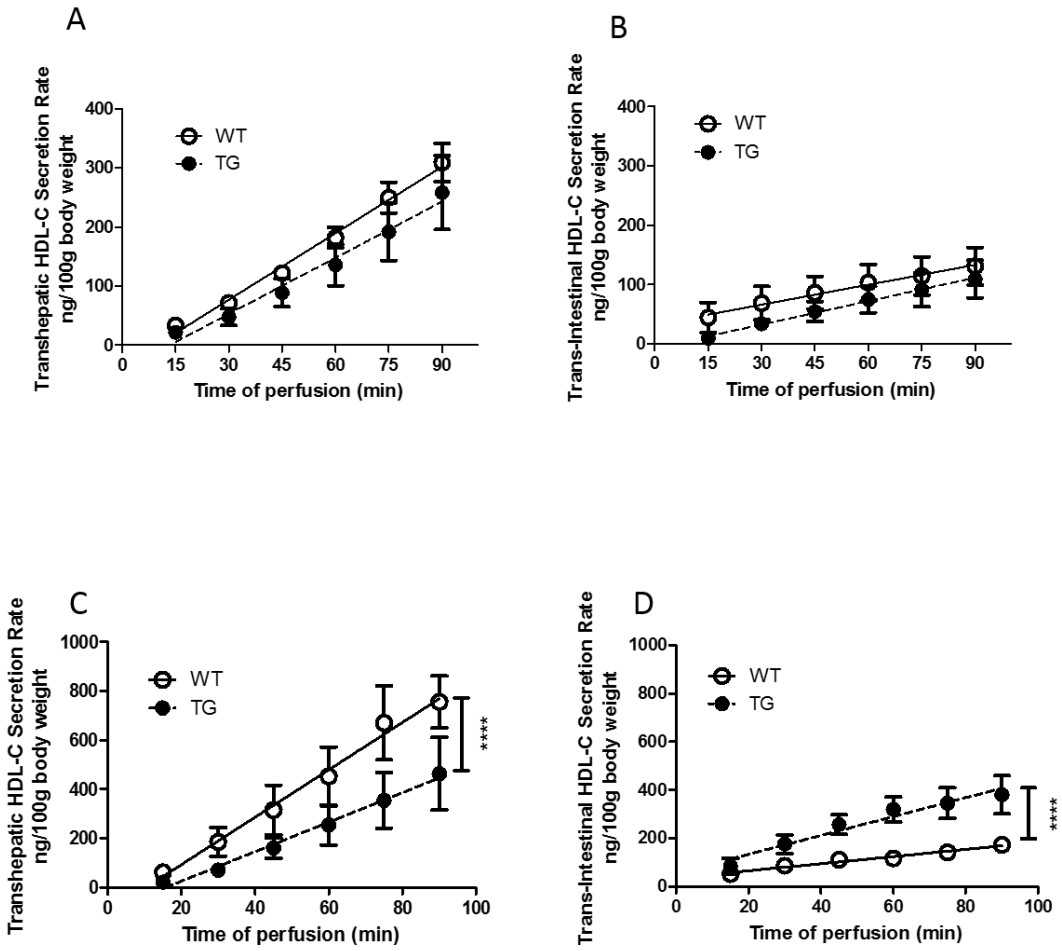

Figure 6.6. CETP altered hepatobiliary and intestinal HDL-derived cholesterol secretion rates in mice fed with Western Diet but not Chow Diet. 0.6 µCi/mouse of human [^{3}H]-cholesteryl oleate HDL was injected into 12 weeks male WT mice 30 minutes before surgery. (A) cumulative biliary [^{3}H]-cholesterol secretion rates in mice fed with chow diet. (B) cumulative intestinal [^{3}H]-cholesterol secretion rates in mice fed with chow diet. (C) cumulative biliary [^{3}H]-cholesterol secretion rates in mice fed with Western Diet. (D) cumulative intestinal [^{3}H]-cholesterol secretion rates in mice fed with Western Diet. n=4~5 per genotype. Data are means±SEM. Data were analyzed by linear regression. ****$p<0.0001$.

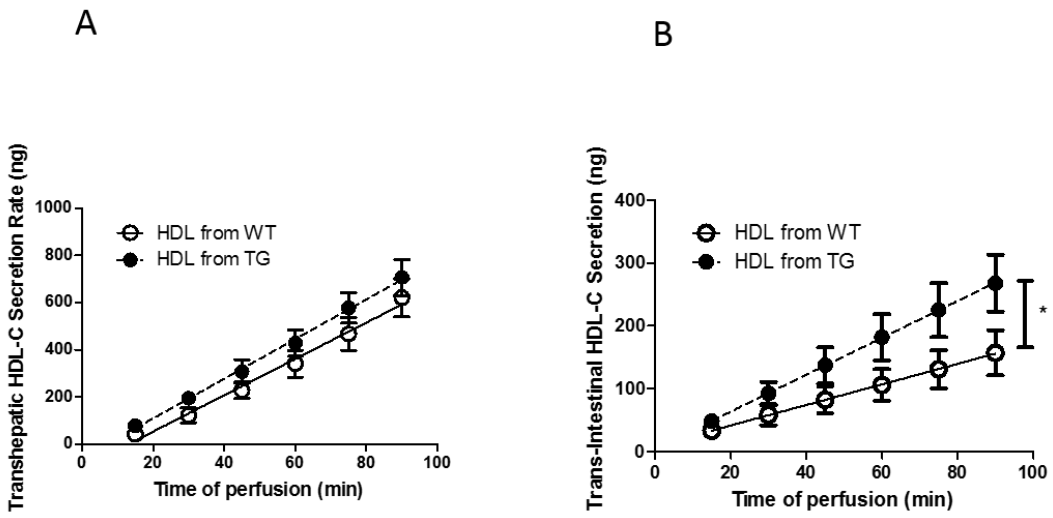

Figure 6.7. CETP-modified HDL favored intestine for cholesterol elimination. 0.6 µCi/mouse of [^{3}H]-cholesteryl oleate HDL extracted from WT and Tg mice fed with Western Diet for two weeks was injected into 12 weeks male WT mice 30 minutes before surgery. (A) Cumulative biliary [^{3}H]-cholesterol secretion rates. (B) Cumulative intestinal [^{3}H]- cholesterol secretion rates. n=6 per genotype. Data are means±SEM. Data were analyzed by linear regression. *$p<0.05$.

