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COMPARISON OF RETINOIC ACID (ORAL AND SUBCUTANEOUS) AND VITAMIN A (ORAL) EFFECTS ON IMMUNE RESPONSE IN VITAMIN A DEFICIENT MICE

A Thesis in

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by

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ABSTRACT

Vitamin A deficiency is widely present in developing countries, affecting human's health especially children with high morbidity and mortality. Vitamin A deficient (VAD) animals have the impaired immunity to many infections. In vaccines models VAD animals show depressed antibody and cytokine production, reduced cytotoxic T lymphocyte activity and impaired T lymphocytes trafficking to the gastrointestinal tract. Vitamin A (VA) supplements have been shown to play an important role in reducing mortality, improving recovery from measles, and decreasing the severity of malaria infection in children. Oral VA supplementation as well as retinoic acid (RA) administration restores the impaired mucosal immune response and vaccine efficiency in VAD mice model. RA subcutaneous (sc) injection has been shown to induce the homing of T and B cells to the gut and to stimulate immunoglobulin A (IgA)+ plasma cells generation.

Ovalbumin (OVA) is a widely used oral antigen for immune and oral tolerance studies. Tetanus toxoid (TT) is a protein antigen used as a vaccine against tetanus, to prevent the infection of *Clostridium tetani.* Cholera Toxin Subunit B (CTB), as a possible nontoxic adjuvant shown in animal studies, was mixed with OVA as a mucosal adjuvant in my project.

Here, we compared the different supplementation (VA orally versus RA orally or by subcutaneous injection) in the VAD mice model, with OVA and TT as antigens delivered through oral challenge or subcutaneous injection. RA sc supplementation enhanced the plasma and fecal OVA specific IgA and TT specific IgG production in secondary immune response and plasma OVA specific IgA after third immunization. RA oral supplementation only showed its effect on

increasing plasma OVA specific IgA response after the third immunization. Whereas VA supplementation did not affect the OVA mucosal immunization, it only strengthens TT specific IgG response after the secondary immunization. From these results, RA, especially the RA sc injection demonstrates stronger effects on mucosal immune response than VA supplementation.

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LIST OF ABBREVIATIONS

ADH: alcohol dehydrogenases

APC: antigen presented cell

ARAT: acyl CoA:retinol acyltransferase

BALT: bronchus-associated lymphoid tissue

CCR9: CC chemokine receptor

CPs: Cryptopatches

CRABP: cellular retinoic acid binding protein

CTB: cholera toxin B subunit

CTLs: CD8+ cytotoxic T lymphocytes

CTX: cholera toxin

DCs: dendritic cells

D-MALT: diffuse mucosa-associated lymphoid tissue

ELISA: enzyme-linked immunosorbent assay

FAE: Follicle associated epithelium

GALT: gut-associated lymphoid tissue

HPLC: high performance liquid chromatography

IELs: intraepithelial lymphocytes

IgM/G/A: Immunoglobulin M/G/A

IL: interleukin

ILFs: isolated lymphoid follicles

ip: intraperitoneously

LNs: lymph nodes

LP: lamina propria

LRAT: lecithin retinol acyltransferase

LT: heat-labile enterotoxin

MAdCAM1: mucosal vascular addressin cell adhesion molecule 1

MALT: mucosa-associated lymphoid tissue

MHC: major histocompatibility complex

MLN: mesenteric lymph nodes

NOD1: nucleotide-binding oligomerization domain containing 1

O-MALT: organized mucosa-associated lymphoid tissue

OVA: albumin from chicken egg white or ovalbumin

PBS: phosphate buffered saline

PIC: polyriboinosinic:polyribocytidylic acid

PPs: Peyer's patches

RA: retinoic acid

RDH: retinol dehydrogenase

RALDH: retinal dehydrogenases

RAR: retinoic acid receptor

RT: room temperature

RXR: retinoid X receptor

RBP: retinol binding protein

RE: retinyl ester

RARE: retinoic acid response element

S-3: pneumococcal polysaccharide specific for serotype 3

sc: subcutaneous

TECK/ CCL25: thymus-expressed chemokine

TGF-β: transforming growth factor–β

Th1/2/17: T helper 1/2/17

TLRs: toll-like receptors

Treg: T regulatory

TT: tetanus toxoid

VA: vitamin A

VAD: vitamin A deficient

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CHAPTER 1

INTRODUCTION AND HYPOTHESIS

Vitamin A (VA), the essential micronutrient, plays an important role in vision, cell proliferation and differentiation, bone growth embryo development and immunity [1]. Retinoic acid (RA), the active metabolite of VA, has been proven to mediate the major functions of VA by binding the nuclear receptors, retinoic acid receptor (RAR) and retinoid X receptor (RXR) to regulate transcription [2].

Vitamin A deficiency is associated with the higher risk of infection such as measles and diarrhea [3]. The impaired innate and adaptive immune system in VAD mice could be one explanation for the increased susceptibility of animals to infection. VA supplements have been proven to play an important role in regulating infections by reducing mortality, improving recovery from measles, decreasing the severity of malaria infection in children [4]. In mouse models, VA, as RA form, is necessary for maintaining the immune response by regulating immune cell differentiation and proliferation, especially important for the mucosal immunity.

RA injected subcutaneously induces the class switch to IgA and imprints T cells and plasma cells' gut homing capacity by upregulating the gut homing receptors in inguinal lymph nodes (LNs) [5]. RA combined with PIC stimulated the anti-TT immune response such as anti-TT IgG production, TT-specific lymphocytes proliferation and cytokine production in neonates [6]. Oral VA and retinoic acid administration fully restored the abrogated antigen-specific T-lymphocyte homing to the gastrointestinal tract, gastrointestinal cellular immune responses and vaccine protective efficacy in VAD mice [7].

Here we generated a VAD mouse model that was immunized with two different antigens (OVA and TT) by two different routes (oral and subcutaneous) with three different kinds of supplementation (VA, RA oral, RA subcutaneously). In our study, we mainly focus on exploring the role of RA and VA supplementation in regulating gut local and systemic immune response in VAD mice. Our hypothesis is that RA supplementation enhances the systemic and local mucosal immune response to OVA and TT in VAD mice.

CHAPTER 2

BACKGROUND AND SIGNIFICANCE

The first description of VA can be traced back to the use of juice of ox liver applied to the night-blind eyes by ancient Egyptians [8]. E.V. McCollum and Thomas Osborne with Lafayette Mendel discovered VA in the 1910s independently by identifying the fat-soluble factor A, which is essential for animal growth and survival [8, 9]. After the discovery of VA, the researchers focused on exploring the functions of VA based on animal models fed with diet lacking in β-carotene or retinol [8]. Now VA has been proven to play an important role in vision, cell growth and differentiation, immunity, reproduction, bone growth and embryonic development [1].

Vitamin A and Retinoic Acid

The brief introduction of VA and RA's metabolism

VA contains a series of fat-soluble essential retinoids, including retinal, retinoic acid, retinol and retinyl ester (RE) (shown in Figure 1). The food sources of VA are mainly two kinds, precursors carotenoids and retinol and its esterified form (shown in Figure 2). Provitamin A (carotenoids) is abundant in dark color vegetables and fruit such as carrots, broccoli, squash and cantaloupe, while preformed VA (retinol and retinyl esters) is from animal source, such as liver, milk, egg and fish oil [9,10].

The carotenoids from diet are β-carotene, α-carotene, and β-cryptoxanthin, in which β-carotene is the most important, and these carotenoids could be converted to retinol or absorbed

intact [11]. As for the REs, they are hydrolyzed in the intestinal lumen to retinol and then absorbed by enterocytes [12, 13]. The enzymes involved in the hydrolysis are lipases secreted by the pancreas or associated with intestine cells. Following uptake by the enterocytes, the free retinol is reesterified to RE by lecithin retinol acyltransferase (LRAT) for incorporation into chlylomicrons with other lipid esters. These chlylomicrons then can be absorbed into the lymphatics and then efflux into the vascular circulation, where they are hydrolyzed by lipoprotein lipase into the chylomicron remnant containing most of the newly absorbed RE. Then chylomicron remnants are absorbed into the liver where the RE are hydrolyzed and reesterified (shown in Figure 2). The storage form of VA in liver is RE (predominately retinyl palmitate), which can be hydrolyzed into retinol. The retinol then enters the circulation by binding to retinol binding protein (RBP) to meet the need from other tissue [12]. The remaining retinols are reesterified and stored in stellate cells in liver for future use. The alcohol dehydrogenases (ADH) expressed can oxidize retinol into retinal, which can be further oxidized by retinal dehydrogenases (RALDH) into RA (shown in Figure 1).

RA, the active metabolite of VA, exists in two isomeric forms: all trans RA and 9-*cis*-RA. All trans RA is the major form in mice and humans, while 9-*cis*-RA is at significantly lower concentration [2]. RA binds to cellular retinol binding protein (CRBPs) within cells and circulates in blood by binding albumin. The concentration of RA are strictly regulated and controlled in vivo.

VA status is determined based on the plasma retinol level. VA inadequacy is the concentration of plasma retinol level is less than 0.70 micromoles/L, and the marginal could be

0.70~1.05 micromoles/L for some people [9]. VA deficiency is the main contributor to children (age under five) and pregnant women's morbidity and mortality in low-income developing countries, especially in Southeast Asia and Africa [3,14,15]. VA deficiency can start from infancy by not receiving sufficient breast milk or colostrum. The main cause of VA deficiency is the inadequate intake of VA in diet, which leads to lower VA store in body. Insufficient VA intake can cause xerophthalmia, which is detectable by symptom like night blindness. In the world, it is estimated by WHO that 5.2 million pre-school age children are affected by night blindness [3]. However, the night blindness is the first observable ocular symptom and can be reduced by VA supplementation. A higher risk of infection, such as measles and diarrhea, has been correlated with exacerbated VAD children in developing countries [3]. Because VA is fat soluble, it can accumulate in the liver. When body stores the excess amount of VA, it can be toxic and result in hypervitaminosis A. The symptoms of hypervitaminosis A are nausea, headache, skin irritability, blurry vision, coma, liver damage or even death [8, 9].

