SERUM AMYLOID A IS A RETINOL BINDING PROTEIN THAT TRANSPORTS RETINOL DURING BACTERIAL INFECTION

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Dedicated to my husband Mitko and my Mom and Dad

for all your unwavering love and support.

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by

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Retinol plays a vital role in the immune response to infection, however it remains unclear which proteins mediate retinol transport during infection. Serum amyloid A (SAA) proteins are produced by the liver following acute systemic infection and are also induced by bacteria in the intestine. SAAs have been proposed to play a role in the inflammatory response to infection and injury, but their exact functions have not been well defined. In this dissertation, I present data that demonstrates the acute phase protein SAA is a novel retinol binding protein that transports retinol during infection. SAA proteins are induced by bacteria and additionally require retinol for their expression. I demonstrate that SAA's requirement for retinol is not restricted to the

small intestine, as mice on a vitamin A deficient diet have reduced SAA expression in the liver as well. Additionally, I demonstrate in fluorescence based binding assays that SAAs are capable of binding retinol at nanomolar affinities, which is comparable to a known retinol binding protein. I also found that SAA proteins associate with retinol in the serum following a bacterial challenge in wild-type mice. This phenotype was not observed in SAA*1/2*$^{-/-}$ mice following bacterial challenge. Furthermore, SAA*1/2*$^{-/-}$ mice have greater bacterial loads in their spleens and livers following bacterial infection. In parallel with my studies, Dr. Mehabaw Derebe, a post-doctoral researcher in the Hooper lab, recently solved the mSAA3 crystal structure, demonstrating the protein oligomerizes to form a tetramer. This tetramer unit contains a central pore-like cavity, lined with hydrophobic amino acid residues, which would allow a lipophilic ligand to bind. A single amino acid mutation within this hydrophobic core resulted in reduced mSAA3 retinol binding. This structural insight describes how SAA, as a small and mostly alpha-helical protein, can protect a lipophilic ligand from the aqueous environment. Altogether, these data demonstrated that SAAs are a family of microbe-induced retinol binding proteins, reveal a unique protein architecture involved in retinol binding, and provide insight into the acute response to infection.

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LIST OF ABBREVIATIONS

α: alpha

ABCA1: ABC transporter A1

ADH: alcohol dehydrogenase

AID: activation-induced deaminase

AP-HDL: acute phase high density lipoprotein

Apo: apolipoprotein

ARAT: acyl-CoA:retinol acyltransferase

Abx: antibioctic

β: beta

BCMO1: β-carotene 15,15'-monooxygenase 1

B. theta: *Bacteroides thetaiotaomicron*

BCR: B cell receptor

BSA: bovine serum albumin

CCL: C-C chemokine ligand

CCR: C-C chemokine receptor

CM: chylormicron

CRABP-I/II: cellular retinoic acid binding protein I/II

CRBP-I/II: cellular retinol binding protein I/II

CRP: C-reactive protein

Cy3: cyanine 3

CYP450: cytochrome P450

cDNA: complementary DNA

DC: dendritic cell

DM: decyl maltopyranoside

DMEM: Dulbecco's modified eagle medium

DNA: deoxyribonucleic acid

E. coli: *Escherichia coli*

E. faecalis: Enterococcus faecalis

ER: endoplasmic reticulum

ESI: electrospray ionization

FBS: fetal bovine serum

FPRL1: formyl protein receptor like-1

γ: gamma

G-CSF: granulocyte-colony stimulating factor

GM-CSF: granulocyte/macrophage-colony stimulating factor

GWAS: genome wide association study

HDL: high density lipoprotein

HOX: homeobox

HPLC: high-performance liquid chromatography

HSC: hepatic stellate cell

hTrf: human transferrin

huPBL: human peripheral blood lymphocytes

IBD: inflammatory bowel disease

IEC: intestinal epithelial cell

IFN: interferon

Ig: immunoglobin

IHC: immunohistochemistry

IL: interlukin

IPTG: isopropyl-β-D-galactoside

iT_{reg}: induced T_{reg}

κ: kappa

K_d: apparent dissociation constant

LC-MS: liquid chromatography mass spectrometry

LC-MS/MS: liquid chromatography tandem mass spectrometry

LDL: low density lipoprotein

L. mono: Listeria monocytogenes

LPL: lipoprotein lipase

LPS: lipopolysaccharide

LRAT: lecithin retinol acyltransferase

MAMP: microbe associated molecular pattern

MHC-II: major histocompatibility class-II

MLN: mesenteric lymph nodes

MMP: matrix metalloproteinase

MRM: multiple reaction monitoring

mTOR: mammalian target of rapamycin

MyD88: myeloid differentiation primary response gene (88)

NF-kB: nuclear factor κB

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PLB1: phopholipase B1

PP: Peyer's patch

PTL: pancreatic triglyceride lipase

PTX3: pentraxin 3

Q-PCR: quantitative real-time polymerase chain reaction

RA: retinoic acid

RALDH: retinaldehyde dehydrogenase

RAR: retinoic acid receptor

RARE: retinoic acid response element

RBP: retinol binding protein

RBPR2: retinol binding protein receptor 2

RDH: retinol dehydrogenase

RE: retinyl ester

REH: retinyl hydrolysis enzyme

RNA: ribonucleic acid

RXR: retinoid X receptor

SAA: Serum Amyloid A

SAD: single wavelength anomalous dispersion

SAF: SAA-activating sequence binding factor

SCID: severe combined immunodeficiency

SD: standard deviation

SDR: short-chain dehydrogenases/reductase

SEM: standard error of the mean

SR-B1: scavenger receptor class B group 1

S. typhimurium: *Salmonella enterica* Serovar Typhimurium

STRA6: stimulated by retinoic acid gene 6

TCR: T cell receptor

TGFβ: Transforming Growth Factor β

TLR: Toll-like receptor

TNFα: tumor necrosis factor α

TTR: transthyretin

UV: Ultraviolet

VAD: vitamin A deficient

VLDL: very low density lipoprotein

Wt: wild-type

CHAPTER ONE

THE ACUTE PHASE RESPONSE AND SERUM AMYLOID A

INTRODUCTION

The acute phase response comprises an immediate set of host reactions to infection or trauma. This response results in a concentrated site of inflammation that triggers additional local and systemic responses.   The overall function of the acute phase response is to quickly neutralize pathogens, prevent the entry of additional pathogens, activate downstream adaptive immune responses, and promote tissue repair processes that can restore host homeostasis. During the acute phase response, blood monocytes and tissue macrophages are activated at the site of inflammation. These cells propagate the response by producing pro-inflammatory cytokines and chemokines such as IL-1, IL-6, IL-8, and TNFα (Fig. 1). These molecules induce both local and systemic effects that help to further drive adaptive immune responses and magnify existing immunity to the pathogen. Chemokines, such as IL-8, generate concentration gradients that direct migrating neutrophils and leukocytes to the site of inflammation. Once present, neutrophils quickly phagocytize pathogens and damaged cells and secrete additional cytokines to further amplify the host response.   The cytokines IL-1, TNFα, and IL-6 promote host physiological changes that are detrimental to pathogen survival. These include fever induction, enhanced vascular dilation to promote leukocyte access to inflamed sites, and the production of acute phase proteins.

ACUTE PHASE PROTEINS

Acute phase proteins are a set of serum proteins produced by the liver whose concentrations increase or decrease by 25% upon cytokine stimulation [1]. To date, 40 acute phase proteins have been identified. While the functions of some acute phase proteins are still not fully understood, they are assumed to act generally in host defense and tissue repair following infection and/or inflammatory challenge [2]. Some prominent acute phase proteins are C-reactive protein (CRP), haptoglobin, α1-glycoprotein, and serum amyloid A (SAA).

Acute phase proteins that are up-regulated in the serum can be further characterized as major and minor proteins. The major acute phase proteins are CRP and SAA, as their serum concentrations undergo the most marked increase following infection. CRP recognizes phosphocholine, which is found on the surface of many prokaryotes as well as on damaged eukaryotic cells [3-5]. This binding interaction ultimately results in complement activation and enhanced phagocytosis, which aid in the removal of bacteria and promote host survival [4, 6].