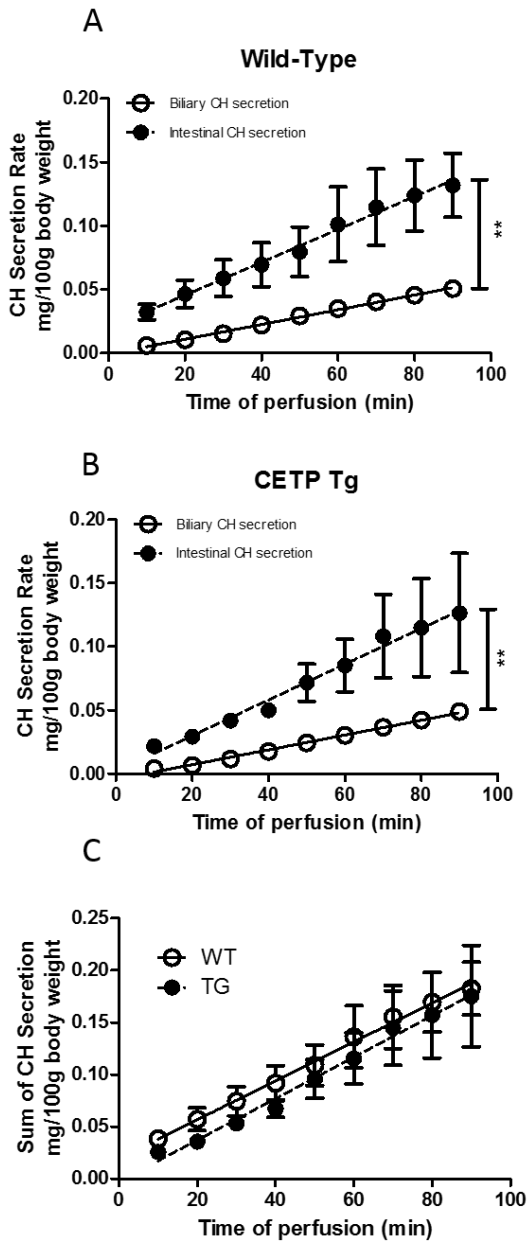

Figure 6.8. Both Tg and WT mice have higher intestinal than biliary total cholesterol secretion rates when maintained on chow diet. (A) Cumulative total cholesterol secretion rates in WT mice. (B) Cumulative total cholesterol secretion rates in Tg mice. (C) Sum of biliary and intestinal total cholesterol secretion rates in both genotypes. n=3 per genotype. Data are means±SEM. Data were analyzed by linear regression. **$p<0.01$.

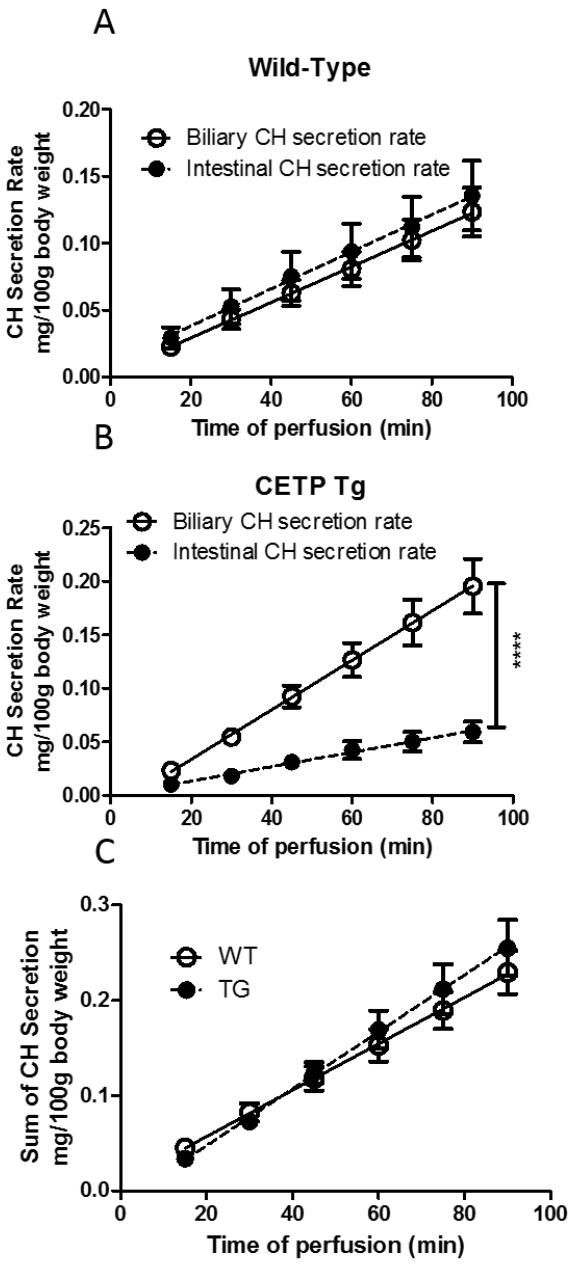

Figure 6.9. Tg but not WT mice had higher biliary than intestinal total cholesterol secretion rates in response to two-week Western Diet feeding. (A) Cumulative total cholesterol secretion rates in WT mice. (B) Cumulative total cholesterol secretion rates in Tg mice. (C) Sum of biliary and intestinal total cholesterol secretion rates in both genotypes. n=5~6 per genotype. Data are means±SEM. Data were analyzed by linear regression. ****$p<0.0001$.

6.3 Discussion

We made several findings within the present study: 1) CETP upregulated biliary and downregulated intestinal total cholesterol secretion rates. 2) HDL-derived cholesterol ester in CETP transgenic mice favored the intestine for cholesterol elimination. This can be explained by the fact that CETP-modified HDL favored intestine for cholesterol elimination. 3) Active cholesterol secretion pathways in both liver and intestine appear to work corporately to maintain cholesterol excretion routes.

Firstly, we reported a method for simultaneous measurement of intestinal and biliary cholesterol secretion for at least 90 minutes to compare cholesterol elimination rates between hepatobiliary and transintestinal pathways. In previous TICE studies, intestinal perfusion assays were widely used to measure the average rate of transintestinal cholesterol secretion. In some of the studies, bile was cannulated for 15 minutes to measure the basal bile flow and cholesterol concentration, yet hepatobiliary cholesterol secretion rates were not measured at the same time with TICE[137, 151]. We developed an assay that allowed us to compare the relationship between the rates of hepatobiliary and transintestinal cholesterol secretion. During intestine perfusion, we ligated the bile duct and cannulated gall bladder for bile collection in 90 minutes period. Because the intestine is the main site for bile salt reabsorption, the bile duct ligation in the classic intestine perfusion assay cut off the enterohepatic circulation of bile salts and other non-lipid components found in bile. As amphipathic molecules with hydrophobic and hydrophilic regions, conjugated bile salts function as cholesterol acceptor in cholesterol secretion process [236]. To solve the bile salts exhaustion issue during the assay, we infused the mice with taurocholate through the tail vein to supplement exogenous bile salts to maintain normal biliary cholesterol secretion. To mimic the rate of physiological bile acids secretion to the intestinal lumen, the bile acid amount in the intestinal perfusate we used was at the similar range of what

is in the real bile (Figure 6.10). Thus, this assay allows us to compare the hepatobiliary and transintestinal cholesterol secretion rates simultaneously so that we can have an idea of the potential relationship between the two pathways and how they cooperate in whole body cholesterol homeostasis.

TICE is a specific pathway that accounts for up to 70% of daily total body neutral sterol secretion in mice[140]. However, the possible lipoproteins that deliver cholesterol to this pathway remain controversial. It was reported that HDL does not mediate TICE based on the evidence that ABCA1-/- mice did not show altered TICE rates as compared to WT mice[140]. Furthermore, TICE increased about two-fold in SR-B1-deficient mice[137]. However, contrary to the liver, Cédric Le May *at el*. found that the acute repression of TICE was dependent on the LDLR when PCSK9 was present, yet LDLR-/- mice tended to have increased TICE[139]. Thus, the extent on the lipoproteins contributed to TICE remains unclear and needs to be explored. Mice lack CETPs and have much lower biliary cholesterol relative to bile salt secretion as compared with humans[237]. CETP transgenic mice overcame the issue and humanized the biliary lipid secretion. CETP transgenic mouse strains have shown altered plasma lipoprotein pool composition[233], and plasma CETP activity can be further stimulated by high fat diet (Figure 5.3D). Therefore, CETP transgenic mice are proper models to study the role of the contributions of different lipoproteins to TICE.