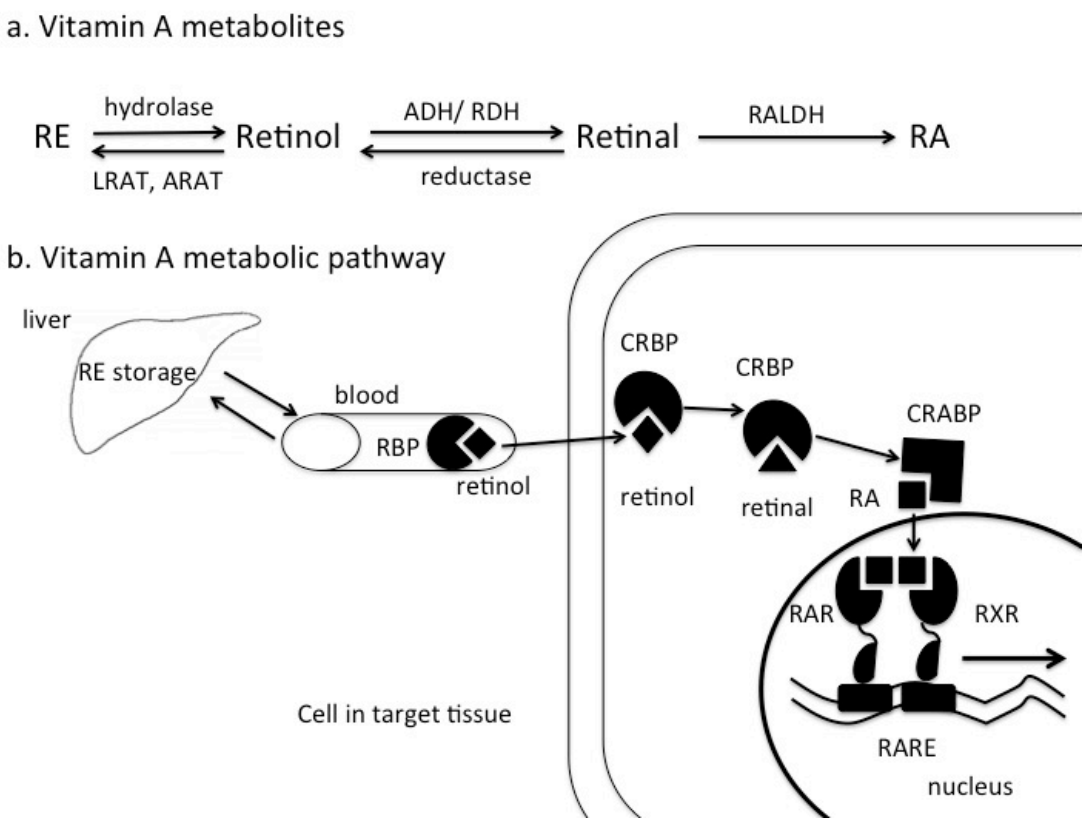

Figure 1. a. Vitamin A metabolites. RE (retinyl ester) can be hydrolyzed into retinol which can reversely be esterified into RE by LRAT (Lecithin retinol acyltransferase) or ARAT (acyl CoA:retinol acyltransferase). Retinol can be converted to retinal (retinaldehyde) catalyzed by ADH (alcohol dehydrogenase) or RDH (retinol dehydrogenase). And reductase could catalyze retinal back to retinol. Retinal can be further oxidized into RA (retinoic acid) by RALDH (retinaldehyde dehydrogenase). b. Vitamin A function pathway. Liver is the major storage site for VA as the RE form. RE can be hydrolyzed into retinol, which enters the circulation by binding to retinol binding protein (RBP) to meet the need from other tissue. When the retinol enters the target cell, it can bind to cellular retinol binding protein (CRBP) and be converted to retinal by RDH. The RALDH can catalyze the oxidization of RBP binding retinal further into RA, which binds to CRABP (cellular retinoic acid binding protein). After RA gets into the nucleus, it can binds to RAR and RXR to form a heterodimer, which can function through RARE (retinoic acid response element).

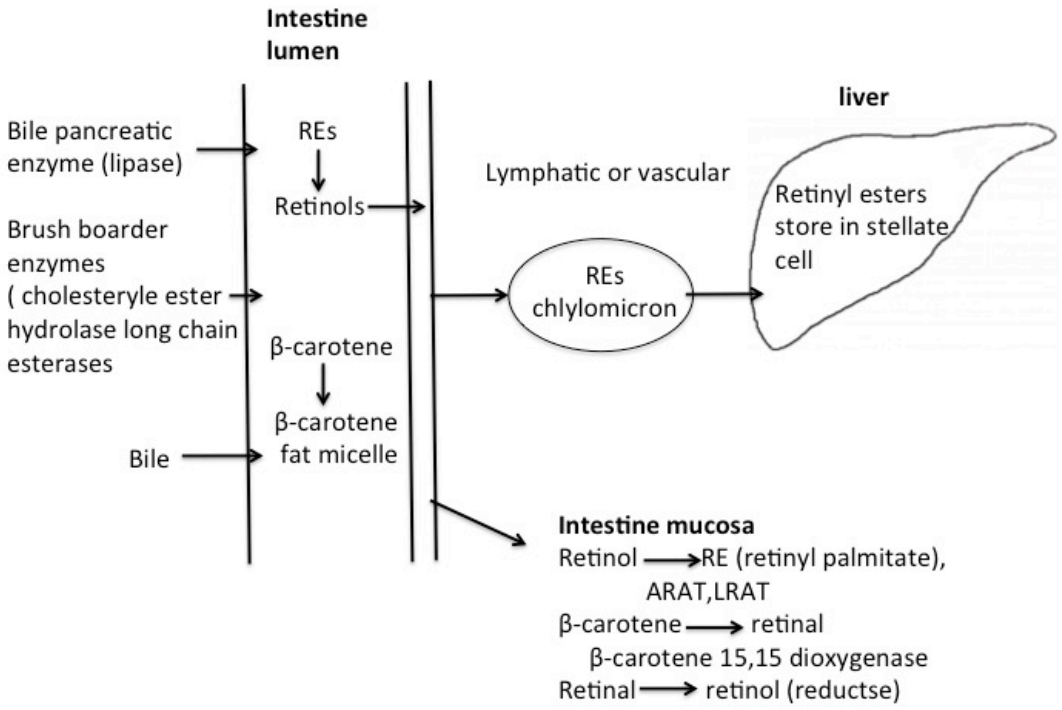

Figure 2. The absorption and metabolism of vitamin A. The dietary source of VA mainly has two kinds, provitamin A like carotenoids and preformed Vitamin A such as retinol and retinyl esters. β-carotene, the main carotenoids could be converted to retinol or absorbed intact into human body. Retinyl esters should be hydrolyzed in intestine lumen into retinol and then be absorbed by enterocytes. Retinols need to be reesterified to retinyl esters by lecithin retinol acyltransferase (LRAT) for incorporating into chlylomicrons with other lipid esters. These chlylomicrons then can be absorbed into the lymphatics or efflux into the vascular circulation where they can be hydrolyzed by lipoprotein lipase into chylomicron remnant containing most retinyl esters. Then chylomicron remnants are absorbed into liver where REs store.

Basic functions of vitamin A

VA has various functions, and the earliest discovered one is to maintain the normal vision. RA form is required for cornea and conjunctival membranes' normal differentiation. The 11-*cis*-retinaldehyde (retinal) form of VA is necessary for binding to opsins (rhodopsin and iodopsin). When light comes into eyes, the isomerization from *cis* to *all trans* leads to dissociation of retinal with opsin and then induces the signal to the visual cortex of brain [8]. Night vision and low light require rhodopsin binding and dissociation with retinal, so this is why deficiency in VA can result in night blindness.

Another function of VA, especially the RA form, is to maintain the normal skin health. RA, the active metabolite of VA, functions through binding to retinoic acid receptor (RAR) in nucleus. The RAR with retinoic X receptor (RXR) can form a heterodimer, which binds to DNA in retinoic acid response elements (RAREs) region (shown in Figure 1). The binding of RA to RAR leads to alteration of the RAR conformation, which affects the binding of other protein related to gene transcription. And RA regulates various genes related to epithelial cells, such as genes encode for skin keratins, laminin (the major protein in basal lamina) [8, 16].

VA, specifically RA, also regulates the embryonic development and is critical to the normal development of heart, lungs, vertebrate, and urogenital system [17,18,19]. RBP in yolk sac placenta involves in transferring retinol from maternal retinol pool to the embryo.

VA, as RA form, is also necessary for maintaining the immune response by regulating immune cell differentiation, proliferation and migration, which will be discussed later in VA and RA's effects on immune system.

Mucosal immunity

Recently mucosal surfaces have been explored for the potential for the development of more effective vaccines and immunotherapies. Unlike the parenteral vaccines, the mucosal vaccines do not need needles, so they can reduce the risks of other blood borne infections. Due to the safely and easily administration, especially for the infants and elder persons, vaccine via mucosal route is very attractive [20]. What is more, the mucosal immunization can induce both local and systemic immune responses [20,21].

However, there are some problems and limitations of mucosal vaccines, such as the weak immune response of the mucosal antigen and mucosal tolerance especially oral tolerance to prevent the intestine disorders such as food allergy and celiac disease [20]. Most nonviable antigens are diluted in the mucosal secretion, segregated by epithelial barriers and attacked by proteases and nucleases in gastrointestinal tract, which leads to ineffective enteric immunization [22,23]. Some food antigens are resistant to degradation and enters intestine. Then oral tolerance is critical for preventing the intestine disorders with reduced T-cell proliferation, cytokine production and serum antibody responses to food antigens. Now a number of different mechanisms have been implicated in oral tolerance, including clonal deletion and clonal anergy of T cells as well as regulation by Tregs with the IL-10 production [24]. So orally administrated protein immunogens induce only moderate immune responses, even when given repeatedly in large doses [22].

Only a few mucosal vaccines have been approved for human use: poliovirus, *Salmonella typhi*, *V. cholerae* and rotavirus vaccine for oral use and a nasal vaccine against influenza virus

[23,25]. Most mucosal vaccines involve live attenuated pathogens to induce the stronger and longer-lasting protection. However, in developing countries, the oral vaccines' protecting effects are still poor compared to industrialized countries. This situation may be due to the poor sanitation, VA deficiency, intestinal flora overgrowth and malfunction of digestive and absorptive system [5]. These all suggest the urgent need for developing effective and longer-lasting mucosal vaccines.

The architecture, cell type and trafficking of mucosal immune system

The respiratory, digestive and genitourinary tracts constitute the mucosa membranes, which is 200 times larger than the skin with $400m^2$ area [26,27]. It is obvious that mucosal surfaces face various antigens everyday and represent the most important portal of entry for estimated 70% of infectious agents and allergens [27,28]. A large number of immunological activities happen at the mucosal sites everyday to protect the body from the external environment.

The organized mucosa-associated lymphoid tissue (O-MALT) as the inductive site and the diffuse mucosa-associated lymphoid tissue (D-MALT) as the effector site constitute the whole mucosal immune system [27]. The O-MALT, including gut-associated lymphoid tissue (GALT) such as Peyer's patches (PPs) in intestine and bronchus-associated lymphoid tissues in respiratory tract, is responsible for the initiation of antigen specific immune response [28]. The genitourinary tract such as vagina does not have its own O-MALT, so it relies on the mucosa-draining lymph nodes that recognize the transported antigens [27,28]. The O-MALT gathers around the lymphoid follicles and resides below the mucosal epithelium [28,29]. Taking the PPs as an example, there

are almost 100 follicles in PPs in human ileum. Each follicle contains a light germinal center in the center and a dark peripheral region. Follicles contain most B cells and some macrophage and DCs, while T cells reside in para-follicular and inter-follicular regions [29]. Except for the higher percentage of B cells than T cells, the O-MALT is similar to peripheral lymph node [28].