Haptoglobin and α1-acid glycoprotein are both categorized as minor acute phase proteins. Although their expression is increased during the acute phase response, they do not reach the levels observed for CRP or SAA. Haptoglobin binds free hemoglobin in the plasma and thus sequesters hemoglobin-bound iron and limits hemoglobin loss through kidney filtration [7]. During the acute phase response, haptoglobin is thought to promote host survival by sequestering iron, thus limiting bacterial access to this essential nutrient [8]. Another minor acute phase protein is α1-acid glycoprotein, which binds lipopolysaccharide (LPS) and other lipophilic small molecules, and also down-regulates neutrophil function [9-12].

While most acute phase proteins show increased expression during the acute phase response, a small group of proteins exhibit reduced expression. In general, this is thought to

divert essential nutrients to processes that are necessary for host survival. Some of these proteins, which mainly function as transport proteins, include retinol binding protein (RBP), transferrin, albumin, and transthyretin [2]. For example, the serum concentrations of RBP normally range from 30-50 μg/mL, but drop by 50% during infection [13].

SERUM AMYLOID A

As mentioned previously, serum amyloid A is a major acute phase protein whose concentrations increase more than 1000-fold during an inflammatory response [14]. At steady state, SAA can be found in serum at concentrations of 1-10 μg/mL, but its concentration increases to ~1 mg/mL during the acute phase response [15]. At roughly 12 kDa, SAA proteins are small apolipoproteins composed of between 104 and 112 amino acids. During an acute phase response, SAA proteins are secreted by hepatocytes and associate with high density lipoprotein fraction 3 (HDL_3) in the serum [16, 17]. As an incorporated part of HDL, SAA replaces apolipoprotein A1 (ApoA1) as the major apolipoprotein during the acute phase response.

SAA genes are highly conserved across all vertebrate species. SAA homologs are encoded in the genomes of many mammal species including human, mouse, rabbit, mink, sheep, horse, cow, and marsupials [15, 18, 19]. In addition to mammals, duck, geese, soft-shelled turtles, and a variety of boney fish species all encode SAA homologs [20]. Recently, the invertebrate sea cucumbers were also found to encode a SAA homolog [21]. The presence of SAA homologs across vertebrate and invertebrate species suggests that SAA proteins participate in essential host functions. While four SAA isoforms are present in mice, only three SAA proteins are found in humans. SAA1 and SAA2 are considered acute phase proteins because their transcription in the liver is induced by cytokine stimulation. SAA1 and SAA2 also share

close to 90% amino acid identity, while SAA3 shares roughly 70% identity with SAA1 and 2. Not all species that encode for SAA in their genome have multiple SAA isoforms. The existence of several SAA isoforms in some mammalian species suggests that the SAA gene underwent gene duplication events, giving rise to multiple SAA isoforms. Gene duplication of SAA in higher order mammals is another indication that SAAs likely participate in functions that are advantageous for host survival. Interestingly, human *SAA3* is considered a pseudogene due to a single nucleotide insertion that results in a frameshift mutation and premature stop codon. As a result, human *SAA3* does not produce a protein product [22]. As mentioned previously, mice harbor a fourth *Saa* gene, *Saa4*, which encodes a constitutively-expressed SAA homolog that it is very weakly induced during the acute phase response and not believed to contribute to SAA concentrations during the acute phase [23, 24]. SAA4 is produced in the liver and can be found associated with HDL at steady state [25]. Additionally, SAA4 shares roughly 50% amino acid similarity with the acute phase SAA family members. Therefore, the remainder of this work will focus on the acute phase SAA proteins: SAA1, SAA2, and SAA3.

The tissue expression profile of SAAs varies depending on the isoform. This is likely due to differences in molecular triggers and expression of necessary transcription factors. In fact, a number of molecular triggers have been noted to stimulate or enhance SAA transcription in a variety of cell types. The cytokines IL-1β, IL-6, TNFα, and INF-γ were noted to up-regulate a human *SAA2* promoter construct transfected in mouse L cells, a fibroblast cell line of subcutaneous connective tissue [26]. Several studies also observe that incubation of liver cells with both IL-1β and IL-6 results in enhanced *SAA* transcription [15]. In addition to cytokines, LPS and turpentine can also stimulate *SAA* expression in rabbit liver cells [27]. Interestingly,

glucocorticoids, which act as anti-inflammatory mediators, actually enhance IL-1β and IL-6 dependent *SAA* transcription [28].

Following exposure to these molecular triggers, SAA1 and SAA2 are expressed in liver hepatocytes, as well as extra-hepatic tissues such as the small intestine and colon [29]. Studies of HepG2 cells, a human liver cell line, identified several binding sites for NF-kB and C/EBP in the promoter regions of *SAA1* and *SAA2* [30]. These binding sites appear to be important in order to mediate cell responsiveness to cytokine stimuli. In order to achieve maximal *SAA* transcription, both IL-1β and IL-6 are needed, as IL-6 only up-regulated C/EBP expression in this system [30]. In rats, the *SAA1* promoter contains several *cis*-regulatory elements where binding of NF-kB and C/EBP can occur upstream of the *SAA1* transcription start site [15]. One of these C/EBP sites appeared to be more involved in mediating higher basal levels of *SAA1*, while a second site, in conjunction with NF-kB, seemed to be important for liver cells to up-regulate *SAA* in response to cytokines [31].

Expression of *SAA* in the liver appears to also have an additional level of transcriptional control executed by the YY1 transcription factor. YY1 is found constitutively expressed in all mammalian cells and can be found bound to promoter regions of the liver *SAA1* gene, and in this way, represses *SAA1* transcription [32]. However, following cytokine stimulation, activation of NF-κB occurs and allows it to enter the nucleus to displace YY1 from the *SAA1* promoter. Indeed, negative regulation of *SAA* transcription is not unique to the liver, as AP-2, which is expressed in extra-hepatic tissues, appears to act as a dominant negative factor involved in rat *SAA* expression. In this case, an AP-2 binding site overlaps the NF-κB binding site and allows AP-2 to displace NF-κB, resulting in disruption of gene transcription [33].

In extra-hepatic cells, *SAA1* and *SAA2* transcription is also responsive to IL-1β and IL-6 cytokines, LPS, and turpentine. Injection of rabbits with either LPS or turpentine resulted in activation of liver *SAA* expression, with turpentine maintaining C/EBP activity longer than LPS, but this stimulation was insufficient to activate NF-κB [27]. Non-hepatic expression of *SAA2* in rabbits also appears to depend on the SAA-activating sequence binding factor (SAF), an IL-6 responsive transcription factor. Following stimulation by LPS or IL-6, SAF-DNA binding increases and promotes *SAA* expression [34]. As SAF is expressed in a variety of tissues including brain, heart, lungs, kidney, spleen, testis, and skeletal muscle, this represents an alternative transcription pathway that likely contributes to *SAA* induction [15]. Additionally, in rabbit synovial fibroblasts, the transcription factor Sp1 was found to heterodimerize with SAF following stimulation by LPS, IL-1β, or IL-6 to enhance *SAA2* promoter activity [35].

Unlike SAA1 and SAA2 proteins, SAA3 is expressed only in extra-hepatic tissues, including the colon, small intestine, lamina propria macrophages, lung, and adipose tissue [36]. It remains unclear which molecular triggers are responsible for non-hepatic *Saa* expression, as colonic mouse *Saa* expression was found to be constitutive, while expression in the small intestine is up-regulated by the microbiota [29, 37, 38]. This up-regulation depended on signaling through the Toll-like receptor (TLR) adapter MyD88 [37]. MyD88 is known to activate NF-κB, a transcription factor that drives the expression of many genes involved in host immune defense and also activates *Saa* transcription [15] (Fig. 2). As IL-1 receptor signaling also utilizes MyD88 as an adaptor, a potential role for IL-1β signaling in inducing intestinal *Saa* transcription cannot be ruled out.