In the first study, we did not see many differences in physiological features of CETP transgenic mice fed with chow diet, following no difference in both hepatobiliary and transintestinal cholesterol secretion rates as compared with WT mice (Figure 6.1&6.2). Although CETPs were highly expressed in the plasma of transgenic mice, HDLs are still the main components in the plasma lipoprotein pool[232]. Indeed, studies with the human hepatoma cell line HepG2 show that the efficiency of CETP on the uptake of HDL cholesteryl ester is high only in the presence of

LDL[238]. Therefore, the results we saw from mice fed with chow diet may be due to the lack of a certain amount of proper substrate (LDL) and triglycerides pool (Figure 6.1D) for CETP to work with. Secondly, LDLR is ubiquitously expressed in mammals and plays an important role in maintaining cholesterol homeostasis[239]. LDLR recognizes ApoB-100 of LDL particles through a stoichiometry of a single copy of apoB-100 per one LDL particle per receptor monomer[239]. The low content of plasma LDL might be due to the efficiency of LDLR uptake in transgenic mice.

It is known that cholesterol upregulates CETP activity and hepatic CETP gene expression in transgenic mice expressing human CETP, and a high fat content diet further enhances this effect [234]. We found that the CETP transgenic mice had lowered HDLs and increased LDLs and other altered prominent physiological features when stimulated with a 2-week Western Diet feeding (Figure 6.3). Under this condition, CETP transgenic mice showed increased biliary total cholesterol secretion rates and decreased transintestinal cholesterol secretion rates compared to WT mice (Figure 6.4). This result might be explained by the different favored pathways of lipoproteins for cholesterol elimination as a result of the various lipoprotein pools that the two groups of mice have (Figure 6.3D). Interestingly, if the cholesterol secretion amount from both biliary and transintestinal pathways were added up, the total cholesterol secretion rates remained the same between two genotypes (figure 6.8). This result indicated that both liver and intestine active cholesterol secretion pathways appear to work corporately to maintain the cholesterol excretion route. This finding is consistent with many reports that the patients or mammals which experienced deficiency in normal biliary cholesterol secretion maintained normal levels of fecal neutral sterol[128, 130]. This finding further illustrates the importance of the intestine as a potential therapeutic target for cholesterol elimination when this function of the liver is deficient.

However, it is still not clear if the increased biliary and decreased transintestinal cholesterol secretion rates in CETP transgenic mice were caused by the increased plasma LDL content in transgenic mice. Compared to LDLs, HDLs are still the main components of the lipoprotein pool in the plasma of transgenic mice (Figure 6.3D). Next, human HDLs labeled with ^{3}H-Cholesteryl oleate were used to determine the role of HDL in biliary and transintestinal cholesterol secretion pathways. Interestingly, we found decreased biliary and increased transintestinal HDL-derived cholesterol (HDL-C) secretion rates in CETP transgenic mice, which are in contrast to the total cholesterol secretion rate trends (Figure 6C&D). According to this result, it is likely that HDL-C did not contribute to the increased biliary cholesterol secretion rates as much as to the transintestinal ones. Additionally, this result is consistent with the finding that the HDL features were modified by CETP and hepatic uptake of HDL-ester is delayed after treatment with CETP in rats[235]. Therefore, we hypothesized that HDL from transgenic mice was modified by the plasma CETP and favored the intestine rather than the liver for cholesterol elimination. HDL from both WT and transgenic mice (untreated HDL and CETP modified HDL) were collected and labeled with ^{3}H-cholesteryl oleate and injected back into WT mice retro-orbitally to track their elimination pathways. We found no differences in biliary HDL-C secretion rates from two treatments (Figure 6.7A), which is in agreement with the finding that the biliary HDL-C secretion was not different between CETP-treated and untreated HDL administration in rats[235]. Not surprisingly, the mice had higher transintestinal CETP modified-HDL-C secretion rates as compared to untreated HDL-C (Figure 6.7B). This is consistent with our previous finding that HDL favored the intestine for cholesterol elimination in transgenic mice (Figure 6.6D). We can assume that some remodeling took place on HDLs in the plasma which affected their uptake by liver and intestine.

The findings showed that CETPs upregulated biliary and downregulated intestinal cholesterol secretion rates, but HDL-derived cholesterol ester favored intestine for cholesterol elimination in CETP transgenic mice. This can be explained by the fact that CETP-modified HDL favored intestine for cholesterol elimination. More importantly, the perfusion assay we developed allowed us to compare the biliary and intestinal cholesterol secretion rates simultaneously, by which the relationship between the two pathways were studied for the first time. Both liver and intestine active cholesterol secretion pathways appear to work corporately to maintain cholesterol excretion routes.

CHAPTER VII. Summary and General Discussion for chapter II and III

7.1 Summary

The purpose of this study is to determine the hypothesis that fatty acids with different chain lengths and saturabilities affect allergic sensitization and anaphylaxis. We demonstrated that the absorption pathways of food allergens are varied by absorbing together with different fatty acids, leading to different sensitization and anaphylaxis statues. Our findings demonstrated that MCT diets promote allergic sensitization to ingested antigen in both acute and chronic feeding model whereas LCT played a suppressive role. Similarly, this result can be repeated when a chylomicron inhibitor, PL-81, when added to LCT. Unlike LCT, MCT do not form chylomicrons that might play a protective role in allergen sensitization and anaphylaxis phases. Formation of chylomicron induces macrophage cytokine production that may prevent Th2-biasing phenotypes in antigen-presenting cells (APC) and may promote oral tolerance. Instead of processing antigen into chylomicrons, we found that MCT promote antigens into Peyer's Patches, in particular, the local dendritic cells, more compared to LCT. The role of Peyer's Patches in food allergy is unclear although some studies suggested their roles in oral tolerance[240, 241]. Interestingly, LCT promote antigen to Peyer's Patches more than MLN, despite less than MCT. This result may be explained by the possibility that chylomicrons traffic through the MLN but prevent the uptake or processing of associated antigens by MLN dendritic cells. Besides the protective role in sensitization phase, chylomicron formation also plays a suppressive role in anaphylaxis phase of food allergy. Gavage of antigen with LCT in sensitized mice did not cause anaphylaxis, unless PL81 was added. In contrast, gavage of MCT in sensitized mice caused clinically significant anaphylaxis. The possible explanation is that chylomicrons prevent the access of ingested antigens to IgE associated with mast cells and basophils. This

hypothesis is proved by in vitro basophil activation tests that the presence of chylomicrons reduced the effects of antigen on basophils significantly.

Besides the effects on antigen absorption pathway, LCT and MCT have different pharmacological effects on intestinal epithelial cells, which play important roles in food allergy because of their barrier function and active participation in immune responses to microbial and dietary antigens like mentioned in section 1.1.2.. We found that MCT via acute gavage or via chronic dietary enrichment increased intestinal-epithelial expressions of three Th-2 biasing cytokines: TSLP, IL-25 and IL-33, among which, TSLP was shown to be important for the induction of experimental food allergy11. The effect is greatest in the jejunum, the major site for fat absorption. This may explain why MCT exacerbate food allergy in both sensitization and anaphylaxis phases in another way. MCT is one type of saturated fatty acids. Likewise, we found that other saturated fatty acids have the similar effects on intestinal epithelial cells. We found that acute palmitate oil feeding promoted allergic sensitization to concomitantly ingested antigens, as evidenced by increased antibody production and anaphylaxis following antigen re-exposure. This result may also be explained by the finding that palmitate oil can induce the same Th-2 biasing cytokines in the intestinal epithelium, which was confirmed by our *in vitro* experiment.