D-MALT distribute all over the mucosal surface, including lymphocytes and plasma cells that acquire the effector and memory characteristics after encountering the antigen, such as plasma cells in lamina propria (LP) and intraepithelial lymphocytes (IELs) [29].

Forty percent of the lymphocytes in LP are fully differentiated IgA-producing plasma B cells, and 25% are T cells of which most are CD4+ T helper cells [29]. LP is responsible for most of the IgA antibody production. The lymphocytes are originated from O-MALT but disperse and migrate to distant mucosal sites after antigen stimulation. IELs are most γδ T cells with T cell receptor made up of one γ-chain and one δ-chain. γδ T cells do not need the APCs and MHC molecules to initiate the activation. The ontogeny and functions of IELs are different from the lymphocytes in LP, for IELs do not need priming [29]. Once encountering the antigens, IELs can kill the infected cells immediately by secreting cytokines.

In intestine, the epithelium barrier is composed of only single layer cells with the self-renewing ability. Though the epithelium is replaced every 2 ~3 days, the integrity of this single layer cells is key to maintain the health life. The stem cells near the base of the crypt of Lieberkuhn are continuously proliferating and differentiating into four different cell lineages： enterocyte, goblet cells，enteroendocrine cells and paneth cells [30]. The enterocytes are the main cell type for nutrient absorption and electrolytes secretion; goblet cells secret mucins;

enteroendocine cells produce hormones and paneth cells protect the stem cells by secreting alpha-defensins, lysozyme, phospholipase A2 and tumor necrosis factor-alpha [30,31]. Follicle associated epithelium (FAE) is different from the normal villus epithelium, for FAE contains few goblet cells, low level of digestive enzymes and less pronounced brush borders but with special M cells (Microfold cells). M cells do not have microvilli as the normal enterocytes and do not secret mucus or enzymes. Instead, M cells deliver the antigens from the intestine lumen to the DCs or lymphocytes with its phagocytic ability due to its thin overlying glycocalyx [29,30]. B cells and T cells clusters have higher frequencies near the M cells. The B cells near the M cells have a memory phenotype like the germinal center B cells.

Another important immune cells in mucosal epithelium are the IELs. IELs are mostly CD3+ and CD8+ with natural killer ability and antitumor ability. Different from conventional T cells, majority of the IELs are γδ T cells differentiated locally in epithelium or independent of thymus. Besides releasing cytokines and causing killing of infected target cells, γδ T cells are shown to mediate the host microbial homeostasis in intestine by producing antimicrobial factors to the bacterial pathogens [32]. There is no B cell or macrophage in epithelium.

The PPs, the secondary organized lymphoid nodules underneath the FAE, have the follicle center, which contains macrophages, DCs, small B cells, plasma cells in dome zone and surrounded by small lymphocytes that merge into the dome zone [31]. While PPs T cells, mostly CD4+, are shown high intensity in parafollicular area surrounding the follicles where a large amount of high endothelium venules reside [30]. The germinal center within PPs contains B cells that can proliferate, differentiate, somatic hypermutate and isotype switch. The germinal center,

which is important to adaptive humoral immunity, develops fast after T cell dependent antigen exposure to B cells. And finally, B cells become plasma cells secreting antibodies or memory B cells that could be reactivated by the same antigen. The isolated lymph follicles (ILFs), with prominent B2 cells from bone marrow, has also been assumed to have similar structure and functions as PPs [33]. With the similar function of supporting antigen-specific IgA production after oral immunization, PPs and ILFs' formation both require lymphotoxin receptor (LTR) dependent events but differ in timing and source of lymphotoxin [34]. Another organized lymphoid structures are cryptopatches (CPs), which are small cluster of immature lymphocytes such as T cell precursors and DCs. CPs now is considered as the extrathymic site for the IELs differentiation [35]. These intestine lymphoid structures are shown in Table 1.

The microflora in intestine recently gain interests of researchers, for the diverse microbial community forms a mutually beneficial relationship with the host. The microflora can help the metabolism of nutrients, the development of vasculature and intestine epithelium [36]. Also the formation of lymphoid tissue such as mature isolated lymphoid follicles needs the microflora induction [37]. However, the tissue invasion could happen if the homeostasis of microflora and host is broken. And the inappropriate activation of the immune responses may lead to the inflammatory bowel diseases with chronic inflammation in intestine [36]. So the epithelium is critical to maintain the sequestration of microflora, while the gut needs the some activation of immune system for protection. The activation of TLRs (Toll-like receptors) and NOD1 (nucleotide-binding oligomerization domain containing 1) are shown to be important to protect the gut from injury and regulate the intestine homeostasis [36,37]. IELs also can mediate the

homeostasis of host and microflora by secreting antimicrobial factors such as Reg III [32].

Structure	Types	Lynphocytes Composition	Appearance	Location
Lamina propria	Tertiary	B cells (Plasma cells), T cells (most CD4+)	A thin layer of loosely connective tissue	Beneath the epithelium
Intraepithelial compartment	Tertiary	T cells (most CD8+γδ T cells)	Scattered isolated cells	Epithelial layer of mammalian intestinal linings
Peyer's patches	Secondary	B cells (60%), T cells (35%)	Organized lymphoid nodules with multiple domes	Lamina propria layer of the mucosa of ileum, lowest portion of the small intestine
Cryptopatches	Primary	T cells and T cell precursors (few/no B cells)	Small clusters of lymphoid cells	Base of villi throughout the small intestine
Isolated lymphoid follicles	Tertiary	B cells (B220+), few T cells	1–2 domes	Distal>proximal

Table 1. Lymphoid structures in the intestine. Lamina propria and Intraepithelial compartment are the loose and diffuse mucosa-associated lymphoid structures. Peyer's patches, cryptopatches and isolated lymphoid follicles are the organized mucosa-associated lymphoid structures.

IgA synthesis and secretion

IgA is the most abundant antibody that plays a role in mucosal immunity. Above 80% IgA secreting plasma cells are located in the intestinal LP, and each day 3 to 5 grams IgA are secreted into the intestinal lumen [36]. IgA can be found in most mucous secretions, including saliva, tears, colostrum and secretions from the genitourinary tract, gastrointestinal tract, prostate and respiratory epithelium. The secretory IgA is critical for mucosa effective protection from external environment, for it can neutralize many pathogens in the intestine in despite of the protein digestive enzymes due to its protease resistant ability [28]. IgA is secreted in a dimeric form across the epithelium through the active transport mechanism.

The study of exploring IgA producing plasma cells existing site can be traced back to 1970s when researchers used the rabbit model [38,39]. With allogeneic cell transfer to irradiated rabbits, Susan group defined that the precursors of IgA-producing cells are in PPs [38]. PPs are considered the major inductive site for the generation of the IgA secreting plasma cell, and ILFs as well as LP outside the PPs are the main effector sites, which include the final differentiation of IgA-producing plasma cells and secretion of IgA [33].

With high level in PPs and none in lymph nodes, IgA+ B cells develop mainly in germinal center in PPs [40]. And they proposed that the reason why PPs are the prominent sites for generating IgA-producing plasma cells is the chronic and continuous activation by the microenvironment in intestine [40]. This unique microenvironment and special Treg cells make germinal centers in PPs unique and promote the class switch to IgA. The development of IgA-producing B cells requires antigen stimulation, germinal center induction and the cytokines

secreted by LP stromal cells. With the help of follicular DCs and CD4+ T cells to trap and present antigens, B cells go through proliferation, class switch recombination and somatic hypermutation [33]. Besides the PPs, the ILFs have also been proposed to involve in producing antigen specific IgA production [34].

The peritoneal cavity has been shown as another source of intestine IgA+ B cells in mice but not in human. With the chimeric mice model of reconstituting irradiated mice with bone marrow cells and peritoneal cells, the staining showed that half of plasma cells including IgA, IgM and IgG in gut and lymph organs are originated from peritoneal cells [41]. Unlike the natural IgM produced even in germ-free environment, IgA production by B1 cells in peritoneal cavity needs antigen stimulation such as commensal microflora [33]. With previous evidence that intestine IgA exist in T cell deficient mice together, Andrew group proved that B1 cells in peritoneal cavity, different from the induction of IgA in PPs, produce the IgA through the T cell-independent pathway [42].

After the induction in PPs, the IgA+ B cells migrate into mesenteric lymph nodes (MLN) for further proliferation and differentiation into plasmablasts, which mainly home to the gut LP [33]. The interaction between the ligand expressed on target tissue and receptors on lymphocytes guide the migration of lymphocytes. Mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1) expressed on vascular endothelium in the LP can interact with $\alpha_4\beta_7$, an integrin on lymphocytes, to direct the homing of lymphocytes [43]. This is the main ligand-receptor pair for directing lymphocytes homing to gut LP but not specific for IgA-producing plasma cells. In mice, thymus-expressed chemokine (TECK, CCL25), selectively and highly expressed in gut epithelial

cells, interacts with CC chemokine receptor 9 (CCR9) to recruit the IgA-secreting cells, but not IgM/IgG-producing cells from MLNs, PPs or spleen [44,45].

T cell dependent antigens

T cell dependent antigens are a group of antigens that cannot directly activate the B cells to produce antibodies without the T helper cells. After the antigen is digested by antigen presented cells (APCs) such as DCs and macrophages, the small fragment binds to the MHC Class II protein. T cells can be stimulated through recognizing the protein complex presented by APCs and produce autocrines. After T cells proliferate and differentiate into effector and mature T cells, T helper cells can activate B cells to produce specific antibodies with the recognition of both CD40 to CD40L and MHC class II to TLR [46]. Most antigens are T cell dependent, and the antigens used in my study are as follows:

Ovalbumin:

As the main constituent protein of the egg white, OVA is the glycoprotein used for immunization and protein structure and function study. Although it belongs to the Serpin family, it does not have the protease inhibitor function [47].

OVA is the mild immunogen widely used in many mouse models of immunology study. Some papers utilize OVA models to study oral tolerance [48,49]. The oral tolarence is really important for maintain the normal life, because the gastrointestinal system faces many food proteins or other bacterial antigens in mucosa flora everyday. While other studies use OVA as an

immunogen injected subcutaneously or intraperitoneally to induce the immune response to study other effect or functions, such as the RA can induce the homing of T and B cells to intestine in immunized mice [5].

In my experiments, OVA in high dose is mainly used as the oral immunogen to induce the immune response to study the effect of RA or VA supplementation.

Tetanus Toxoid (TT):

Tetanus is caused by the exotoxin produced by *Clostridium tetani*, the bacteria that can enter the body through the wound of skin and secret poison tetanospasmin, which can attack the nerve system and block the signal. This disease is acute and lethal [50].

The inactivated TT, first developed in 1924, is the successful vaccine used in World War II. DTP (vaccine of diphtheria, tetanus and pertussis) was used for more than 60 years, and now two new types of vaccine (TdaP and DTaP) have been developed and applied since 2005 [50]. DTap and Tdap are still combined vaccines but the pertussis component is acellular, and DTap and Tdap differ in dosage with capital letter meaning higher doseage.