Due to the marked elevation of SAA expression during the acute phase response and the phylogenetic conservation of SAAs, these proteins are proposed to promote host survival. However, chronic inflammation or extended production of SAA proteins can result in detrimental effects for the host. Prolonged high concentrations of SAA in the serum can lead to a condition known as AA amyloidosis, where insoluble SAA fibrils deposit and form harmful amyloid plaques in the liver, spleen, or kidneys [39, 40]. In AA amyloidosis, incomplete SAA degradation is thought to liberate N-terminal fragments of the protein that are then deposited in amyloid plaques [39, 41]. SAA has also been implicated in other inflammation-driven diseases like atherosclerosis and rheumatoid arthritis [14]. Additionally, several observational studies have shown that serum concentrations of SAA are increased in patients suffering from a variety of cancers, including cancers of the endothelium, epithelium, lung, ovaries, kidneys, uterus, nasopharynx, and immune cancers such as melanoma [42]. While the high degree of conservation among SAA proteins suggests that they benefit host survival, SAA expression also needs to be carefully controlled in order to avoid serious tissue damage.

PROPOSED FUNCTIONS OF SAA

Since the discovery of SAAs in the 1970s, there have been many proposed functions for SAA that can be broadly divided among pro-inflammatory actions and cholesterol transport functions.

Pro-inflammatory Functions:

As part of the acute phase response, a reasonable hypothesis is that SAA is involved in amplification of the inflammatory response by inducing cytokines or activating complement

cascades. In fact, previous studies have used SAA to stimulate pro-inflammatory responses, like the production of IL-1β, TNFα, IL-8, and G-CSF in a variety of cell culture models [43-46]. In addition to pro-inflammatory cytokines, SAA also induced the production of extracellular matrix degrading enzymes such as matrix metalloproteinase (MMP) 1 and matrix metalloproteinase 13 from human and rat cartilage cells in models of rheumatoid arthritis [47]. Cytokine secretion is frequently attributed to SAA binding to a variety of receptors that activate intracellular signaling cascades. These receptors include formyl peptide receptor-like1 (FPRL1), CD36, scavenger receptor B-1 (SR-B1), TLR2, and TLR4 [45, 48-53]. SAA binding to FPRL1, SR-B1, CD36, TLR2 and TLR4 appeared to trigger signaling cascades that converged on NF-κB to initiate gene transcription [51, 54, 55]. *In vitro* studies of endothelial cells observed SAA binding to SR-B1 and FRPL1, resulting in expression of IL-8 and release of matrix metalloproteinases enzymes that degrade the extracellular matrix, which aids in increased leukocyte adhesion, migration, and vascular invasion to the site of inflammation [47, 52]. SAA also stimulated granulocyte colony stimulating factor (G-CSF) production in monocytes in a TLR2-dependent manner [45]. In lung tissues, SAA binding to TLR2 led to NLRP3 inflammasome activation and IL-1β expression, which was proposed to provide a mechanistic link for SAA derived IL-1β-Th_{17} models of allergic asthma [46].

In addition to stimulating the production of pro-inflammatory cytokines and MMPs, SAA has been suggested to act as a chemoattractant for neutrophils, monocytes, T cells, and mast cells. Addition of recombinant human SAA to T cells, neutrophils, or monocytes, led to directional migration of cells *in vitro* [56, 57]. Studies utilizing huPBL-SCID mice [58], mice with severe combined immune deficiency that have been engrafted with human peripheral blood lymphocytes, demonstrated that a subcutaneous injection of recombinant human SAA resulted in

the infiltration of T cells into the injection site [56]. Additionally, SAA proteins anchored in the extra-cellular matrix induced chemotaxis of mouse and human mast cells [59, 60]. Furthermore, SAA proteins induced CCL2 production, a monocyte chemoattract molecule, from monocytes following binding to FRPL-1 [61]. Together, these findings suggest that SAA proteins exert an influence on cell migration and that could impact the host's immune response.

Another proposed function of SAA concerns the activation and stimulation of innate immune cells. For example, SAA can bind to the FPRL1 receptor displayed on neutrophils and this interaction promoted the secretion of lactoferrin (an antimicrobial protein), increased the cells' ability to phagocytize heat killed *Candida albicans*, and up-regulated the expression of CD11c and CD16, which are involved in adhesion and microbial recognition [62, 63]. In addition, activated neutrophils incubated with SAA showed elevated production of reactive oxygen species [63, 64]. Together these results implicate SAA in activation of neutrophils and suggest that SAA may modulate degranulation and killing functions.

Several additional studies have implicated SAA in activating the complement cascade by promoting the production of another acute phase protein, long pentraxin 3 (PTX3). SAA up-regulates PTX3 expression by binding to FPRL1 and activating a signaling cascade that activates AP-1 and NF-κB [65]. A previous study also suggested that SAA binds to OmpA, a lipophilic protein found in Gram-negative bacterial cell walls [66]. Here, it was proposed that SAA-OmpA binding opsonized *E. coli*, eliciting bacterial clearance by enhancing macrophage phagocytosis [67]. However, studies on human serum, where SAA has been depleted, demonstrated that SAA was a minor contributor to the total opsonic activity observed [67]. It remains possible that SAA functions in part as an opsonin, but that is unlikely to be the only function. Supporting this are the observations that SAA is also up-regulated during sterile inflammation and several types

of epithelial cancers [68-70] and during chronic inflammation in diseases such as atherosclerosis and rheumatoid arthritis. Additionally, high plasma concentrations of SAA have been observed in human carcinomas of the lung, prostate, colon, and kidney, with high SAA concentrations associated with a more advanced disease state and poorer outcome [71].

Despite the extensive efforts toward understanding the functions of SAAs during inflammation, there are conflicting reports as to the effects observed for SAA *in vitro* and those observed *in vivo*. For example, SAA-rich plasma harvested from rheumatoid arthritis patients or mice with diet-induced obesity was unable to induce IL-8 from neutrophils [72], which conflicts with a previous result that endothelial cells secrete IL-8 following incubation with SAA [52, 73]. As many of the early studies involving SAA were conducted using cell culture systems, it is clear that more *in vivo* experiments are necessary to determine the true effects of SAA in the host.

Cholesterol transport functions:

At steady state, numerous proteins comprise HDL at varying levels, with ApoA1 serving as the major apolipoprotein component [74]. During steady state, HDL functions in reverse cholesterol transport, a process that removes excess cholesterol from peripheral tissues for transport back to the liver for excretion [75] (Fig. 3). This process is mediated by the ABC transporter, ABCA1, which is required for HDL biogenesis, as *ABCA1*$^{-/-}$ mice have undetectable levels of HDL [76]. HDL particles are generated when ABCA1 lipidates ApoA1 or other lipoproteins to form lipid poor-HDL. As lipid-poor HDL circulates at steady-state, ApoA1 activates the enzyme lecithin retinol acyltransferase (LRAT), which converts free cholesterol

obtained from peripheral cells to cholesteryl esters during transport [76]. These cholesteryl esters are incorporated into HDL particles that transport cholesterol back to the liver for disposal, effectively re-generating lipid-poor HDL, which is critical for effective cholesterol efflux.

During the acute phase, HDL undergoes large scale remodeling of its protein and lipid content [77]. SAA replaces ApoA1 as the major apolipoprotein component of HDL and free cholesterol, triglycerides, and phospholipids are enriched in the acute phase HDL (AP-HDL) core [75]. Other proteins involved in reverse cholesterol transport are decreased during inflammation as well, including LRAT and ABCA1 [78]. Enrichment of free cholesterol is likely due to the decrease of LRAT activity within the HDL particles. Despite the importance of cholesterol homeostasis in the host, the physiological significance of the changes that occur in AP-HDL is poorly understood.