In conclusion, our study suggested that MCT may have a previously unappreciated effect on immune responses to dietary antigens, both on sensitization and anaphylaxis, by affecting antigen absorption and by promoting a Th2 bias. Other saturated fatty acids have similar effects as MCT due to the pharmacological effects on intestinal epithelial cells.

7.2 Insights of effects of fatty acids on food allergy and role of MCT

7.2.1 The importance of altering antigen absorption pathway by fatty acids

Our diet contains great amount of potentially antigenic proteins. Most of those proteins are going through enzymatically degradation, yet a small fraction of them escape from the degradation and enter body through largely unclear mechanisms. In healthy individuals, the escaped allergen (small amount) usually leads to systemic immunological tolerance ("oral tolerance"). However, in sensitized individuals, these allergens absorption usually cause significant morbidity such as celiac disease or food allergies. Apparently, two questions coming from this phenomenon become interesting and important: What happen after escaped allergens were absorbed? Is this process the key step to determine the immune system to go to direction of oral tolerance or allergy? Our findings that LCT play a protective and suppressive role and MCT play an exacerbated role in both sensitization and anaphylaxis phases successesfully answer the first question, which is the first time to propose the idea that other dietary components other than food antigen itself, fatty acids with different chain lengths, determine the induction of food allergy through altering the absorptions of food antigens. Like mentioned in 1.2.2, MCT is absorbed into the portal circulation, passing through the liver and then immediately into systemic circulation. In contrast, LCT is absorbed via chylomicron formation and enters lymph circulation first and then systemic circulation, suggesting that LCT spend approximately 4 hours in contact with mesenteric immune system before reaching the systemic circulation through the thoracic duct[242]. Based on that, our finding that MCT, instead of LCT, decreases systemic allergen absorption but increased Peyer's Patches absorption and IgE formation establishes a foundation for the future researches on locating where the antigens are "seen" by APC and how they "choose" to induce Th1 or Th2-bias immune responses. Besides, this is also a further confirmation for our previous finding[168].

7.2.2 Pharmaceutical effects of fatty acids on intestinal epithelial cells- from the angle of evolution of physiological system

Our findings of the pharmacological effects of fatty acids on intestinal epithelial cells also illustrate the importance of fatty acids in food allergy induction. MCT and other saturated fatty acids such as palmitate oil induce production of Th2 cytokines in intestinal epithelial cells, which put the individuals and animals in a “ready state” for being sensitized when food antigen is present. Besides, fatty acids released in large amounts from dietary triglycerides in the upper gastro-intestinal tract have potent detergent properties and may induce transient mucosal inflammation, damage and gut leakiness[74] which, theoretically, would allow ingested soluble food antigens to enter the body. It was reported that in mice lacking Angptl4, a lipoprotein lipase inhibitor, saturated fat induces a severe phenotype characterized by fibrinopurulent peritonitis, ascites, intestinal fibrosis, and cachexia[80], which further demonstrated the inflammatory effect of fatty acids itself on intestinal epithelial cells. These findings led to an important concerning about food industrialization. It has been said, “Nothing in biology makes sense except in the light of evolution”[243]. In that light, the digestive, metabolic, and immune systems of humans and animals have evolved over millions of years and would be affected by the sudden changes in the energy source. Only around 600 years ago, most of human population was still living in hunter-gathered societies and consuming the “Paleolithic diet”[244]. However, the current Western diet contains most of the calories from seeds, dairy products, refined plant oils and refined sugar[244]. None of which were available and consumed during the majority of human evolution. The mechanical processing of foods has occurred from around 100 years ago and greatly accelerated in the last 50 to 60 years. This phenomenon probably induce the inadaptation of the physical system that experienced slowly evolution, thus leading to some new emerging diet-induced diseases.

Instead of the human evolutionary diet, in which foods were consumed whole, the vegetable-derived cooking oils are separated from seeds, nuts and other plants by mechanical

extraction or screw-press or by organic solvent extraction[245]. The intake of such oils has increased in the past century, and exponentially since 1950, to more than 50 lb per person yearly (figure 7.1[245]). It is worth noting the negative effects of solid hydrogenated (to make the oil more stable due to the improvement of saturability) trans-fatty acids. About 25% of dietary essential fatty acids are not intake as refined oils rather than in whole foods. Simply speaking, corn oil does not equal corn and olive oil does not mean olives[245]. Therefore, the pro-inflammatory effects of oil on intestinal epithelial cells are likely due to the food processing rather than food itself. As to peanut products, peanut butter consumption has dramatically increased since 1950 as well, with the average child in the U.S. eating 1500 peanut butter sandwiches through high school. A study from 30 years ago reported that fecal fat excretion decreased when changing from ingesting whole peanuts to an equal weight of peanut butter, suggesting the peanut butter consumption result in a hyperabsorption of fat[246]. Combined with our findings, the role of fatty acids in food allergy induction appears to be more significant when consuming peanut products instead of whole peanuts. Another example is baby food. MCT is easier to be digested and absorbed compared to LCT and is the main component of fat (60%) in breast milk during lactating, based on which, MCT is also used as the majority fat source in baby formula processing. However, different from formula, breastmilk is a mixed supply of both nutrients and immune protection in terms of the immunoglobulin A levels. Besides, very small amount of food antigens would transfer from mom to babies which possibly induce oral tolerance to protect the baby instead of being sensitized. According to our finding, lactating is very likely the key time window for induction of certain food allergy in babies which may become their lifelong burden. That’s to say, without immune protection, consumption of MCT only is likely the reason for some babies to induce development of certain food allergy which may last the whole life. More evidences need to be provided to test this hypothesis.

7.3 The insight of MCT oral sensitization mice model

7.3.1 Significance of the model in food allergy studies

In researches of food allergy, several strains including C3H/HeJ, BALB/c mice have be investigated as murine animal models for food allergy[247]38. These animals have more capacities to produce the anaphylactic antibodies such as IgE and/or IgG1 than others, and strains can be divided into high or low IgE responders39. One of the most challenging obstacles is to develop murine model of ORALLY induced food allergy due to the tendency for the immune system to develop oral tolerance to ingested antigens[248]. We have the similar find that the gavage mice with combination of peanut protein and water would not induce sensitization. Several rodent models have been developed by using distinct sensitization routes, such as intraperitoneal[248, 249] or subcutaneous[250, 251] injection of allergen or genetically modified bacteria expressing food antigens[252]. Apparently, these models have several limitations that they do not reflect the pathogenic mechanism that induces food allergy, and thus could underestimate the importance and involvement of mucosal immune system. With the regard, the ideal food allergy rodent model should mimic human food allergy by inducing food hypersensitivity by oral ingestion. To achieve this goal, it is necessary to bypass the tendency of mice to develop oral tolerance and to ensure the induction of a Th2 response by antigen after administration. It is found that toxins such as cholera toxin (CT) have the capability to orally stimulate Th2 response and the production of IgG1 antibodies[253, 254]. Recently, other bacterial toxins such as staphylococcal enterotoxin B[255] or some approaches based on anti-acid treatment[256, 257] are developed as well. However, these orally sensitization models still have drawbacks. The adjuvants of toxins are used to disrupt the tolerogenic potential of the oral route which could underestimade the natural Th2 immune response by food antigen and which cannot truly reflect how individuals are sensitized by food antigen in the daily lives. Based on the

food allergy models, our finding that MCT, a common dietary fatty acids, would induce sensitization successfully without intentionally disruption of tolerogenic potential of immune system, which is an oral food allergy model that is truly reflect how Th2 response is induced by food antigen itself and is the model most close to the induction of human food allergy by pure food components.