Tetanus mouse models are used in many immunization studies. Ross group study the adjuvant effect of RA combined with PIC in neonatal mice immunized with tetanus [6]. In TT immunized mice model, VA and RA oral supplementation can lead to stronger antibody production [51].

In my study, the TT was injected subcutaneously to study the effects of supplementation.

Cholera toxin subunit B (CTB):

Cholera toxin (CTX), an enterotoxin produced by bacterium *Vibrio cholerae,* can cause massive diarrhea and vomiting symptoms out of cholera infection. CTX is an oligomeric protein complex consisting of six subunit. Subunit A is enzymatic and toxigenic with adenosine-diphosphate-ribosyl-transferase activity; subunit B is pentameric and responsible for binding to the cell membrane receptor, including five subunits [52,53].

CTX is now considered as the potential immune adjuvant via different routes, such as mucosal, parenteral or transcutaneous [53]. Now as the mucosal vaccines gains more and more attention, many researchers focus on exploring the potential of CTX as the mucosal adjuvant when orally or nasally administrated. The mechanism how CTX enhance the mucosal immunity is not well identified. One study illustrated that CTX can activate DCs through interaction with the GM1 ganglioside receptor in DCs [54].

However, since CTX may be toxic, some researchers use CTB as a safe adjuvant for studies [20,21,22,52]. There are some doubts about the efficiency of CTB as the mucosal adjuvant, but it may depend on the antigen type, dosage, animal species and age, immunization route and method of conjugation [52]. Tochikubo showed an elevated bovine serum albumin (BSA)-specific IgG and mucosal IgA response when immunized with BSA combined with CTB as adjuvant in mice [50]. Repeated nasally administered tetanus toxoid or diphtheria toxoid with CTB induces the high level of serum antigen specific IgG and secreted IgA in mice [20]. There is an interesting study in rhesus monkeys. The authors compared monkeys that intranasally immunized with CTB chemically conjugated with antigen to CTB, mixed with antigen, and detected the systemic and

mucosal antibody responses in saliva, nasal, genital tracts [22]. Although two groups did not show a significant difference, the authors successfully generated the strong mucosal immune response in rhesus monkeys model that is more close to humans.

These studies suggest the potential role of CTB as a mucosal adjuvant. In my experiments, CTB is used as the mucosal adjuvant orally administrated with OVA.

Vitamin A and retinoic acid's role in immunity

VA and RA's effects on immune system

VA plays an important role in regulating both innate immunity and adaptive immunity, mainly through its active metabolite form RA.

VA is critical for maintaining the normal skin, which is the primary barrier to infection. VA deficiency in rats compromise the mucosal barriers in intestine caused by loss of goblet cells and epithelia of cornea and conjunctiva of the eye by altering the expression of mucin genes in rats [55,56]. Mucus plays an important role in resisting to infection by trapping the pathogens and being swept away out of body [4]. RA has also been shown to have various roles in innate immunity. RA successfully reestablished the circulating lymphocytes such as natural killer cells, which have lower number in VAD status [57]. RA, by binding to RAR and modulating target genes, could regulate the neutrophil maturation [58]. As for the macrophage, the total cell number of the macrophage increased in VAD mice [59]. And retinoids such as 9-*cis*-RA inhibits LPS-induced IL-12 production in primary macrophage in vitro [60]. With some other studies together, VA deficiency may cause stronger inflammation by increasing the IL-12 and IFN-γ production from macrophage with an impaired phagocytic ability [4].

Besides innate immune system, VA has many functions in adaptive immune system. VA influences T helper 1(Th1)/T helper 2(Th2) cells balance, and in brief VA deficiency could enhance Th1 response but impair Th2 immune response. Th1 cells work with macrophage to produce IFN-γ and IL-2 when encountering with intracellular pathogens, such as virus. Th1 cells activate the CD8+ cytotoxic T lymphocytes (CTLs) response and stimulate macrophage. While

Th2 cells can produce IL-4, IL-5, IL-10 responding to extracellular antigens, which promote humoral immune response such as IgG1, IgA and IgE production. Th1 cytokines can downregulate the Th2 activities, and reverse is the same. RA treatment leads to a reduction of IL-2 gene expression in mice but in vitro it increases the secretion of IL-2 by human peripheral blood T cells [61]. CD4+ T cells produce increased IFN-γ in VAD mice than VA sufficient mice, and after supplement of RA the secretion of IFN-γ decreased [62]. Besides IFN-γ, the frequency of IL-5-secreting cell is lower in VAD mice and with RA supplement in vitro the population doubled [63]. And IL-10 secretion also is lower in VAD mice than control [63]. These studies suggest that VA promotes the Th2 cell response and downregulate Th1 cell response.

Treg cells, which are suppressor T cells, regulate the immune system by preventing recognizing self-antigen and abolishing the autoimmune diseases. In 2007, Mucida et al found that retinoic acid helps TGF-β to promote the T regulatory cells differentiation from naïve T cells in vitro; LP DCs and mucosal CD103+ DCs promote conversion of CD4+ T cells to Treg cells dependent on RA and TGF-β [64,65,66] (Figure 3). Both the antagonist of RAR and the blockade of TGF-β can diminish Treg cells differentiation [64,65,66]. What's more, RA counteracts IL-6 activity and inhibits the IL-6 and TGF-β dependent Th17 cell differentiation [64]. These studies suggest RA, the VA metabolite, can balance the pro and anti inflammation response by regulating Treg and Th17 cells.

Besides T cells, RA also affects B cells differentiation and antibodies production. With RA treatment, the human tonsillar B cells showed an increased plasma cell phenotype in vitro cell culture, which indicate that RA could induce the maturation of B cells into plasma cells [67].

High VA level diet mice group showed the increased saliva IgA production after influenza A virus Infection, and anti-CT IgA level is significantly lower in VAD rats than control when immunized orally with cholera vaccine [68,69]. IgG1 response is poor in VAD mice immunized with purified protein antigen or infected with *Trichinella spiralis* [4,70]. In human study of mild vitamin A deficiency children in Indonesia by Semba et al, the VA supplemented group has a stronger immune such as IgG response to tetanus vaccine than control, similar finding has been reported in infants received diphtheria vaccine by Rahman group [71,72].

VA supplements have been shown to play an important role in some infections by reducing mortality, improving recovery from measles, decreasing the severity of malaria infection [4].

Impaired immunity in VAD animals and human

VAD animal model has been applied in many studies to investigate the immune functions of the VA. Many studies suggest that VAD animal models showed some symptoms of impaired immunity. Enlarged spleen and lymph node were observed in severe VAD mice, along with impaired cell-mediated imunity and less IgM antibodies production in primary immune response [59]. In diarrheal diseases study, VA deficiency impaired the recovery from infection; CTLs activity was compromised, by showing the diminished CTLs pool, in VAD chickens infected with newcastle disease virus [7]. Moderate VA deficiency leads to the failure of antigen-specific T lymphocytes trafficking into gastrointestinal tract, impaired cellular immune response in gastrointestinal tract [73]. As described before, VAD mice also have a poorer IgG1 antibody response compared to VA sufficient when infected with *Trichinella spiralis.* These studies

suggest that VAD animals and human have impaired immunity with the higher morbidity and mortality rate than VA sufficient one.

RA's role in gut mucosal immunity

RA has been shown to play a critical role in mediating the immune and tolerance in intestine immune system. DCs in intestine but not from extraintestinal sites can produce RA with the expression of RALDH, which convert retinol to RA. These intestine APCs mainly exist in PPs, LP and MLNs [74].

One study suggests that RA function through a positive feedback loop in gut DCs to induce its own production, which implies another source of RA to start this loop [75]. Intestinal epithelial cells have been shown to produce RA with high expression of RALDH1 [76]. Different from RALDH2, RALDH1 expression is not affected in VAD mice [77]. This may suggest the intestinal epithelial cells maybe the intrinsic and primary source of RA. This positive feedback loop requires MyD88 signaling which is conventionally correlated with Toll-like receptor and IL-1/18 signaling [75]. Another study also suggests that RA with TGF-β derived from intestinal epithelial cells can induce DCs to become the Treg cell-promoting type of DCs [78]. In conclusion, RA from intestinal epithelial cells induces the intestinal DCs to produce more RA and promote the Treg cell differentiation and IgA-secreting cells.

RA plays an important role in regulating the balance of Th17 and Treg cells as discussed before in VA and RA's effects. Recently a study showed that the local environment, especially the intestinal epithelial cells, drive the special phenotype of the intestine DCs [78]. The TGF-β

and RA derived from intestinal epithelial cells are required for the conversion of DCs to CD103+ Treg cells-promoting DCs [78]. What's more, RA can counteract IL-6 activity and inhibit the IL-6 and TGF-β dependent Th17 cell differentiation [64]. However, another recent study suggests that that RA in a lower concentration (1 nM), secreted by CD11c+CD11b+ LP DCs, induced the Th17 cell differentiation [79]. From above results, we can conclude that RA regulates the Th17 cell in a dose dependent manner. These studies suggest RA, the VA metabolite, can balance the pro and anti inflammation response by regulating Treg and Th17 cells.

RA also regulates the gut tropism such as the homing of the T cells and B cells to intestine. After the priming by antigens in MLN or PPs, T cells express the homing receptors, such as integrin α4β7 and the CCR9 [80]. These α4β7+ or CCR9+ T cells preferentially migrate the intestine epithelium expressing TECK/CCL25 and the MAdCAM-1 [81]. The expression of α4β7 and CCR9 on activated T cells was upregulated by RA, which could further mediate the homing of T cells [82]. VAD mice showed a depletion of T cells in LP and a reduction of activated T cells in lymph organs [82]. Not only activated T cells but also IgA-secreting B cells can be regulated the homing to intestine by RA [83]. DCs from GALT can induce the homing receptors expression and IgA production of B cells [83]. RA alone induces the gut tropism of B cells by enhancing the expression of α4β7 and the CCR9, but RA needs synergistically working with IL-6 or IL-5 derived from GALT DCs to promote IgA secretion [83]. VAD mice showed a significant reduction of IgA-secreting B cells in gut [83]. In sum, RA induces the homing of activated T cells and B cells by increasing the expression of homing receptors, and VA is critical for maintaining the normal immune cells in intestine. RA functions in intestine are shown in Figure 3.