Because of the increased levels of SAA in AP-HDL, it has been proposed that SAA is involved in cholesterol efflux during the acute phase by binding to SR-B1, a HDL receptor important for cholesterol uptake [79, 80]. SR-B1 binds to a broad range of lipophilic proteins including various apolipoproteins, lipoproteins from LDL and VLDL, and SAA [81, 82]. Two possibilities have been suggested for how SAA affects cholesterol efflux: either SAA is involved in cholesterol removal from sites of cellular destruction or SAA contributes to the delivery of phospholipids and cholesterol to sites for tissue repair. Despite numerous studies that have tried to address SAA contributions to cholesterol efflux, none have presented experimental evidence to support SAA delivery of cholesterol to damaged tissues during the acute phase. In fact, several studies have observed lower cholesterol efflux for AP-HDL compared to steady state HDL; however, no evidence was presented that connected this observation with SAA [83, 84]. Instead, lower cholesterol efflux was linked to lower LRAT protein levels that resulted in a

73% decrease in plasma LRAT activity [84, 85]. Since HDL undergoes extensive remodeling, it is impossible to determine if differences in cholesterol efflux are due to changes in SAA levels found in HDL during the acute phase. The best way to resolve this speculation would be to induce an acute phase response in SAA*1/2*$^{-/-}$ mice and determine if changes occur in cholesterol efflux compared to the cholesterol efflux observed in wild-type mice. Since SAA*1/2*$^{-/-}$ mice have been recently generated, perhaps this can now be explored.

Following an acute phase challenge, SAA proteins are highly up-regulated in the serum. This observation, coupled with widespread species conservation and the many proposed functions described for SAA, all suggest an important role for this protein in host survival. Although SAA was discovered over 50 years ago, we still do not have a clear understanding of its functions, and despite the many suggested functions of SAA, there has not been a proposed function that unifies all of the characteristics and observed behavior of SAA during the acute phase response.

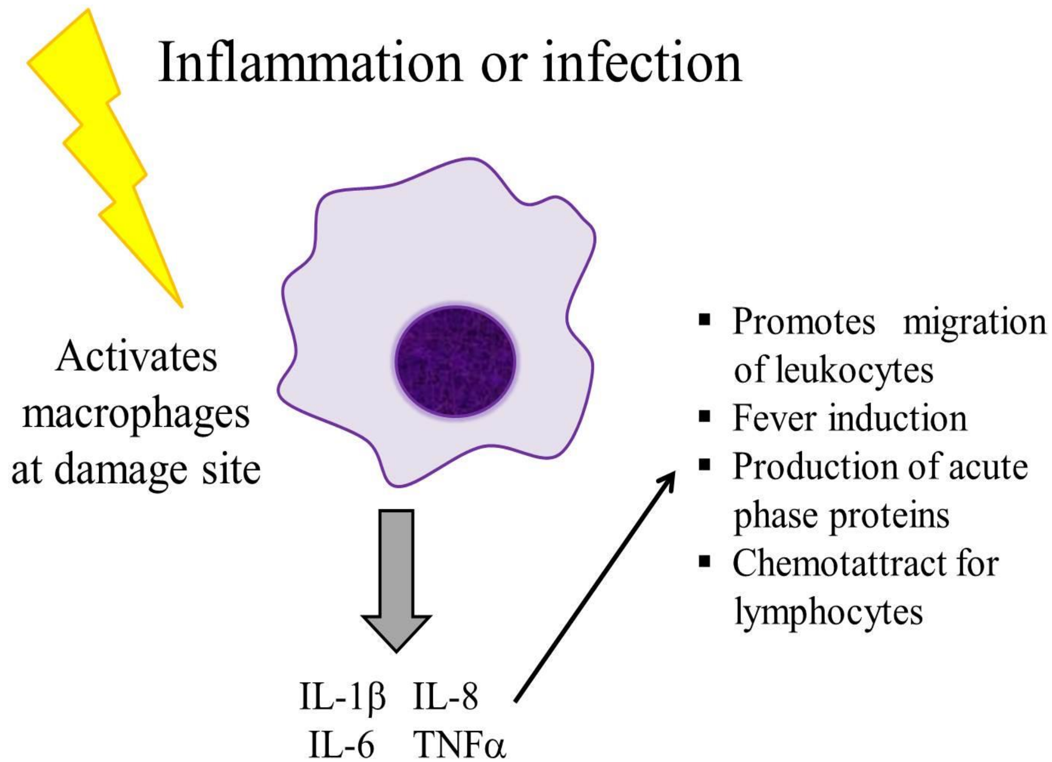


Figure 1: The acute phase response. Following inflammation or infection, resident macrophages are activated and secrete inflammatory cytokines. These cytokines, IL-1β, IL-6, IL-8, and TNFα help to neutralize the pathogen, activate downstream immune responses, and promote tissue repair by: inducing fever, promoting the migration of leukocytes, attracting neutrophils to the damage site, and inducing the production of acute phase proteins.

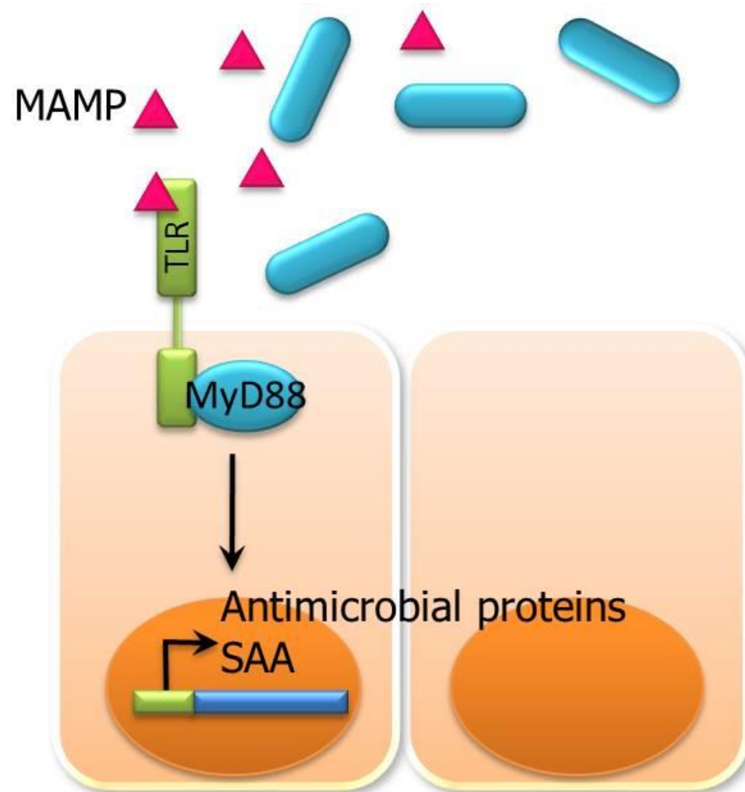


Figure 2: Commensal microbiota trigger anti-microbial responses through Toll-like receptor signaling in intestinal epithelial cells. (*Adapted from Vaishnava S, et al. PNAS. 2008 Dec 30;105(52):20858-63.2008*). Conserved microbe associated molecular patterns (MAMPs) found on commensal bacteria are detected by intestinal epithelial cell Toll-like receptors (TLRs). These signals require the adaptor protein MyD88 in order to induce the production of antimicrobial proteins that help prevent bacteria attachment to the epithelium. MyD88 signaling is necessary for production of SAA in the small intestine as well.