7.3.2 Significance of the model in development of oral vaccination

The idea of “sensitization” is the similar idea as “vaccination”, the purpose of both of which are to generate great amount of antibodies with stimulation of proteins or peptides. As early as 900 years ago, the bedousin of the negev desert were reported to feed a dog-bitten person the liver of a rabid dog to cure rabies for several days. In ancient China, physicians there prescribed the medicines made from fleas collected from sick cows to prevent smallpox. With full evidences, in 1900, the Nobel laureate, Charles Richet, demonstrated that the raw meat feeding can cure tuberculous dogs which he termed as zomotherapy[258]. Today, most of the vaccines are administered by injection with limitations that recipients especially children hate needles. In the current times, there are very few licensed oral vaccines and more candidates are under development[258]. Based on that, our MCT oral sensitization model may provide a new insight of developing oral vaccination by using food-borne component only.

7.4 Experimental limitations

We recognize that our studies have some limitations. In study of protective role of chylomicron in effector phase of food allergy, we did an *in vitro* experiment using intralipid as a substitute of chylomicrons. The emulsions contain particles with similar size and lipid content as chylomicron but lack Apolipoprotein B48 although intralipid particles would acquire other apolipoproteins from serum [259] and have similar metabolic clearance rates as

chylomicrons[260]. Using chylomicron separated from mice as carrier might be more relevant to physiological conditions.

Secondly, in the same experiment, we did not determine if the inhibition effect on activation of basophils is due to chylomicron particles or their sequestration function for antigens. Nevertheless, we demonstrated from this experiment that postprandial lipid transport via lamina propria has important effects on the effector phases of food allergies.

We also found that ingestion of antigen with water rather than fat fail to induce anaphylaxis. We currently cannot distinguish the possibility that antigen ingested with water is poorly absorbed from the possibilities that antigen ingested with water becomes associated with chylomicrons or is otherwise protected from access to mast cell and basophil IgE. Regardless of the mechanism involved, our finding is still potentially relevant for optimization of oral desensitization, as currently attempted in small clinical trials[261].

7.5 Clinical implications

Besides the significance on food industrialization discussed in 7.2 and on development of oral vaccination discussed in 7.3, our finding that feeding antigen with LCT might prevent anaphylaxis can be an alternative approach during oral treatment while boosting its effectiveness by promoting oral tolerance. Please refer to 1.2.1.3 for the introduction of clinical treatment on food allergy by inducing oral tolerance.

7.6 Future directions

7.6.1 Peyer's Patches role in food allergy induction

Peyer's patches have been studies as a major site for mucosal IgA responses yet less in oral tolerance. The results of present study provide direct evidence for the role of Peyer's patches in the induction of oral tolerance. It was reported that ovalbumin (OVA) transgenic mice

had significant increase in transgenic T cells in Peyer's Patches of mice tolerized by OVA, indicating the importance of Peyer's patches in the development of oral tolerance[262, 263]. Peyer's patches were also found essential for the induction of systemic hyporesponsiveness to ingested antigen. We found that both LCT and MCT promote antigen to Peyer patches more than MLN, among which MCT would enhance the effect more. It is well accepted that APC, such as dendritic cells, macrophages and MHC II B cells, play major roles in the induction of mucosal immune responses. Therefore, it is worth determining the role of functioning immune cells in Peyer's patches and how they direct the immune response to oral tolerance or sensitization responses.

7.6.2 Studies on chylomicrons and anaphylaxis

Chylomicrons are large, spherical triacylglycerol-rich lipoproteins which surface is covered with phospholipid monolayer and contains free cholesterol surrounded by a large protein, apoB48. Besides, several exchangeable apolipoproteins can be found on the surface of the chylomicron, in cluding apoAI, apoAIV, and apoCs. Like discussed in 2.4, to investigate the role of chylomicron in shielding epitopes of food antigen, it is more closed to physiological conditions by using chylomicrons separated from animals for the in vitro experiments.

Secondly, chylomicron assembly begins with apoB48 translation. The insufficient supply of lipids or microsomal triglyceride transfer protein (MTP) induce degradation of the nascent polypeptide. MTP can interact with and transfer lipids to the nascent apoB physically[264]. With help from MTP, apoB fold into a configuration which can accept more lipids[265]. Therefore, MPT plays an important role in assembly and secretion of chylomicrons. To know better about the role of chylomicrons in sensitization and anaphylaxis phases, MTP deficient mice model will be good approach to study on.

7.6.3 Mechanism studies by using TSLP deficient mouse model

As discussed in 2.3, the effects of MCT and saturated fatty acids on intestinal epithelial cells include stimulating expression of Th2 bias cytokines, TSLP and IL-33 in particular. TSLP and IL-33 are critical cytokines responding at the interface between the environment and the body with local and systemic immune responses, most of which are thought to be Th2 type[266]. Mast cells are distributed everywhere and particularly abundant in epidermis, epithelial cells and endothelial cells where TSLP and IL-33 are produced most. Besides, mast cells respond to IL-33 and TSLP rapidly due to the high expression of the receptors like ST2[267]. In addition, TSLP and IL-33 affect other cell biology of mast cells such as growth, development and attachment which affect the inflammatory response and probably of significant for the pathology of different diseases that is regulated by IL-33/TSLP-mast cell link. To better understand the hypothesized link of MCT/Saturated FA-TSLP/IL-33-mast cell-sensitization, TSLP deficient mice are worth using by determining the effects of MCT and saturated FA on induction of sensitization in this mice model.

CHAPTER VIII: Summary and discussion for chapter IV and V

8.1 Summary

In this study, we want to determine the role of cholesterol transporter ABCG5/G8 in TICE and the possible cholesterol donors in TICE based on CETP transgenic mice models.

In the first study, we found a sexually dimorphic cholesterol elimination in mice that females have higher biliary cholesterol concentration and biliary cholesterol secretion rate but a lower intestinal cholesterol secretion rates than male mice. This finding suggested that female mice preferentially eliminate cholesterol through biliary pathway where male mice depend on intestine. Our finding is consistent with the clinical phenomenon that females tend to secrete more cholesterol into bile than males[221]. Furthermore, we confirmed that female mice have more liver ABCG5 protein expression than male mice fed with PSF diet, which might explain the

result above from the angle of cholesterol secretion. Interestingly, the sum of total cholesterol secreted from both biliary and transintestinal pathways, suggesting a complimentary relationship between these two pathways. Secondly, we found that the differences of biliary cholesterol levels between WT and G5G8 KO mice were greater in females than in males when fed with PSF diet. The difference between genotypes in male mice was recovered when transferred to chow diet (plant sterol-containing diet). These data suggest that accumulation of plant sterol may be partially responsible for the blockage of cholesterol secretion in G5G8 KO mice due to its effect of inhibiting cholesterol synthesis[217]. Simultaneously, we found that female KO mice have increased transtintestinal cholesterol secretion rate compared to their WT littermates, indicating a possible G5G8 independent cholesterol elimination. However, the possible transporter responsible for that need to be further explored.