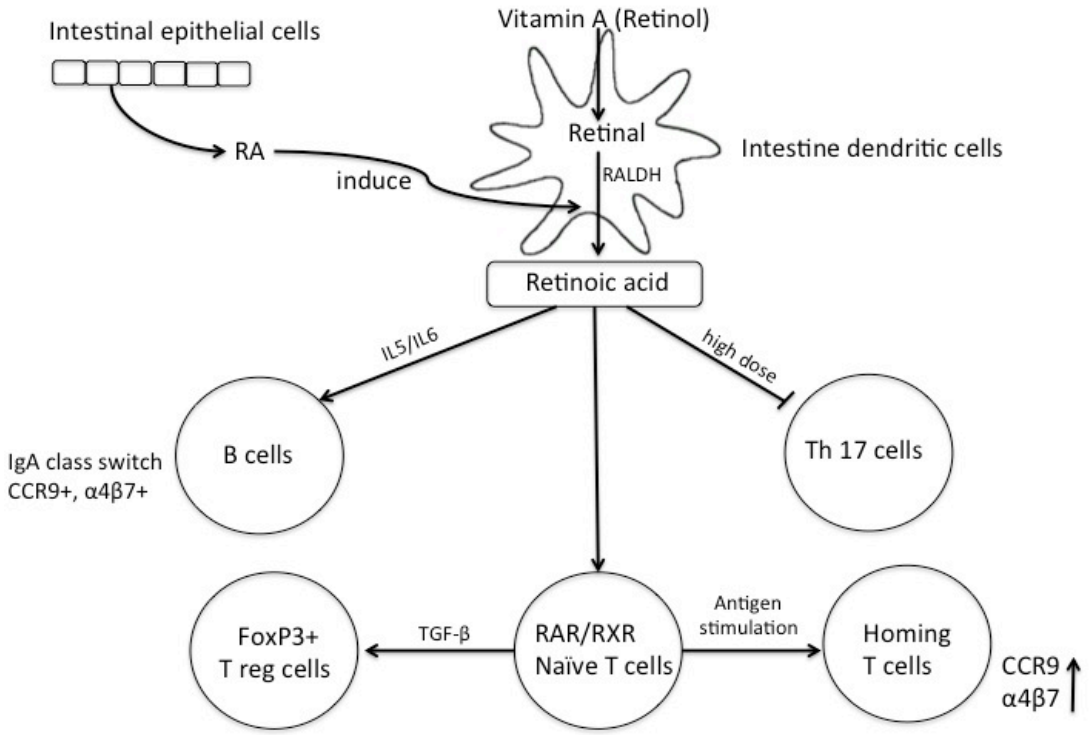

Figure 3. RA's multiple functions on intestine immune system. RA, secreted by intestinal epithelial cells, induces the positive feedback loop in dendritic cells to produce more RA. RA in high dose suppresses the Th17 cells differentiation, while with TGFβ together RA promotes the differentiation of FoxP3+ T reg cells. RA is necessary to imprint gut-homing lymphocytes such as B cells and T cells by increasing the expression of homing receptors CCR9 and α4β7. RA and IL5/IL6 synergistically induce the IgA class switch and secretion.

CHAPTER 3

EXPERIMENTS AND RESULTS

Materials and Methods:

Animals: Animal protocols were approved by the Institutional Animal Use and Care Committee of Pennsylvania State University. 8 weeks old BALB/c mice purchased from Jackson Laboratory were bred under specific pathogen-free conditions. All the mice used in study were bred from adult Balb/c mice. Breeding was conducted by putting one male and one female in one cage with the special diet. After ten days, separate the male mice with female mice and female mice were still fed with special diet. Dates of birth were recorded and weaning was conducted at day 21 after birth. During the pilot study, mice were fed with VA marginal diet (Modified AIN-93G rodent diet with 1.34mg retinyl acetate/kg, Research Diets. INC. NJ). For the supplementation study, all mice were fed vitamin A deficient diet (AIN-93G growing rodent diet without added vitamin A with soybean oil, Research Diets. INC. NJ).

Experiment design and Immunization:

Pilot study: All mice are on VA marginal diet starting from breeding to the euthanization. The detailed experiment plan is shown in table 2. Mice are divided into 6 groups, no immunization with canola oil treated as control or with RA supplementation, orally immunized with canola oil treated or RA supplementation and intra peritoneal injection with oil or RA treated. At day 9 after birth, pups were immunized orally with 17mg OVA (albumin from chicken egg white, Sigma-Aldrich) mixed with 170μg pneumococcal polysaccharide specific for serotype 3 (S-3)

dissolved in PBS, 20μg OVA mixed with 0.2μg S-3 in PBS for ip injection. For the second immunization at day 42, mice were immunized orally with 50mg OVA mixed with 50μg S-3 in PBS, and 50μg OVA mixed with 0.5μg S-3 in PBS for ip injection. RA was given 12.5μg per mouse per day from day 9 to day 13. Canola oil was given the same volume as control.

Supplementation study: As shown in Table 3, newborns were divided into 5 groups. Control group is without the immunization. Other 4 groups were all immunized orally with 20mg OVA and 20μg CTB (Cholera Toxin B Subunit from vibrio cholera, Sigma-Aldrich) as adjuvant and subcutaneously injected with 5μg tetanus toxoid (Connaught Laboratories) per mouse at day 22 after birth. One group is fed with canola oil as control of other supplementation, other three groups were supplemented with vitamin A (retinyl palmitate dissolved in canola oil, 50μg, three times, once a day), retinoic acid (RA was dissolved in canola oil, 25μg each time per day, three times, once a day) orally and retinoic acid (RA were dissolved in Polyethylene glycol 400(Sigma-Aldrich), 100μg each time per day, two times) subcutaneously injected into the dorsal flanks. Time for feces and blood collection are shown in Table 2. At day 44 of the second immunization, the immune dose changed to 50mg OVA and 50μg CTB by oral gavage and 10μg TT by subcutaneous injection per mouse. For the supplementation, 100μg VA and 50μg RA were orally treated per mouse per day from day 44 to 46 and 150μg RA was subcutaneously injected two times. And for the third immunization, the doses of immunization and supplementation were the same as the second immunization. At day 79, all mice were euthanized.

Day 0	Date of birth
Day 9	Randomly divided into six groups: no immunization (oil or RA), orally immunized (oil or RA) and intra peritoneal injection (oil or RA). Primary immunization. Supplementation was given from day 9 to day 13 once a day. Oil is for control.
Day 20	Euthanized half of the mice in each group
Day 21	Weaning
Day 42	Second immunization without supplementation
Day 49	Euthanized the rest mice

Table 2. Experiment plan for pilot study. Total 43 mice, 8 for no immunization with oil, orally immunization with oil and no immunization with RA groups; 5 for both ip injection with oil and RA groups; 9 for ip injection with RA group.

Study of mice 1st/2nd/3rd immune response to OVA and CTB-orally and TT s.c. in VAD fed mice with RA or VA supplementation					
Day	Vitamin A Deficient diet				
Day 0(DOB)	Oil N=6	VA N=7	RA orally N=7	RA s.c. N=7	Control (no immunization) N=4
Day 22(1^{st})	1^{st} Immune. TT sc, OVA+CTB orally Supplementation from day 22 to day 24 Wean to VAD diet				No immunization Wean to VAD diet
Day 25	Collect feces				Feces as control
Day 28	Collect feces				Feces as control
Day 29	Retro-orbital bleeding for serum ELISA				Blood as control
Day 31	Collect feces				Feces as control
Day 44(2^{nd})	2^{nd} Immune. TT sc, OVA+CTB orally Supplementation from day 44 to 46				No immunization
Day 47	Collect feces				Feces as control
Day 49	Collect feces				Feces as control
Day 51	Bleed for serum ELISA				Blood as control
Day 52	Collect feces				Feces as control
Day 71	Bleed for ELISA				Blood as control
Day 72（3^{rd}）	3^{rd} Immune. TT sc, OVA+CTB orally Supplementation from day 72 to 74				No immunization
Day 74	Collect feces				Feces as control
Day 76	Collect feces				Feces as control
Day 78	Collect feces				Feces as control
Day 79	Euthanize: blood, small intestine and spleen OCT fix etc.				

Table 3. Experiment plan for supplement study. The feces from individuals were collected continuously after each immunization. Every immunization, the subcutaneously injected RA supplementation and TT injection were on the same leg. Bleeding was conducted through orbital sinus for live mice and posterior vena cava for the final euthanization. The doses of first and second immunization are given differently for the significant different body weight of day 22 and day 44.

Sample collection: Blood were collected by orbital sinus bleeding for live mice between immunizations and posterior vena cava bleeding for final euthanization. Mice were adequately anesthetized by isoflurane (Phoenix pharmaceutical) before commencing the orbital sinus blood collection procedure and given one drop of tetracaine hydrochloride ophthalmic solution USP (0.5%, sterile, Bausch&Lomb) after the bleeding.

Feces samples were collected for 30min~50min individually. The weights of the feces were recorded after collection. And then immerse the feces in PBS (1 ml of PBS solution per 100 mg feces) at 4°C for 2-4 h, then vortex intensely to homogenize it. After centrifugation at 15,000 × g for 10 min, the supernatants were collected and kept at -80°C until use.

Intestine IgA extraction: small intestine (5cm ileum) were excised and immediately placed in a cocktail of protease inhibitors (protease inhibitor cocktail tablets, Complete Mini) and then frozen in PIC at -20°C. After thawing, the tissues were disrupted with a tissue homogenizer (IKA T-10 basic, ultra turax) and saponin (saponin, from quillaja bark, Sigma) was added to 1% final concentration. Extraction was performed for 2 h at 4°C. After centrifugation at 12,500 g for 10 min at 4°C, the supernatants were collected and further clarified by another round of centrifugation [83].

Enzyme-linked immunosorbent assay (ELISA) for mouse OVA specific IgA detection: Dissolved the OVA into 150μg/ml with OVA specific coating buffer. Added 100 μl coating OVA to the wells of an enhanced protein binding ELISA plate (10μg/well, Nunc Maxisorb). Sealed plate to prevent evaporation. Incubated overnight at 4°C. Removed the capture antibody solution by

washing 3 times with Wash Buffer (0.05% Tween 20, 0.015 M Tris, pH 7.6, 0.135 M NaCl). Added 100ul Blocking Buffer (1% BSA in 0.15 M Tris Buffer, store at 4°C) per well to block nonspecific binding. Sealed plate and incubate at RT for 1h. Purified mouse IgA (BD Biosciences) as standards and samples were diluted in Blocking Buffer at 100ul per well (use 0.25μg/100ul as start for standards, better titrate first). Sealed the plate and incubated it for 2-4 h at RT or overnight at 4°C. Wash plate 3 times with wash buffer. Diluted the alkaline phosphatase-conjugated goat anti-mouse IgA detection antibody (1: 2000 dilution, titrate first, BD Biosciences) in Blocking Buffer. Added 100ul of diluted secondary antibodies to each well. Sealed the plate and incubate it for 1h at RT. Wash 3 times with Wash Buffer. Added substrate (p-nitrophenyl phosphate, Sigma-Aldrich, 1mg/ml), 100μl/well, RT for 30 min. Read Plate at 405 and 570nm and did calculation.