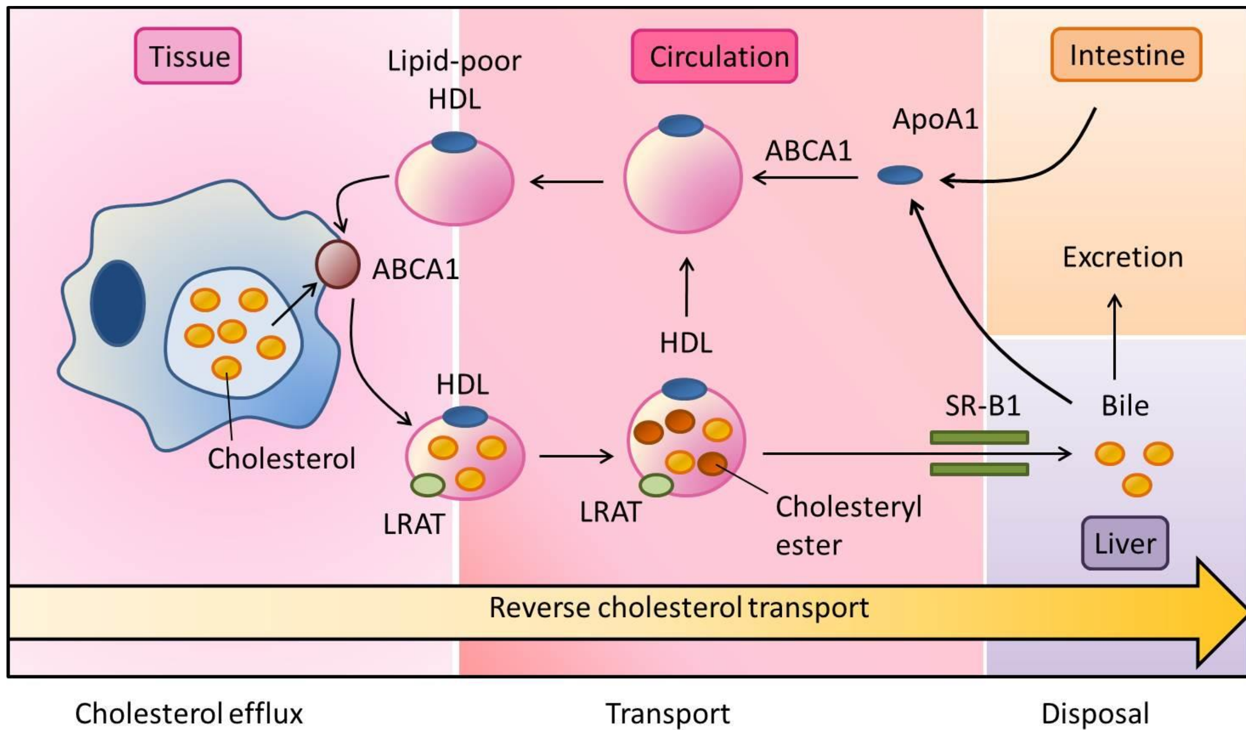


Figure 3: Reverse cholesterol transport. (Adapted from *Heinecke, J. Nat Med. 2012 Sept 12; 18(9):1346-7.*) During steady state, HDL functions in reverse cholesterol transport, a process that removes excess cholesterol from peripheral tissues for transport back to the liver for excretion. HDL particles are generated when the ABC transporter ABCA1 lipidates ApoA1 or other lipoproteins to form lipid poor-HDL. As lipid-poor HDL circulates, ApoA1 activates the enzyme lecithin retinol acyltransferase (LRAT), generating cholesterol esters that are transported back to the liver for disposal.

CHAPTER TWO

VITAMIN A: METABOLISM, TRANSPORT, AND STORAGE

INTRODUCTION

Vitamin A (retinol) is an essential nutrient that plays a key role in many biological processes. As a dietary nutrient, the main sources of vitamin A come from food products. These include retinyl esters (REs) found in animal fats or dairy products and pro-vitamin A carotenoids in yellow or orange vegetables. The most common form of carotenoids found in the human diet is β-carotene [86]. In order for a host to utilize and store these sources of vitamin A for health and survival, epithelial cells must first collect these compounds from the diet.

INTESTINAL EPITHELIAL CELLS TAKE UP VITAMIN A

Retinyl esters and pro-forms of vitamin A must be converted to retinol before downstream retinol metabolism can proceed. Intestinal epithelial cells can take up dietary retinyl esters or their retinol byproducts following retinyl ester hydrolysis (Fig. 4). Retinyl ester hydrolysis is carried out by either the pancreatic triglyceride lipase (PTL), which is secreted by the pancreas in the gut lumen, or phospholipase B (PLB1), located in epithelial brush borders [87]. The retinol produced by retinyl ester hydrolysis is then taken up into epithelial cells by facilitated diffusion [88, 89]. β-carotene enters enterocytes through scavenger receptor class B group 1 (SR-B1) mediated endocytosis [90, 91]. Once in the cell, β-carotene 15,15'-monooxygenase 1 (BCMO1) cleaves β-carotene into two molecules of retinal, which are then converted into retinol [92, 93]. Epithelial cells can use retinol in one of two ways: it is either

converted into retinoic acid (RA) for cellular use or into retinyl esters for transport and storage in the liver.

VITAMIN A BINDING PROTEINS

Due to the lipophilic nature of retinoids, they are always transported within and among cells by retinoid binding proteins. Within a cell, retinoids associate with two classes of retinol binding proteins: cellular retinol binding proteins (CRBP-I and CRBP-II) and cellular retinoic acid binding proteins (CRABP-I and CRABP-II). It is thought that these classes of proteins perform two functions, including protecting retinoids from the aqueous environment and providing a structural platform for enzymes to dock and access the retinoids. Both CRBPs and CRABPs belong to the fatty-acid binding protein family and are described as globular proteins that share a similar folded structure [94].

The two main isoforms of CRBP are CRBP-I and CRBP-II. CRBP-I and CRBP-II share more than 50% sequence identity and both proteins have approximately 95% identity across species [95, 96]. CRBPs are mostly composed of 10 anti-parallel beta-sheets folded around a central cavity that functions as a binding pocket [96]. Both CRBP-I and CRBP-II bind retinoids in a 1:1 protein to retinoid ratio [94]. However, CRBP-I displays a much tighter binding affinity to retinol than CRBP-II, despite both proteins displaying similar binding affinities to retinal [94]. This difference in retinol binding could be a consequence of the specific tissue expression patterns for CRBPs. CBRP-I is expressed in a multitude of tissues including the liver, kidney, lung, brain, eye, and reproductive organs [95, 97]. In contrast, CRBP-II is exclusively expressed in the intestinal epithelium. It’s possible that the weaker retinol binding affinity of CRBP-II

allows for easier release of retinol for packaging and transport for storage. Studies utilizing CRBP-*I*$^{-/-}$ mice reveal a 50% loss of retinyl ester stores in the hepatic stellate cells (HSCs), which was associated with a decrease in retinyl ester synthesis [98]. This evidence suggests that CRBP-I contributes to retinyl ester synthesis and potential maintenance of retinoid stores.

Like CRBPs, CRABP (-I and -II) are highly conserved among species and expressed in many, although not overlapping, cell types. CRABP-I can be found in almost all tissues, while CRBAP-II is restricted to the skin and reproductive organs [95, 99, 100]. CRABPs are generally thought to function in protecting retinoic acid from degradation in the aqueous cytosolic environment. In addition, studies have suggested that CRABPs also deliver retinoic acid to the nucleus for regulation of gene transcription. Supporting this idea, both CRABP isoforms have been located in the cytosol and nucleus [101-103]. However, CRABP-II appears to be more effective in delivering retinoic acid to the retinoic acid receptor (RAR) transcription factors (discussed in detail in Chapter 3) in the nucleus [101]. CRABP-II passes retinoic acid to RAR by direct protein-protein interactions and enhances RAR driven transcription in cells overexpressing CRABP-II. This effect was not observed in cells over-expressing CRABP-I [104, 105]. Studies of overexpressed CRABP-I demonstrated increased generation of retinoic acid-derived polar metabolites, indicating that CRABP-I may play a role in retinoic acid catabolism [106, 107]. These results suggest that CRABPs may be specialized for different functional roles concerning retinoic acid. While CRABP-II is more effective in driving retinoic acid-directed transcription, CRABP-I participates in retinoic acid degradation.