In the second study, we used CETP transgenic mice as models which have altered plasma lipoprotein profile to determine the role of HDL in TICE. When fed with western diet, we found that CETP mice had increased biliary total cholesterol secretion rates and decreased transintestinal cholesterol secretion rates compared to WT mice. This result might be explained by the different favors of different lipoproteins for cholesterol elimination. In addition, we also found the same result that sum of total cholesterol secreted from biliary and transintestinal pathways were the same between genotypes, which further confirmed out finding in the first study that these two pathways work corporately with each other for cholesterol elimination. Secondly, we found that HDLs did not contribute to increased biliary cholesterol secretion rate as much as TICE. This result may be explained by the finding that uptake of CETP modified HDL was delayed in the rats[235]. We next collected and labeled HDLs from both WT and CETP mice and injected them back to WT mice retro-orbitally to track their elimination pathways. We found that mice had higher transintestinal CETP modified HDL-C secretion rates compared to

untreated HDL-C and we did not detect any difference of those in bile. We assumed that some remodeling took place on HDL in the plasma that affect their uptake by liver and intestine.

In conclusion, we found sexually dimorphic cholesterol elimination in mice and ABCG5/G8 independent cholesterol secretion pathway in female TICE and male biliary pathway when fed with PSF diet. Plant sterol is the possible factor to affect biliary cholesterol secretion rate in ABCG5/8 KO mice. Based on CETP transgenic model, we found that CETP transgenic mice have higher biliary cholesterol secretion rate, which is not contributed by HDL. Furthermore, HDL modified by CETP may favor intestine for cholesterol elimination.

8.2 Cross-talk between liver and intestine on cholesterol metabolism

In our studies on TICE, the most prominent finding is that no matter that either biliary or transintestinal cholesterol secretion pathway was altered, the sum amount of cholesterol secreted from both pathways remained similar, which happened in almost all of our mice model such as between female and male mice, WT and G5G8 KO mice and between WT and CETP transgenic mice. These findings suggested that liver and intestine work corporately with each other on cholesterol elimination, illustrating and further confirming the cross-talk between liver and intestine on cholesterol metabolism.

As discussed in 1.3.2, the liver controls plasma cholesterol levels via a complete metabolic network of a series of lipoprotein receptors, sterol transporters and nuclear receptors, which can translates the signals by cholesterol levels alteration into transcriptional regulation of gene expression. Among these factors, LDLR, SR-B1, LXR- and SREBP-targeted pathways that functioning significant this network have been extensively reviewed[239, 268-271]. Linked to liver by bile duct, the intestine, which is the main site for cholesterol absorption and excretion, is important in regulation of cholesterol metabolism (see 1.1.3). Therefore, it is important to point out that the balance of cholesterol uptake, synthesis and excretion requires extensive

cross-talk between liver and intestine. It is known from chapter 1.3.2 that RCT is delivery of cholesterol from peripheral cells or macrophages to the liver and eliminated in feces from the body. After uptake by liver, some cholesterol is used to synthesis of BS through the process of addition of a hydroxyl group on position 7 of the steroid nucleus by the enzyme CYP7A1 as neutral sterols or bile acids (BS). Intestine would absorb certain amount of cholesterol and BS that is secreted into intestinal lumen as described in 1.1.3. It is interesting that the complete absence of cholesterol absorption does not have any symptoms. However, the absence of intestinal bile can cause deficiencies of essential lipids, leading to clinical symptoms such as increased fecal fat loss, malnutrition and a retardation of growth and development in children[272]. Therefore, the conversion of cholesterol to BS have considerable significances. For one thing, BS is one of the major route for sterol removal from the body and is the important cholesterol acceptor for cholesterol transport assistance. Another thing is that BS have been found to play an important role in signal transduction pathways.

8.2.1 BS and signaling

BS may play a role in regulation of energy homeostasis through the activation of nuclear bile acid receptor FXR[273, 274] and the membrane-bound G-protein coupled receptor TGR5[274]. In the recent years, it has become clear that BS are not only the major constitution of bile and facilitation of dietary fats absorption, but also involved in energy metabolism in both genomic and non-genomic ways. BS are the natural ligands of FXR, which regulate BS synthesis and transport downstream genes within enterhepatic circulation directly, or by induction of FGF15/19 in intestinal epithelial cells. FGF15 and FXR are indicated in control of energy metabolism. Furthermore, BS signal through another pathway in peripheral tissues and intestinal L cells involving activation of TGR5, which is reported to stimulate energy expenditure in brown adipose tissue and skeleton muscle. In colon, TGR5 activation stimulate GLP-1

secretion from L-cells which promotes pancreatic insulin secretion, thus improves hepatic and peripheral insulin sensitivity[275].

8.2.2 BS and cholesterol

The connection between cholesterol and BS metabolism including some other important processes are manifold. It is believed in the literature that BS excretion reflects the main route for cholesterol removal from the body. However, in other species including humans, this opinion is not quite true. It was reported the ratio of fecal neutral sterol/BS excretion of 2:1 in humans[276-279] and closer to 1 in rodents yet with largely variations between strains and depending on different experimental conditions[280]. It is demonstrated that fecal BS loss contributes to cholesterol turnover significantly due to the physiological functions related to the amphiphatic nature of the BS molecules, but 95% of BS showed up in the intestinal lumen is reabsorbed so that BS pool is maintained in the enterohepatic circulation[281, 282]. From these findings, it is clear that removal of excess cholesterol is not the main role of BS synthesis in cholesterol metabolism in the whole body. There is still 5% of BS pool lost in the feces per cycle and humans have 4-5 cycles per day. Quantitatively, the BS loss is compensated by hepatic synthesis from cholesterol to maintain BS pool size, which is a significant way to maintain whole body cholesterol homeostasis.

8.2.3 FGF15/19 and BS synthesis

Fibroblast growth factor 15 (FGF15) is highly expressed in the terminal ileum and is the target for FXR. FGF15 protein was demonstrated to suppress BS synthesis in the liver by regulating the expression of the genes involved including CYP7A1. It was shown that the upregulation of hepatic BS synthesis was associated with low levels of circulating FGF19 in humans[283] and an absence of ileal FGF15 mRNA in mice. However, the concentration of this protein in the circulation is very low that it is likely that FGF15/19 may not reach the central

zone of liver acini. A recent study failed to prove a relation between FGF15/19 levels in circulation and CYP7A1 gene expression in rats or rabbits[284]. Therefore, the contribution of FGF15/19 to regulation of BS synthesis is still debated compared to other classic pathways (FXR-SHP mediated suppression of CYP7A1, LXR, insulin) and may vary in different physiological conditions. However, in rats with bile duct ligation induced cholestasis model, absence of FGF15 might explain the upregulation of hepatic CYP7A1 gene expression[285]. But based on the reports that in intestine-specific FXR null mice, only slight upregulation of hepatic CYP7A1 expression was detected at the specific time window during day-light cycle[286], this question is still argued. Therefore, it should be appreciated that the regulation of BS synthesis do not by definition represent the opposite sides of the same coin but may actually reflect separate other processes[275].