ELISA for Mouse anti-TT antibody IgM/IgG detection: Diluted the TT antigen to 10 μg/ml in 0.15M Tris buffer, pH 7.6. Added 100 μl of diluted antigen to wells of an enhanced protein binding ELISA plate. Sealed the plate to prevent evaporation and incubated overnight at 4°C. Removed the capture antibody solution by washing 3 times with Wash Buffer. Added 100ul Blocking Buffer per well to block nonspecific binding. Sealed plate and incubate at RT for 1h. Standards and samples were diluted in Blocking Buffer at 100 μl per well. 1:1000 dilution to start with. Sealed the plate and incubated it for 2-4 hours at RT or overnight at 4°C. Washed plate 3 times with wash Buffer. Diluted the alkaline phosphatase-conjugated goat anti-mouse IgG/IgM detection antibody (1:5000 for IgM, 1:10000 for IgG) in Blocking Buffer. Added 100 μl of

diluted antibody to each well. Sealed the plate and incubate it for 1h at RT. Washed 3 times with Wash Buffer. Added substrate (p-nitrophenyl phosphate, Sigma-Aldrich), 100µl/well, RT for 30 min. Read Plate, 405 and 570nm and did calculation. For the IgG detection, the titers were calculated based on the standard curve with a series of dilution folds. One unit of titer represents the maximal optical density for the standard sample.

High performance liquid chromatography (HPLC): Liver samples were collected at day 79 and retinyl ester and retinol levels were detected by HPLC [84].

Statistics: Statistics analysis was performed with Prism software (GraphPad Software, Inc). Data are reported as mean±SE. All significant values were determined by 1-way ANOVA, followed by Tukey's post hoc test. P value less than 0.05 was considered significant.

Results:

Part 1: pilot study

After primary immunization, RA supplementation did not enhance the mucosal immune response to OVA immunized orally, but it improved the IgA antibody response to ip injected OVA than the group without supplementation.

Comparing the control group to the oral group in Figure4a and 4b, almost no change has been found in the plasma total IgA and OVA specific IgA, which means the weak or none mucosal response to the oral immunization in VA marginal mice. RA supplementation has no effects on total IgA response in serum [Figure 4a]. As for the OVA specific IgA in plasma, RA+ip group shows a significant increase compared to the ip group, which indicates that RA supplementation boosts the OVA-specific IgA production with ip injection (Figure 4b). The significant increased OVA-specific IgA level did not influence the total IgA level, because the mice produce large amount of IgA everyday and the amount of antigen specific IgA was very small compared to the total IgA. For the intestine total IgA, no significant changes have been found in supplementation groups with the big variations, which may be due to the differences of the intestine samples. Though we took the same length and location of the small intestines, the intrinsic structures such as the numbers of PPs may be different within each group. So for the next supplementation study, we measured the feces IgA instead of homogenizing intestine. For the weak primary response of OVA specific IgA, it is normal for the mucosal immunization, especially it is the primary response and OVA is the mild immunogen. In addition, the mice were immunized at day 9 when mice were neonates with immature immune system.

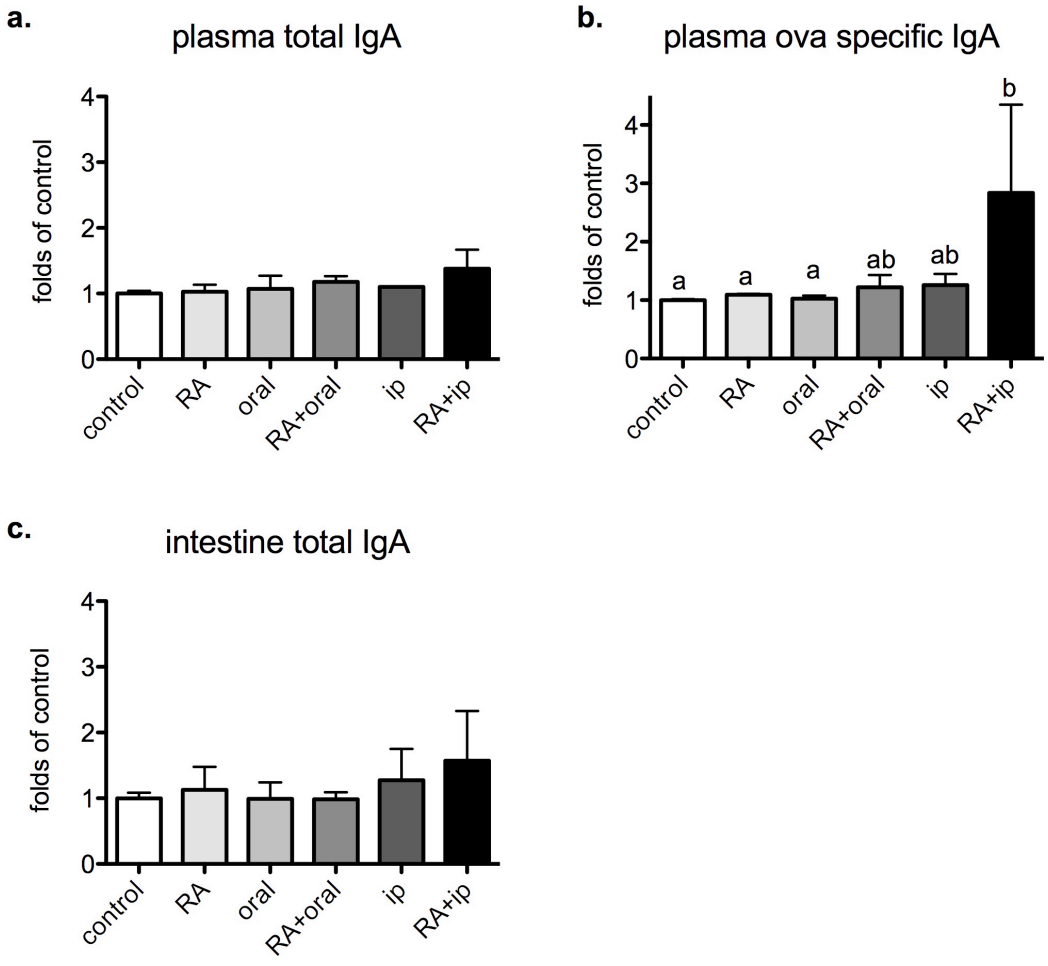

Figure 4. IgA detection after primary immunization. a. Plasma total IgA detection for six groups. No significant differences between each two groups. b. Plasma OVA specific IgA detection. c. Intestine total IgA detection. ELISA was conducted to detect the IgA level. 17mg OVA and 170μg S-3 for oral immunization; 20μg OVA mixed with 0.2μg S-3 in PBS for ip injection. RA was given 12.5μg per time per day for three times from the day of immunization. Mice were all on VA marginal diet. Control group (n=4) is without immunization and supplementation; RA (n=4) is the group RA supplemented without immunization; oral (n=5) is oral immunization without supplementation; RA+oral (n=5) is with both the oral immunization and RA supplementation; ip (n=3) is OVA and S3 intraperitoneally injection; RA+ip (n=3) group is positive control both with ip injection and RA supplementation. S-3 specific IgA was too weak to detect. The data are presented as the folds of the control group ($a < b$, $P < 0.05$).

In secondary immune response, oral immunization still cannot induce the mucosal IgA response, even with the RA supplementation.

There are still no significant differences between the control group without immunization and the oral group with immunization for the plasma total IgA and OVA/S-3 specific IgA of the secondary immunization (Figure 5a, b, d). This indicates that even for the secondary immunization the OVA or S-3 still cannot generate the strong mucosal immune response in VA marginal diet fed mice. Although the ip group showed some changes compared to the control group, it is still not significant (Figure 5a,b). And RA supplementation cannot rescue the weak immune response in VA marginal diet fed mice either (Figure 5a,b). As for the intestine total IgA, we found an increase in RA+oral group than oral group but the difference is not significant. In sum, the results are still not significant, but they show some potential changes in pilot study. So in the next supplementation study, we applied the mucosal adjuvant CTB and changed the primary immunization date from day 9 to day 22 when mice are not considered as neonates. We expect to get stronger mucosal immune response such as elevated antigen specific IgA response.

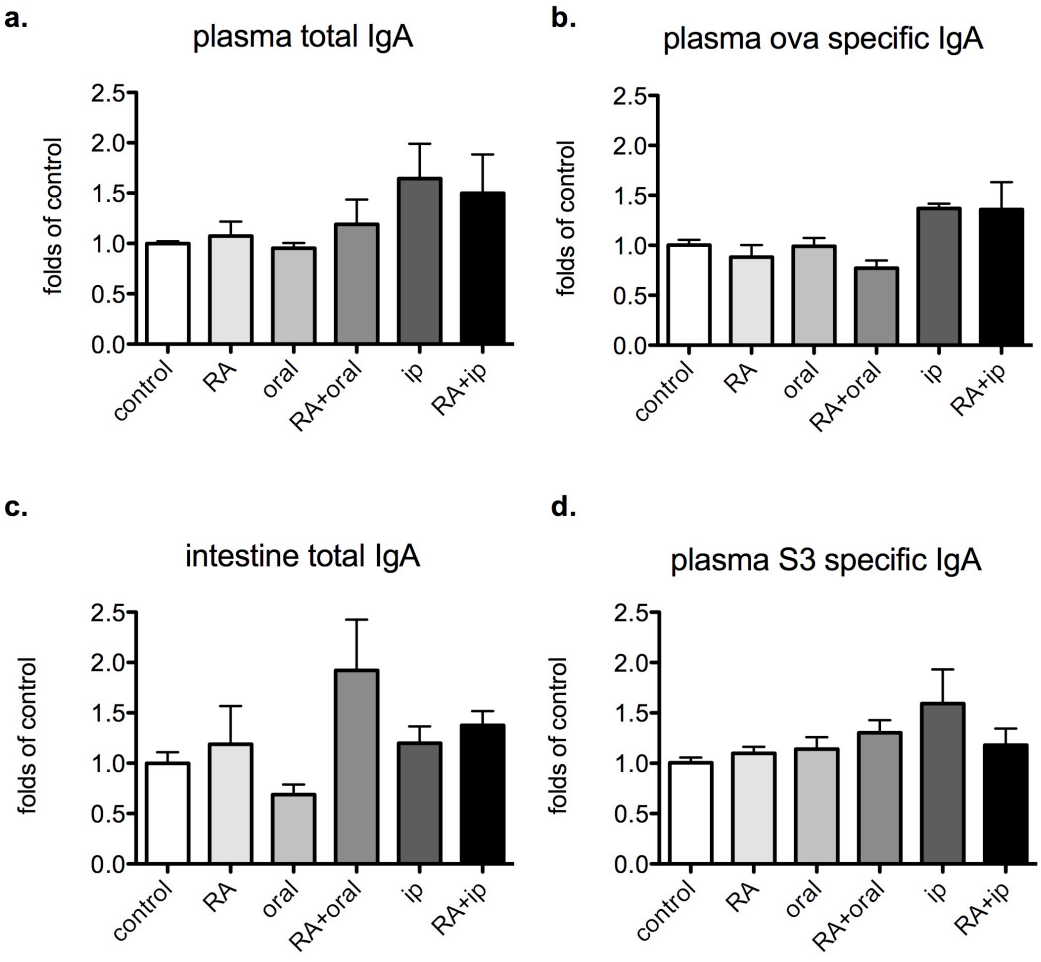

Figure 5. IgA detection for the second immunization. a. Plasma total IgA detection. b. Plasma OVA specific IgA detection. c. Intestine total IgA detection. d. Plasma S-3 specific IgA detection. 50mg OVA and 500μg S-3 were orally immunized; 50μg OVA mixed with 0.5μg S-3 in PBS were for ip injection. No supplementation was given at this time, and RA means the supplementation given during neonatal time. Control group (n=4) is without immunization and supplementation; RA (n=4) is the group RA supplemented without immunization; oral (n=5) is oral immunization without supplementation; RA+oral (n=5) is with both the oral immunization and RA supplementation; ip (n=3) is OVA and S3 intraperitoneally injection; RA+ip (n=3) group is positive control both with ip injection and RA supplementation in primary immunizaiton. The data are presented as the folds of the control group.