Retinol binding protein (RBP) represents a third class of retinol binding protein. RBP functions extracellularly to transport retinol in the plasma from storage locations such as the liver to peripheral tissues. RBP belongs to the lipocalin protein family, a group of secreted proteins

that bind hydrophobic ligands. RBP has been crystallized from a variety of species including chicken, human, bovine, and mouse. All forms demonstrate a conserved structure composed of 8 anti-parallel β sheets that fold into a barrel-like structure [108-113] (Fig. 5). The amino terminus of the protein is flexible and seals one end of the beta barrel cylinder to create a central binding cavity. Retinol is oriented within the cavity pocket with the hydroxyl end closer to the protein surface and the β-ionone ring half-way down the protein barrel. Binding occurs with a favorable free energy of -86.83 kcal/mol [114]. RBP displays a strong binding affinity for retinol (K_d=190 nM) and binds with a stoichiometry of 1:1 [115]. *In vitro* studies have shown that retinoic acid binds with a similar affinity; however, given the location of and requirements for RBP secretion, retinoic acid is not likely to be a physiological ligand.

While hepatocytes are the main site of RBP production, RBP can also be expressed in adipose tissue, kidney, lung, heart, skeletal muscle, spleen, eye, and testis [116-120]. RBP acquires its retinol cargo following completion of its folding in the endoplasmic reticulum (ER). Binding of retinol triggers the translocation of RBP to the Golgi, and ultimately its secretion into the serum [121, 122]. After secretion into the serum, RBP associates with transthyretin (TTR), a large globular protein that limits removal of RBP from the serum by glomerular filtration in the kidney [123]. RBP serum concentrations are held fairly constant at approximately 30-60 μg/mL in humans and 20-30 μg/mL in rodents [124, 125], except during extreme vitamin A deficiency or infection when RBP levels can drop to 7-17 μg/mL [126]. During vitamin A deficiency, it is likely that the low observed RBP levels are due to an accumulation of the protein in the ER of hepatocytes because RBP cannot be secreted in the absence of retinol. *RBP*$^{-/-}$ mice have also been generated and largely appear normal, except for abnormal vision which resolves ~2 months after birth. Correction of vision in *RBP*$^{-/-}$ mice indicates that retinol can be delivered by another

mechanism when RBP is limiting or absent. In addition, *RBP*$^{-/-}$ mice quickly reach vitamin A deficiency (VAD) within 1 week when placed on a vitamin A deficient diet, which is a consequence of being unable to mobilize retinol stores from the liver [124].

In conclusion, CRBPs, CRABPs, and RBP all play important roles in vitamin A metabolism by protecting their retinoid ligands from degradation in the aqueous environment as well as protecting the host cells from retinoid-induced damage. Additionally, these proteins play key roles in regulating access and docking for enzymes and help to solubilize retinoids for cellular use.

VITAMIN A METABOLISM

Many of the effects of vitamin A on mammalian physiology are mediated through retinoic acid. Upon enzymatic conversion from retinol, retinoic acid can complex with nuclear receptors in order to regulate gene transcription programs in cells [127]. Retinol is converted to retinoic acid in two enzymatic steps, both involving oxidation reactions [128] (Fig. 6). There are several classes of enzymes that have been implicated in catalyzing the two oxidation steps in retinol metabolism. The first oxidation reaction is a reversible reaction that requires NAD-dependent oxidation cofactors. It is carried out by the alcohol dehydrogenase (ADH) and/or the short-chain dehydrogenase/reductase (SDR) families. To date, 18 enzymes have been proposed to participate in this part of enzymatic pathway [129]. Within the ADH family, ADH1, ADH3, ADH4, and ADH7 are critical for retinal generation in early growth and development [130, 131]. Gene disruption studies have shown that *Adh1*$^{-/-}$ mice have no viability defects, but display a substantial reduction in the amount of retinol that is converted to retinoic acid, resulting in a

higher degree of retinol toxicity in these mice [130]. *Adh3*$^{-/-}$ and *Adh4*$^{-/-}$ mice are also viable, however, these deletions become lethal if mice are maintained on a vitamin A deficient diet. This suggests that ADH3 and AHD4 are required for postnatal survival in a vitamin A deficient environment [132, 133]. Additionally, mice lacking both ADH1 and ADH4 displayed 100% lethality 15 days following birth, demonstrating an absolute requirement for these enzymes when mice are maintained on a vitamin A deficient diet. Deletion of both of these genes also increases retinol toxicity in mice fed a vitamin A high diet [133]. During embryogenesis, ADH7 is ubiquitously expressed and is also critical in generating retinal during vitamin A deficiency [134].

Short chain dehydrogenase/reductase (SDR) family members also participate in the conversion of retinol to retinal. Many retinol dehydrogenase (RDH) enzymes from the SDR family have been identified and have been implicated in physiological roles for the first step in retinol metabolism [135, 136]. RDH5 and RDH11, members of the SDR family, play important roles in visual system function in mice [137-139]. Additionally, loss of RDH10 led to an almost complete loss of retinoic acid production and severely reduced the level of retinoic acid signaling in several tissues, which resulted in cardiac, pharyngeal, limb, and organ abnormalities leading to embryonic death [135, 136]. ADH and SDR families, while sharing somewhat redundant and overlapping activities, have distinct cellular localizations that appear to regulate their ability to access free ligand. ADH enzymes are found in the cytosol, while SDR members must be membrane-bound in order to be functional [136, 140-142]. In this way, RDH10 appears to function preferentially to bind retinol within the membrane compartment, which ADH enzymes cannot access.

Retinaldehyde dehydrogenase (RALDH) is an enzyme family that performs the second and irreversible oxidation reaction converting retinal to retinoic acid. These enzymes are highly conserved among vertebrate species and four isoforms of RALDHs have been described in mice: RALDH1, RALDH2, RALDH3, and RALDH4 [128, 129, 143, 144]. Previous studies have shown that overexpression of these enzymes leads to overproduction of retinoic acid *in vivo* and studies utilizing RALDH*2*$^{-/-}$ or RALDH*3*$^{-/-}$ mice resulted in embryonic lethality [145, 146]. In developing mouse embryos, RALDHs are expressed in various tissue, including the lung, stomach, intestinal epithelial, lamina propria, kidney and the urogenital tract [147]. However, in adult mice, RALDH expression is limited to the liver and the following gut-associated tissues: intestinal epithelial cells, mesenteric lymph node (MLN), stromal cells, and gut-associated dendritic cells (DC) [143, 148-152]. These observations further reinforce the absolute necessity of retinoic acid for host development and survival, as well as, establishing that the importance of the gut-associated lymphoid tissues in the production of retinoic acid.

As lipophilic metabolites, retinoids cannot be readily removed from the body and can easily become toxic unless their levels are properly maintained. Excess retinoic acid can lead to a multitude of limb and organ abnormalities including, extensive malformations during antero-posterior body-axis patterning [153-156]. In order to prevent excessive build-up, retinoids are degraded to oxidized metabolites such as 4-*hydroxy* retinoic acid and 4-*oxo* retinoic acid, by a special family of enzymes, the cytochrome 450 (CYP450) proteins [157-159]. CYP450 proteins are responsible for the breakdown of many lipid mediators and xenobiotic drugs [160]. Specifically, the CYP26 subfamily of CYP450s is responsible for the further breakdown and degradation of retinoic acid [153]. CYP26A1 and CYP26B1 catabolize all-*trans* retinoic acid to the polar metabolites 4-*hydroxy*, 4-*oxo*, and 18-*hydroxy* retinoic acid [161]. Retinoic acid

induces expression of both *Cyp26a1* and *Cyp26b1* in different tissues. *Cyp26a1* is highly expressed in the liver, small intestine, colon, and some parts of the brain. Retinoic acid can induce *Cyp26b1* expression during development in the lung, kidney, spleen, thymus and testis, while its expression in adults is limited mainly to the brain, although low expression levels can be found in the skin, liver, lung, and T cells [162, 163]. When T cells isolated from the gut were incubated with physiological concentrations of retinoic acid, they expressed CYP26B1, and this seemed to significantly affect the T cell's ability to up-regulate the CCR9 gut homing receptor; however this only occurred in the absence of TGFβ, indicating that retinoic acid catabolism is an important step in regulating gut T cell populations [164]. Another member of this family, CYP26C1 has also been shown to participate in retinoic acid catabolism by binding both all-*trans* retinoic acid and 9-*cis* retinoic acid [165]. *Cyp26c1* is mainly expressed during embryonic development, but has been found in the lung, spleen, testis, and brain tissues of adults [163].