8.2.4 Summary

Collectively, in BS and cholesterol homeostasis, liver is always been considered as a central place in maintenance. Actually, its role is significant but not without intensive cross-talk with other organs. Our findings that liver and intestine work together and cooperatively in cholesterol elimination reflect part of this point. The mechanism of how they are functioning together needs further exploration. It is largely ignored here because of the space constraints that other potentially relevant organs like brain, adrenals also function significantly in lipid homeostasis. Hopefully the development of technology will allow the detailed mapping these connections. Therefore, working on the cross-talk between liver and intestine on cholesterol elimination will be one of the future directions of our studies.

8.3 Limitations

To directly measure the cholesterol amount secreted from intestine, TICE measurement model is prevalently used in current TICE studies. In this model, bile duct was ligated to cut off

bile secretion in order to measure the net cholesterol secretion from intestine. However, as discussed in 8.2, many components in the bile including BS is of great importance in regulating signals in maintenance of lipid metabolism both in liver and intestine. Cutting off bile secretion path would break the enterohepatic circulation of BS and other lipids, and more importantly, the cross-talk between the two organs. It is true that in intestine perfusion assay, “man-made” cholesterol acceptor including BS (taurocholate) was added for normal cholesterol secretion. However, compared to the BS added into intestinal perfusate, the BS constitution in the natural bile is significantly more extensive. In humans, the major bile acids are cholic acid (CA) and chenodeoxycholic acid (CDCA), which in mice include CA, CDCA and 6-hydroxiylated bile acids, mainly muricholic acid (MCA)[287, 288]. And then they are converted to secondary bile acids, which in human refer to deoxycholic acid (DCA), ursocholic acid (UCA), ursodeoxycholic acid (UDCA) and lithocholic acid (LCA), and in mice include DCA, UCA, UDCA, murideoxycholic acid (MDCA) and hyodeoxycholic acid (HDCA).

The emerging work showed that these transformations are importantly contribute to bile acid signaling and even gut microbiota-induced changes in whole body physiology[289-291]. For example, compared with mice raised conventionally, the bile acids pool in germ-free mice is less “FXR agonistic” because the content of tauro-β-murocholic acids, an endogenouse mouse bile acid functioning as FXR antagonist) increased in the bile and intestinal lumen of these mice[289, 292]. Based on these, the simple Taurocholate in intestinal perfusate cannot represent the bile acids pool that the intestinal lumen are supposed to in normal physiological conditions.

In addition, because of the important host bile acids species difference, it is questionable how to translate many of the recent findings including TICE studies from mouse models to humans. For example, humans do not synthesize 6-hydroxylated bile acids such as

MCA, which makes the mechanisms whereby gut microbiota alterations generated bile acid pool with less FXR activation inoperative in humans.

Therefore, lacking of sufficient and translational bile acids pool in mice models of TICE studies is one limitation, which also point out the future direction on the alteration of intestinal perfusate.

8.4 Future direction

8.4.1 Future directions of experiments

We found that intestinal total cholesterol excretion was increased in female KO mice compared with their WT littermates, indicating another possible G5G8 independent cholesterol elimination. Two possible G5G8 independent cholesterol elimination pathways were reported before. Annemiek Groen at el. reported that the ATP8B1 deficient maintained a high cholesterol output as WT mice did independent of G5G8 activity. The possible mechanism for this phenotype is due to the direct extraction of cholesterol from the canalicular membrane by hydrophobic bile salts caused by ATP8B1 deficiency[214]. Donna at el. reported a G5G8 independent mechanism for basal biliary cholesterol secretion in rats during lactation but a dependence on G5G8 for maximal biliary cholesterol secretion[213]. The possible transporter responsible for increased TICE in female G5G8 KO mice need to be further explored. To achieve this goal, we plan to use Microarray at the University of Kentucky Microarray Core Facility as the approach to detect the possible transporters or other proteins expressed in the intestinal epithelial cells that are involved in accelerating cholesterol efflux in female ABCG5/G8 KO mice.

Secondly, we found that the differences of biliary cholesterol secretion in male mice between genotypes were not as prominent as what was reported before[224] which means that the G5G8 KO mice maintained certain level of biliary cholesterol secretion in the absence of plant sterol. We maintained our mice on PSF diet instead of chow to protect the mice from

sitosterolemia. Thus the possible reason might be the effects of plant sterol accumulation that our experimental mice did not have access to on cholesterol secretion. It was reported that circulating levels of sitosterol and campesterol are correlated with fractional and absolute absorption of dietary cholesterol positively, but are related to whole body cholesterol synthesis inversely. Therefore, accumulation of non-cholesterol sterols may account for the very low rates of cholesterol synthesis in STSL[217]. Yu et al. found there is approximately 50% decline in cholesterols in the livers and plasma of G5G8 KO mice due to low hepatic cholesterol synthesis relative to WT mice[218]. Thus it is very likely that blockage of cholesterol synthesis induced by accumulation of plant sterol made the role of G5G8 in RCT overestimated. Plant sterol is also known as the LXR agonist and FXR antagonists. To figure out the role of plant sterol in the ABCG5/G8 independent pathway is one of our future directions.

We also found a sexually dimorphic cholesterol elimination in both biliary and transintestinal pathways. It was reported that the rates of gallstone disease happen in women two to three times higher than men[219, 220]. One possible mechanism suggests that estrogen increases biliary cholesterol secretion causing cholesterol supersaturation of bile[221]. Besides, female mice expressed more ABCG5 and ABCG8 at mRNA levels stimulated by high cholesterol synthesis or dietary intake via LXRs[222] in the absence of cholesterol[223]. Therefore, it is rational to study the role of estrogen on cholesterol metabolism.

8.4.2 Future directions of projects

Like discussed in 8.2 and 8.3, to study the cross-talk between liver and intestine will be our overall direction. To achieve this goal, development of a TICE measurement model that is more close to the real physiological conditions is required, including to mimic the constitution of the real bile. Since biliary cholesterol is also present in intestinal lumen and the role of which on

TICE is not known. Therefore, to study the effects of cholesterol content itself in TICE is also a significant concern in our future studies.

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Vita

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Educational History

August 2009 – Present	**University of Kentucky, Lexington, KY** PhD Candidate Department of Pharmacology and Nutritional Sciences
August 2007 – July 2009	**Jiangnan University (Southern Yangtze University), Wuxi, China** Master of engineering Department of Food Science and Technology
August 2003 – July 2007	**Jiangnan University (Southern Yangtze University), Wuxi, China** Bachelor of engineering Department of Food Science and Technology

Research Experience

May 2012 – Present	**University of Kentucky**, Lexington, KY • Sexually dimorphic adaptive responses maintain cholesterol elimination in the absence of ABCG5 and ABCG8 • CETP alters routes of total and HDL cholesterol elimination from the body in mice. *(Mentor: Gregory Graf; PhD study, Graduate Center of Nutritional Sciences, University of Kentucky)*
August 2009 – April 2012	**University of Kentucky**, Lexington, KY Effect of dietary triglycerides on peanut protein induced sensitization and anaphylaxis. (*Mentor: Erik Eckhardt; PhD study, Graduate Center of Nutritional Sciences, University of Kentucky*)
August 2007 – June 2009	**Jiangnan University (Southern Yangtze University)**, Wuxi, China The development of multi-element nutritional powder dissolving ability. *(Mentor: Huiming Zhou; MS study, Department* of Food Science and Technology*,* Jiangnan University*)*
Summer 2007 – fall 2007	**Jiangnan University (Southern Yangtze University)**, Wuxi, China The separation and purification of immune active peptide from pilose antler blood, lab of Nutrition Metabolism and Regulation

(Mentor: Guowei Le; Bachelor, Department of Food Science and Technology, Jiangnan University)

Publications

Wang Y, Liu X, Pijut SS, **Li J**, Horn J, Bradford EM, Leggas M, Barrett TA, Graf GA. The combination of ezetimibe and ursodiol promotes fecal sterol excretion and reveals a G5G8-independent pathway for cholesterol elimination. J Lipid Res. 2015 Jan 29. pii: jlr.M053454.