Part 2: Supplementation study

The mice fed on VAD diet from breeding to birth, weaning and final euthanization were depleted of VA storage in liver and retinyl palmitate supplementation can rescue the VA storage levels.

During supplementation study, all mice were fed on VAD diet starting from breeding and are considered as VAD mice. As shown in Figure 6, control group without supplementation shows no retinol or retinyl esters in liver, which indicates that the mice under VAD diet are depleted of VA storage in liver. Only VA supplemented group has a significant rescue of retinol, retinyl esters level in liver, while RA orally supplemented group shows a slight increase of retinol and retinyl esters, which suggests that the mice we generated were truly VAD models. By comparing the Figure 6b and 6c, we can tell that most retinyl esters rescued are retinyl palmitate.

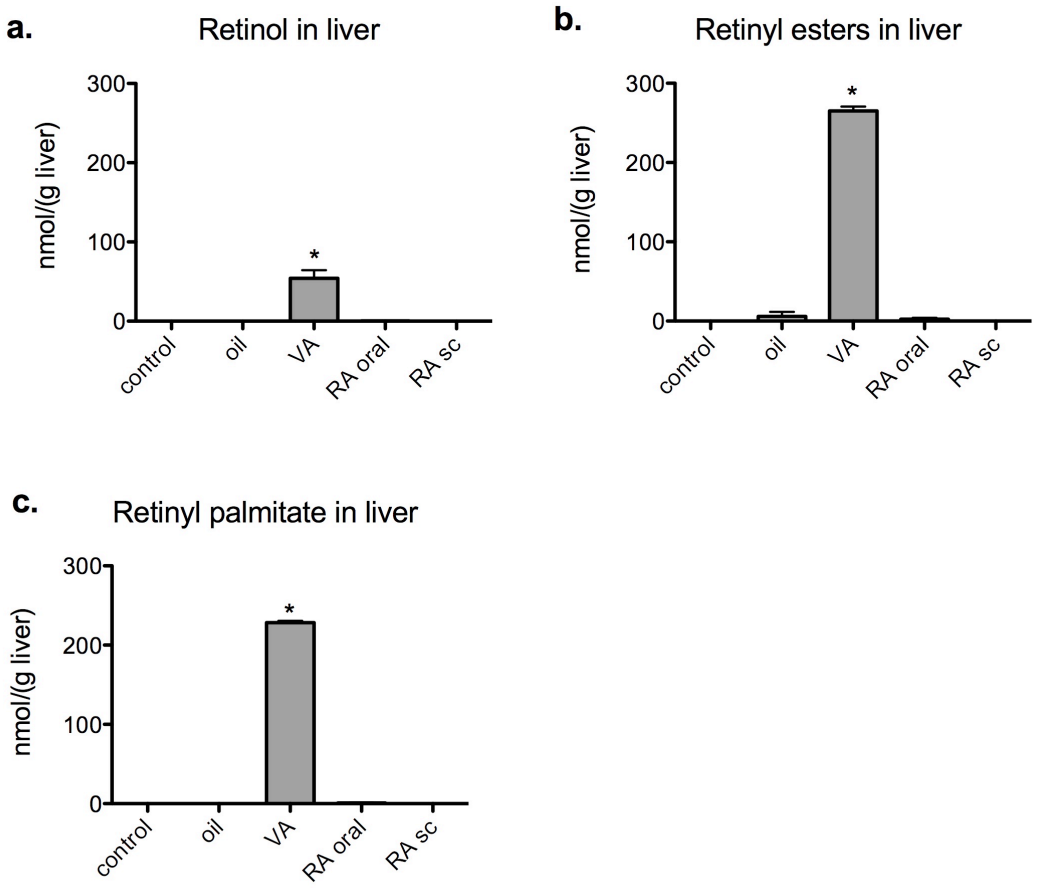

Figure 6. Retinol, retinyl esters and retinyl palmitate detection in liver. a. Retinol concentration of generated VAD mice and supplemented mice in liver. b. REs of generated VAD mice and supplemented mice in liver. c. Retinyl palmitate concentration of generated VAD mice and supplemented mice in liver. Livers were collected at day 79 after euthanization. Retinol and retinyl esters were detected by HPLC. The control group was without supplementation and immunization (n=3); other four groups were all immunized as table 3. The oil group was supplemented with canola oil (n=3); the VA group was supplemented with retinyl palmitate 50μg/day, three times each immunization (n=3); the RA oral group was fed with retinoic acid 25μg each time per day, three times for every immunization (n=7); the RA sc group was RA injected subcutaneously as 100μg each time per day, two times every immunization (n=3). $P<0.0001$.

The weak systemic immune response to oral challenge can be rescued by RA oral supplementation and RA subcutaneously injection.

From Figure 7, we find out that the VAD mice without supplementation did not response to oral immunization at all comparing the control group without immunization and oil group without supplementation. The primary immunization still did not induce strong plasma OVA specific IgA immune response in VAD mice even with VA or RA supplementation (Figure 7a). While after secondary immunization, RA sc group showed the significant increase of plasma OVA specific IgA than the oil group and the VA group, which indicates that RA by subcutaneously injection not the VA supplementation boosts the plasma OVA specific IgA response (Figure 7b). RA orally supplemented group showed elevated OVA specific IgA response though not significant (Figure 7b). As for the third oral immunization, both RA oral group and RA sc group successfully enhanced the plasma OVA-specific IgA when compared to oil group (Figure 7c). Even without the CTB as adjuvant, the mice seemed to be sensitized to the OVA with previous two immunizations with CTB by showing the strong OVA-IgA response after third immunization (Figure 7b,c). However, the VA supplementation did not show any significant effect on IgA response after secondary and third immunization (Figure 7b, c).

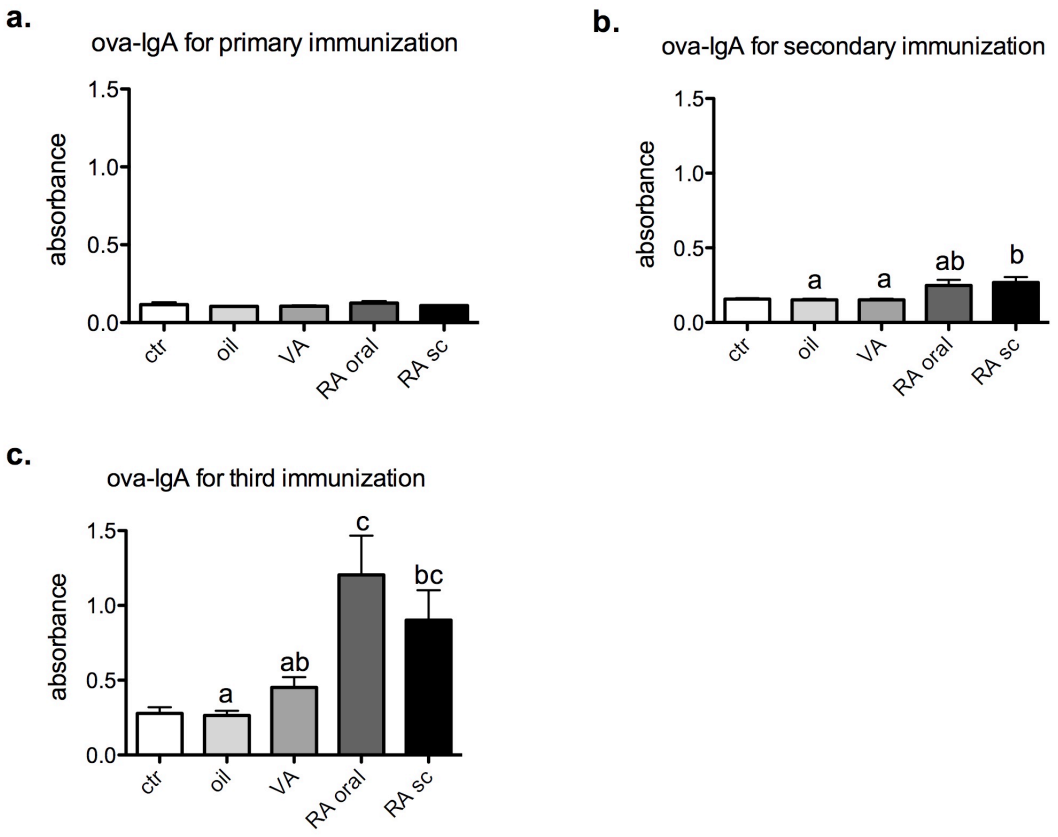

Figure 7. Plasma OVA-specific IgA detection for primary, secondary and third immunization in VAD mice. a. Plasma OVA specific IgA detection for primary immunization (day 22). b. Plasma OVA specific IgA detection for secondary immunization (day 44) ($a<b$, $p=0.003$). c. Plasma OVA specific IgA detection for third immunization (day72) ($a<b<c$, $p<0.005$). Blood were all collected 7 days after immunization. Control group (n=3) is without the immunization and supplementation. Other four groups of VAD mice were immunized orally with 20mg OVA and 20μg CTB for primary immunization and subcutaneously injected with 5μg tetanus toxoid per mouse at day 22 after birth. Oil group (n=5) is fed with canola oil as control of other supplementation, other three groups were supplemented with VA (50μg, three times, once a day, n=5), RA orally (25μg each time per day, three times, once a day, n=6) and RA subcutaneously injected (100μg each time per day, two times, n=5) into the dorsal flanks. Data are presented as absorbance calculated with three dilution folds. One-way ANOVA statistics were conducted with the transformed data and control group is not included.

RA supplementation by subcutaneously injection enhances the mucosal antigen specific IgA response in VAD mice.

Five days after the primary immunization, the fecal OVA specific IgA from other five immunized group did not show any increase compared to the control group, which means the primary immunization fail to induce the strong mucosal immune response even with supplementation (Figure 8a). While after second immunization, RA sc group showed the significant enhanced fecal OVA specific IgA response compared to the oil group, indicating the RA subcutaneous injection has the boost effect to the mucosal immune response to OVA oral challenge (Figure 8b). And RA oral group has an increase IgA response to the oil group but not significant (Figure 8b). However, in the third immunization, though both RA oral group and RA sc group have shown the same pattern as 8b with some enhancing effects on OVA specific IgA response, the differences are not significant.