Together, CYP26 family members play a vital role in retinoic acid degradation, as this activity is important in regulating cellular retinoic acid concentrations in order to maintain proper growth and development, as well as host survival into adulthood [160, 166].

RETINOL TRANSPORT AND STORAGE

Following food ingestion, intestinal epithelial cells as well as other tissues including kidney and lung, can accumulate small stores of retinyl esters in lipid droplets, while the remaining retinoid content is sent to the liver for storage [167, 168]. Before retinol can be delivered for storage, epithelial cells must first convert retinol into retinyl esters, utilizing the enzymes lecithin retinol acyltransferase (LRAT) and acyl-CoA:retinol acyltransferase (ARAT).

LRAT is a microsomal enzyme that catalyzes the esterification of *all trans*-retinol to *all trans*-retinyl esters [87] (Fig. 7). LRAT recognizes CRBP-retinol complexes and is rendered non-functional in the presence of apo-CRBP-I [169]. Interestingly, LRAT is not inhibited by apo-CRBP-II, possibly because CRBP-II is expressed only at retinoid absorption sites where dietary retinoids need to be processed for storage [169]. ARAT seems to only recognize free retinol unbound to CRBP and is not thought to function *in vivo* in retinol esterification when cells express CRBP [170]. It's possible that ARAT serves to esterify excess retinol taken up during food consumption, when all CRBP protein is saturated with retinol. *In vivo*, it appears that LRAT is mainly responsible for retinyl esterification as *LRAT*$^{-/-}$ mice are incapable of storing retinyl esters in the liver and appear to rely completely on dietary retinol intake in order to maintain normal function [171].

Following esterification, retinyl esters are packaged into chylomicrons (CMs) within the enterocytes (Fig. 7). CMs are large lipoprotein complexes that contain aggregates of triacylglycerides, cholesterol, phospholipids and other dietary lipids that are packaged together for transport in the bloodstream [172]. CMs form when dietary triglycerides accumulate in the endoplasmic reticulum until a section of the membrane pinches off, resulting in a lipoprotein particle decorated with apolipoprotein B (ApoB) proteins [173]. As other lipids are incorporated into CM particles, they can grow in size up to 600 nm in diameter [174]. Following exocytosis from enterocytes, CMs undergo remodeling by HDL, where protein components are exchanged that enable lipoprotein lipase (LPL) activity. LPL is localized to the phospholipid protein shell of CMs and functions to hydrolyze triglycerides within the CM for dissemination to peripheral tissues [175]. This process reduces the CM size to a smaller remnant particle that can then be more readily cleared from circulation by hepatocytes. In addition to hepatocytes, CM remnants

can also be stored in adipose, kidney, lung, skeletal, and cardiac tissue [168, 176] Hepatocytes take up CM remnant particles by receptor-mediated endocytosis using the low density lipoprotein (LDL) receptor, which is specific for apolipoprotein E (ApoE) and becomes enriched in CM remnant particles following HDL remodeling [177] (Fig. 8). Interestingly, there are ApoE-independent pathways for CM clearance by hepatocytes, as *ApoE*$^{-/-}$ mice are capable of clearing CM particles as efficiently as wild-type controls [178]. It has been suggested that ApoE-independent clearance of CM could utilize the SR-B1 receptor instead of LDL receptor.

In hepatocytes, CM particles are passed into early endosomes, where retinyl ester hydrolases activity in the plasma membrane and endosomes hydrolyze retinyl esters back to retinol [179]. The presence of apo-CRBP-I also seems to enhance retinyl ester hydrolysis and potentially serves to regulate the enzymatic process [180, 181]. While there are several classes of enzymes that hydrolyze retinyl esters *in vitro*, it is not currently known which enzymes are responsible this activity *in vivo* [182-184]. Newly hydrolyzed retinol is then transferred to the ER, where it can either associate with RBP for re-entry into the circulation or be transferred to hepatic stellate cells (HSCs) for re-esterification and storage (Fig. 8). HSCs house between 50-80% of retinoid stores in the body, and the mechanism for transferring retinol between hepatocytes and HSCs has not yet been elucidated [185, 186]. Both LRAT and CRBP-I are highly expressed in HSCs and appear to be required for optimal HSC accumulation of retinoid stores [187-189]. After the liver, adipose tissue is the second largest retinoid storage site in the body, containing 20% of the total retinyl ester stores [120, 175]. Retinyl ester stores are liberated when peripheral tissues are in need of retinol. In order to utilize retinyl esters, they are converted back to retinol by retinyl ester hydrolase (REH) enzymes, which have been found in a variety of tissues including the liver, adipose, lung, kidney, skin, eye, and intestine [184].

Interestingly, adipose stores are depleted more quickly than liver stores following insufficient vitamin A absorption, indicating a potential mechanism where adipose stores may serve as an immediate source for maintaining serum RBP and retinol levels during early stage vitamin A deficiency [171]. Once retinol has been converted back, it is then able to associate with RBP in the ER before secretion [121]. In this way, all peripheral tissues or cells without retinyl stores and retinyl ester hydrolase activity acquire retinol from circulating RBP:retinol complexes.

Once in the serum, RBP can then deliver its retinol cargo to peripheral cells, however, it is still a matter of debate as to how RBP physically transfers its cargo into a target cell. One potential mechanism involves passive diffusion of retinol through the cell membrane. While this is possible, all other aspects of retinol transport and metabolism are carefully controlled processes, so this seems unlikely. Another possibility is the existence of a RBP specific receptor. This would allow for controlled transfer of retinol into the target cell, where it could be passed to CRBP proteins in the cytosol. The first identified RBP receptor was named stimulated by retinoic acid gene 6 (STRA6), a mulitransmembrane protein expressed in the brain, spleen, lungs, and reproductive organs [190]. STRA6 was shown to bind RBP with high affinity *in vitro* and STRA6 transfected cells were observed to take up retinol, a phenotype that was absent in cells subjected to RNAi knockdown of STRA6 [190]. Recently, a second RBP specific receptor, retinol binding protein receptor 2 (RBPR2), was identified [191]. RBPR2 is highly expressed in the small intestine and liver, and was shown to bind RBP with high affinity in transfected cells [191]. Additional assays demonstrated an increased amount of retinol accumulated in cells expressing RBPR2 [191]. Since STRA6 and RBPR2 display different tissue expression patterns, it has been proposed that these two receptors are responsive to levels of retinoid stores in the liver and thereby direct retinol delivery to peripheral tissues using

STRA6, or back to the liver using RBPR2. While much research has established that metabolism, storage, and transport of retinoids are highly controlled processes, how this pathway functions during an immune response is still poorly defined.