Li J, Wang Y, Tang L, de Villiers WJ, Cohen D, Woodward J, Finkelman FD, Eckhardt ER. Dietary medium-chain triglycerides promote oral allergic sensitization and orally induced anaphylaxis to peanut protein in mice. J Allergy Clin Immunol. 2012 Nov 22. pii: S0091-6749(12)01655-7. doi: 10.1016/j.jaci.2012.10.011.

Yuehui Wang, **Jianing Li**, Lihua Tang, Yu Wang, Richard Charnigo, Willem de Villiers, and Erik Eckhardt. T-Lymphocyte Responses to Intestinally Absorbed Antigens Can Contribute to Adipose Tissue Inflammation and Glucose Intolerance during High Fat Feeding. *PLoS One*. 2010; 5(11): e13951. Published online 2010 November 11. doi: 10.1371/journal.pone.0013951. PMCID: PMC2978720

Jianing Li, Huiming Zhou, Kexue Zhu. Preparation and characteristics of microencapsulated black sesame oil. CHINA OILS AND FATS. 2009; 34(9) QCode : ZGYZ200909002

Gui-fang Chang, Yong-Hui Shi , Guo-Wei Le, Zi-wei Xu, Jin Sun, **Jianing Li**. Lactobacillus plant arum affected genes expression pattern in mice jejuna Peyer's patches. Cell Immunol. 2009; 258(1):1-8. Epub 2009 Apr 26.

YANG Xiaoqing, WANG Chun-guang, **Jianing Li**. Research on the Relationship between Mechanical Properties and Stiffness Refractive Sugar Concentration of Hetao Muskmelon under Compressing. *Journal of agricultural mechanization research.* Jul, 2007. GateGory Index: S652

Manuscripts in Preparation:

Jianing Li, Ailing Ji, Yuhuan Wang, Sonja S Pijut, Deneys Van der Westhuyzen and Gregory Graf. CETP alters routes of total and HDL cholesterol elimination from the body in mice.

Jianing Li, Yu Wang, Lisa Cassis, Yuhuan Wang, Sonja Pijut, Gregory Graf. Sexually dimorphic adaptive responses maintain cholesterol elimination in the absence of ABCG5 and ABCG8.

Jianing Li, Yu Wang, Jerold Woodward, Fred Finkelman and Gregory Graf. Dietary palm oil promotes spontaneous peanut allergy in mice.

Awards and Membership

May 2015	Fourth place of 2015 Barnstable Brown obesity and diabetes research day awards
2014-2014	Member of American Heart Association
November 2014	First place and People's choice awards in the doctoral "Three Minute

	Thesis" competition of University of Kentucky
July 2014	Travel Award for FASEB Science Research Conference entitled "Molecular Mechanisms of Intestinal Lipid Transport & Metabolism."
July 2014	Poster Award for FASEB Science Research Conference entitled "Molecular Mechanisms of Intestinal Lipid Transport & Metabolism."
July 2009	YIN XIANG Scholarship for Excellent Graduate Student, Wuxi, China
2008	Triple-A Outstanding Graduate Student awarded by Jiangnan University
2008	Second Place Scholarship for Graduate Student awarded by Jiangnan University
2004-2006	Distinguished Student Scholarship (3rd), Department of Food Science and Technology, Jiangnan University

Oral Presentations

Jianing Li, Yu Wang, Lisa Cassis, Gregory Graf. Sexually dimorphic adaptive responses maintain cholesterol elimination in the absence of ABCG5 and ABCG8. South East Lipid Research Conference
Callaway Gardens; Pine Mountain, GA

Jianing Li, Yu Wang, Lisa Cassis, Gregory Graf. Sexually dimorphic adaptive responses maintain cholesterol elimination in the absence of ABCG5 and ABCG8. FASEB Science Research Conference entitled "Molecular Mechanisms of Intestinal Lipid Transport & Metabolism." July 2014, Snowmass village, Colorado.

Jianing Li, Yu Wang, Willem de Villiers, Erik Eckhardt. Dietary Long Chain Triglycerides Protect Against Oral Sensitization to Peanut Protein and Promote Oral Tolerance in Mice in a Chylomicron-Dependent Manner. Digestive Disease Week 2011, Chicago, IL.

Poster Presentations

Jianing Li, Ailing Ji, Yuhuan Wang, Sonja Pijut, Deneys Van der Westhuyzen and Gregory Graf. CETP alters routes of total and HDL cholesterol elimination from the body in mice. 2015 BARNSTABLE BROWN OBESITY & DIABETES RESEARCH DAY. Lexington, KY May 2015.

Jianing Li, Yu Wang, Lisa Cassis, Gregory Graf. Sexually dimorphic adaptive responses maintain cholesterol elimination in the absence of ABCG5 and ABCG8. Gill Heart Institute Cardiovascular Research Day; Lexington, KY; October 17, 2014

Jianing Li, Yu Wang, Lisa Cassis, Gregory Graf. Sexually dimorphic adaptive responses maintain cholesterol elimination in the absence of ABCG5 and ABCG8. South East Lipid Research Conference
Callaway Gardens; Pine Mountain, GA; October 2014

Jianing Li, Yu Wang, Lisa Cassis, Gregory Graf. Sexually dimorphic adaptive responses maintain cholesterol elimination in the absence of ABCG5 and ABCG8. FASEB Science Research

Conference entitled "Molecular Mechanisms of Intestinal Lipid Transport & Metabolism." July 2014, Snowmass village, Colorado.

Jianing Li, Gregory Graf. Effect of CETP on Transintestinal Cholesterol Excretion. Barnstable Brown Obesity and Diabetes Research Day; Lexington, KY; May 14, 2014

Jianing Li, Gregory Graf. The sexually dimorphic adaptive cholesterol elimination in mice in the absence of ABCG5/8. Gill Heart Institute Cardiovascular Research Day; Lexington, KY; October 11, 2013

Jianing Li, Yu Wang, Willem de Villiers, Erik Eckhardt. Dietary Long Chain Triglycerides Protect Against Oral Sensitization to Peanut Protein and Promote Oral Tolerance in Mice in a Chylomicron-Dependent Manner. Digestive Disease Week 2011, Chicago, IL.

Jianing Li, Yu Wang, Erik Eckhardt. Dietary Long Chain Triglycerides Protect Against Oral Sensitization to Peanut Protein. South East Lipid Research Conference 2010, Pine Mountain, Georgia.

Teaching Experience

October 2012	One lecture in DHN 311 (Nutritional Biochemistry). Director: Bernhard Hennig. University of Kentucky

Other Activity

March 2015	Be invited by Graduate Student Congress to Dinner With UK President Dr. Eli Capilouto, University of Kentucky.
March 2015	Attend Southern Graduate Schools Regional 3MT Competition in New Orleans representing UK graduate students.
2012-2013	Vice president of Nutritional Science Student Association, University of Kentucky
2005-2009	Volunteer and Bilingual Emcee of Annually International Conference on Food Science and Technology (ICFST), Wuxi, Jiangsu, China.