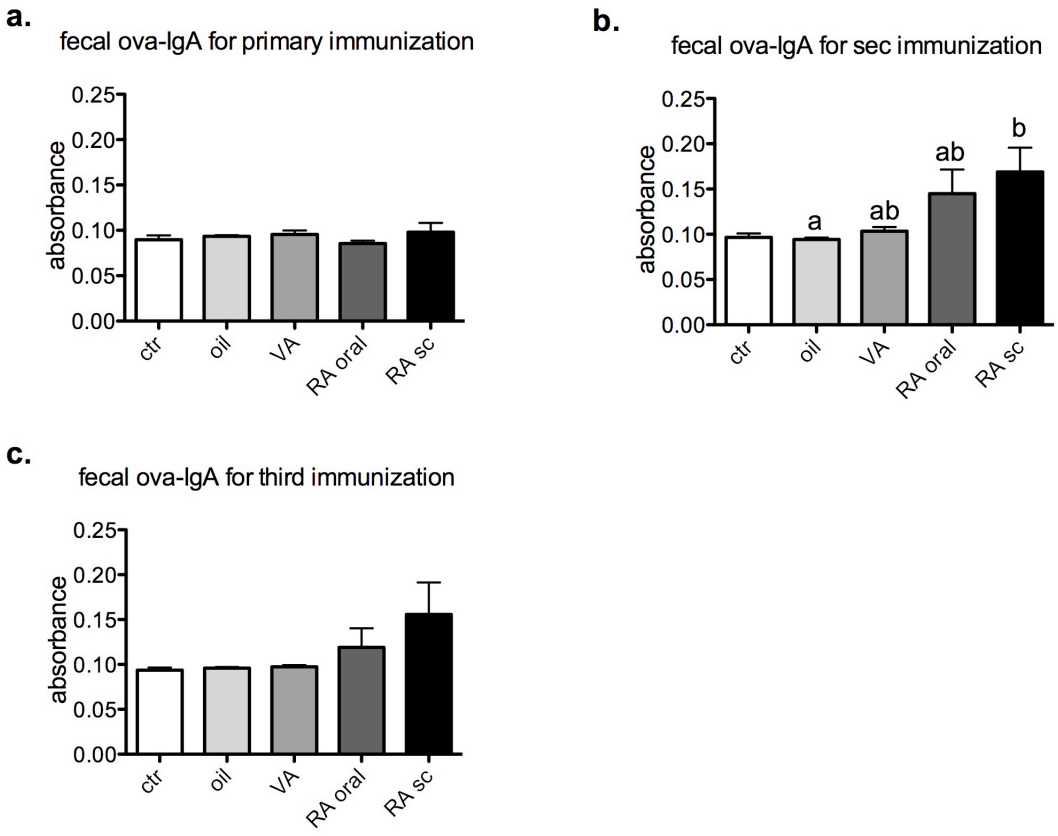

Figure 8. Fecal OVA specific IgA detection after each immunization. a. Fecal OVA specific IgA detection after primary immunization. b. Fecal OVA specific IgA detection after secondary immunization (a<b, $p<0.05$). c. Fecal OVA specific IgA detection after third immunization. All feces were collected 5 days after each immunization. The group treatments are the same as figure 7. Control group (n=3), other four groups n=5. Data are presented as absorbance calculated with three dilution folds. One-way ANOVA statistics were conducted with the transformed data and control group is not included.

VA oral supplementation enhances the immune response to secondary tetanus toxoid subcutaneously injection in VAD mice, while after the third injection the unsupplemented group has the strongest IgG response to tetanus toxoid.

By detecting the TT specific IgM levels in plasma, still no obvious enhanced immune response has been found after primary immunization (Figure 9a). After the second TT injection, all three supplemented groups show some increased TT specific IgG response and only VA group is significant (Figure 9b). However, the third immunization brings out an unexpected result that oil group without supplementation shows the strongest TT specific IgG response with the significant difference to the RA sc group (Figure 9c). We think the antigens in the supplemented groups (VA, RA oral, RA sc) may be neutralized by the existing TT-IgG in secondary immune response and could not induce the strong IgG response.

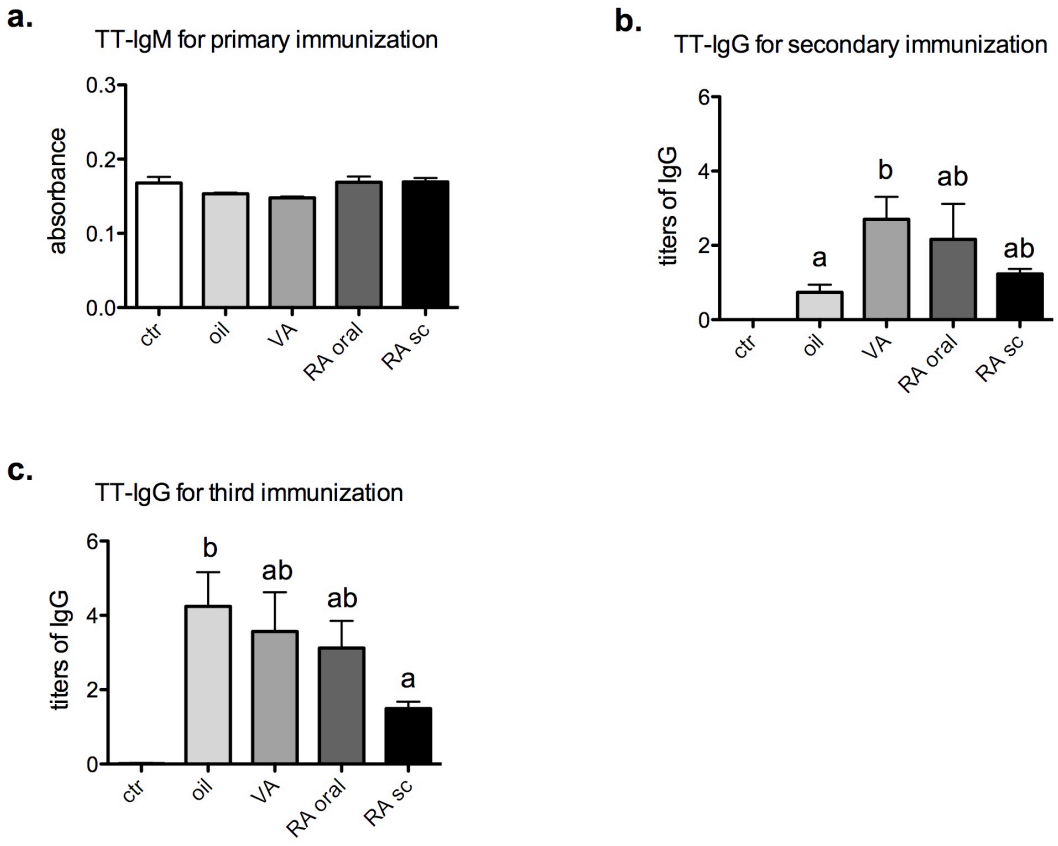

Figure 9. Plasma TT specific IgM/IgG detection. a. Plasma TT specific IgM detection for primary immunization. Control group (n=3), oil group and RA oral and RA sc (n=5), VA group (n=4). b. Plasma TT specific IgG detection for secondary immunization (a<b, $p<0.05$). Control group (n=3), oil group and RA oral and RA sc (n=7), VA group (n=4). c. Plasma TT specific IgG for third immunization (a<b, $p<0.05$). Control group (n=3), oil, VA and RA oral groups (n=7) and RA sc group (n=6). All plasma samples were measured by ELISA. Data are represented as absorbance in 9a and titers of IgG in 9b and 9c. The TT-specific IgG was undetectable in control group. One-way ANOVA statistics were conducted with the transformed data and control group is not included.

CHAPTER 4

DISCUSSION

Now many potential mucosal adjuvants have been studied to enhance the immunogenicity, and the most promising adjuvants are cholera toxin from *V. cholerae* and heat-labile enterotoxin (LT) from *E.coli*, but they are too toxic for human [28]. CTB, nontoxic protein from cholera toxin, shows its potential of mucosal adjuvant in many animal studies. Tochikubo showed an elevated bovine serum albumin (BSA)-specific IgG and mucosal IgA response when immunized with BSA combined with CTB as adjuvant in mice [52]. However, there are some doubts about the efficiency of CTB as the mucosal adjuvant, but it may depend on the antigen type, dosage, animal species and age, immunization route and method of conjugation [52].

In my study, VAD mice did not shown the detectable IgA response to OVA with the CTB as adjuvant in primary and secondary response by comparing the oil group with the control group. Even in the VA group which restored the RE level in liver, OVA immunization with CTB still failed to induce the antigen specific IgA response. Considering we just simply mix the OVA and CTB together, the conjugation could be one explanation. Mixed antigen and adjuvant could be diluted and separated by chyme and mucus in the gastrointestinal tract so that OVA and CTB may not function together to induce the strong immune response. However, one study in monkeys model compared conjugation effect of CTB through intranasally immunization with CTB chemically conjugated with antigen or mixed with antigen, and detected no significant difference in the systemic and mucosal antibody responses in saliva, nasal, genital tracts [22]. Another explanation could be the animal age. Compared to other study of potential mucosal adjuvant CTB

in adult mice, our model generated the primary oral immunization at day 22, which is considered the juvenile age in mice. Maybe the immune system of juvenile mice is still not mature enough to generate strong mucosal immune response especially through oral immunization with OVA, a mild antigen.

RA has been demonstrated to be a potential adjuvant with its multiple functions on the immune response. RA injected subcutaneously imprints T cells and plasma cells' gut homing capacity by upregulating the gut homing receptors in inguinal LNs and induces the class switch to IgA in inguinal LNs [5]. RA combined with PIC stimulated the anti-TT immune response such as anti-TT IgG production, TT-specific lymphocytes proliferation and cytokine production in neonates [6]. Oral VA and retinoic acid administration fully restored the abrogated antigen-specific T-lymphocyte homing to the gastrointestinal tract, gastrointestinal cellular immune responses and vaccine protective efficacy in VAD mice [7].

Based on my results, RA supplementation works better than VA. VA supplemented mice restored the RE levels in liver while VA group still did not shown the strong immune response to the oral challenge. This indicates that VA status may not responsible for the weak mucosal immune response in my study, which could be confirmed with a VA sufficient group in future study. VA could be catalyzed into RA through three enzymatic steps, but the VA group still failed to induce the strong mucosal immune response. We speculate that the enhanced mucosal and systemic immune response requires the immediate exist of RA during each oral immunization. RA could be used to boost mucosal vaccine response but VA cannot.

Compared to Swantje's study in 2011, we reduced the dosage of RA sc injection considering

the mouse health situation and still got the positive results. RA sc supplementation successfully enhanced the plasma and fecal OVA specific IgA production after secondary immunization and plasma OVA-IgA in third immune response. Based on these results and previous studies, RA seems to be the promising adjuvant for the mucosal immunization, especially for the VAD population in developing countries. However, supplementation by subcutaneous injection is not suitable and applicable to human, while the oral feeding is an acceptable way to human. Based on my results, the RA oral group showed its enhancing effect after third immunization, which suggests the repetitive oral supplementation of RA and immunization could be a possible way to enhance the immune response to immunization in humans.

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