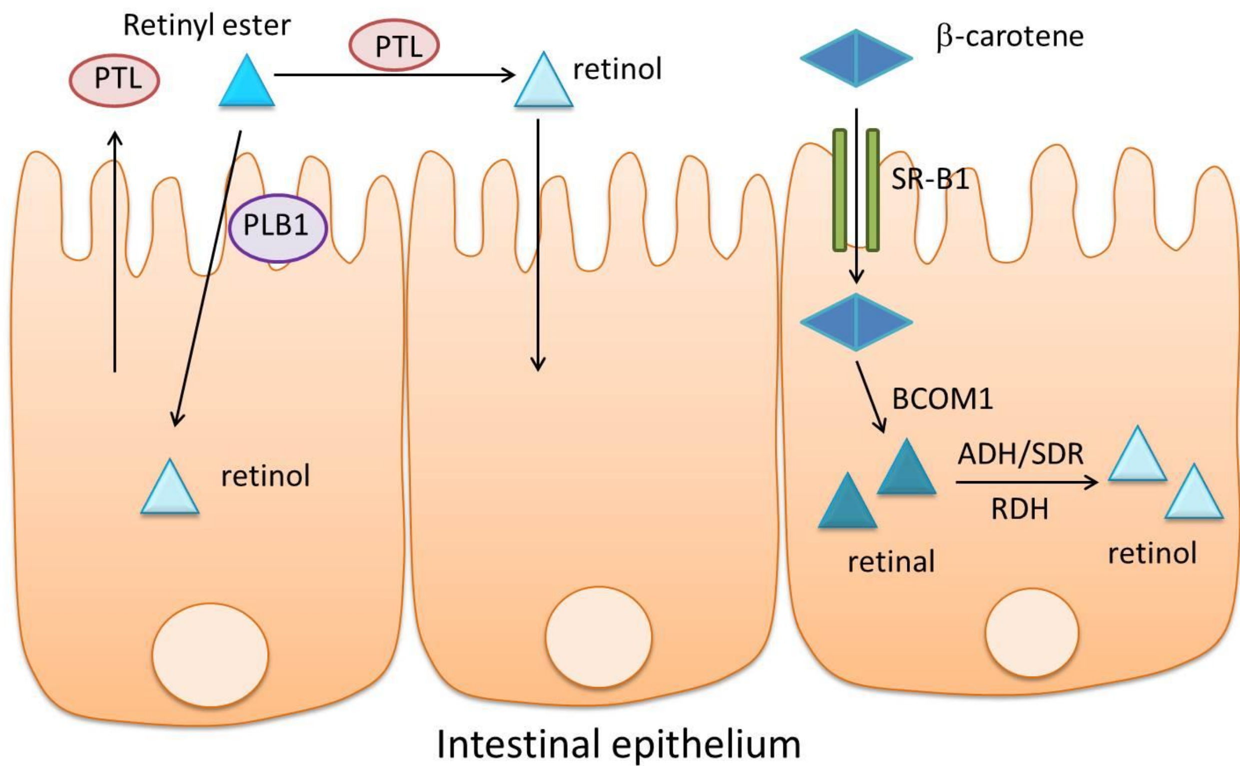


Figure 4: Intestinal epithelial cells take up vitamin A. Intestinal epithelial cells take up retinyl ester or their byproducts, following retinyl ester hydrolysis. Dietary retinyl esters are converted to retinol by secreted pancreatic triglyceride lipase (PTL) in the lumen or by phospholipase B1 (PLB1) located in the brush border before entering the epithelial cell by facilitated diffusion. β-carotene enters epithelial cells through the scavenger class B receptor 1 (SR-B1). Once in the cell, β-carotene is converted to two molecules of retinal by β-carotene 15,15'-monooxygenase 1 (BCOM1), which can then be converted to retinol by alcohol dehydrogenase (ADH), short-chain dehydrogenases/reductase (SDR), or retinol dehydrogenase (RDH) enzymes.

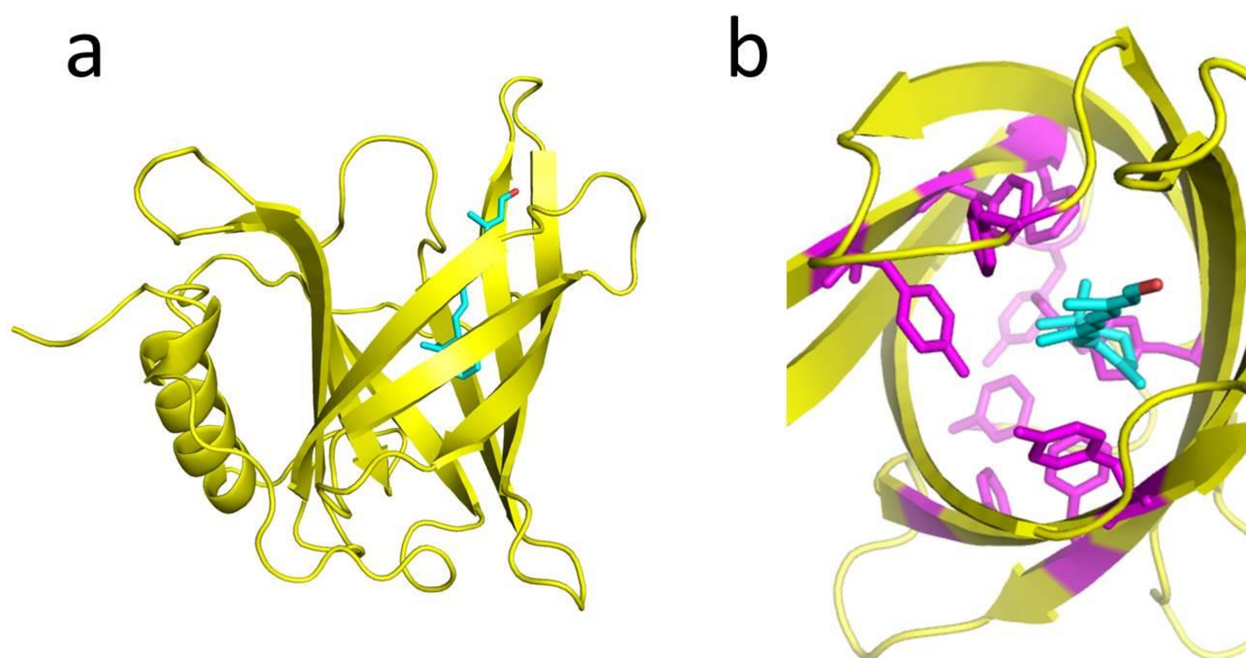


Figure 5: Crystal structure of human RBP. RBP is composed of mostly β sheets that fold together to form a barrel-like structure, creating a binding pocket for retinol. **(a)** The side view of the crystal structure of human RBP [108]. **(b)** The β-ionone ring of retinol is buried in the binding pocket of RBP as seen from this overhead view. RBP protein is colored in yellow and retinol is colored in teal.

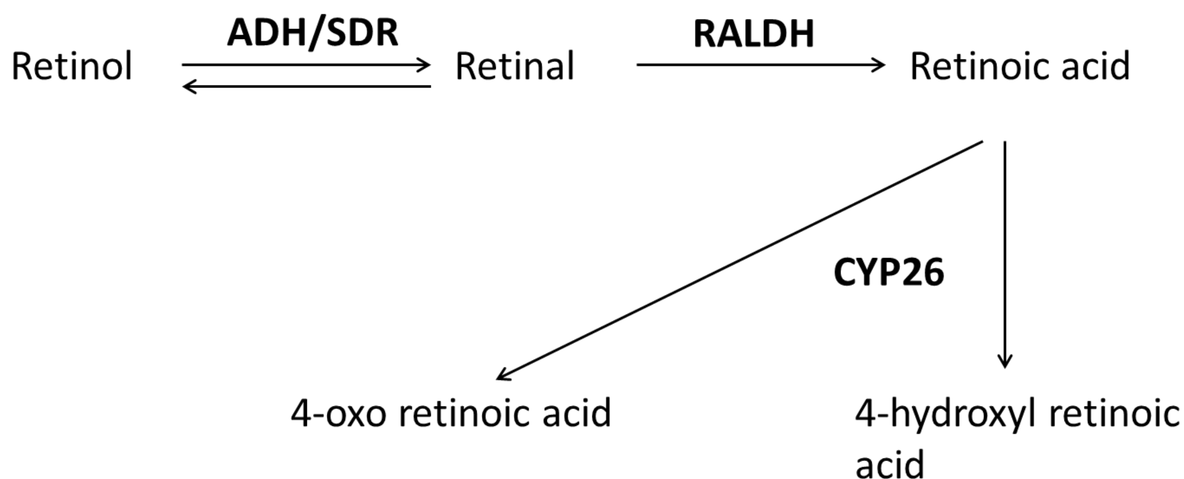


Figure 6: Enzymatic conversion of retinol to retinoic acid. Retinol is oxidized via a reversible reaction into retinal by retinol dehydrogenases belonging to either the alcohol dehydrogenase (ADH) or short-chain dehydrogenases/reductase (SDR) families. Retinal can then be further oxidized into retinoic acid by retinaldehyde dehydrogenase (RALDH) family members. CYP26 of the cytochrome P450 family breaks down retinoic acid into degradation products like 4-oxo retinoic acid and 4-hydroxyl retinoic acid.

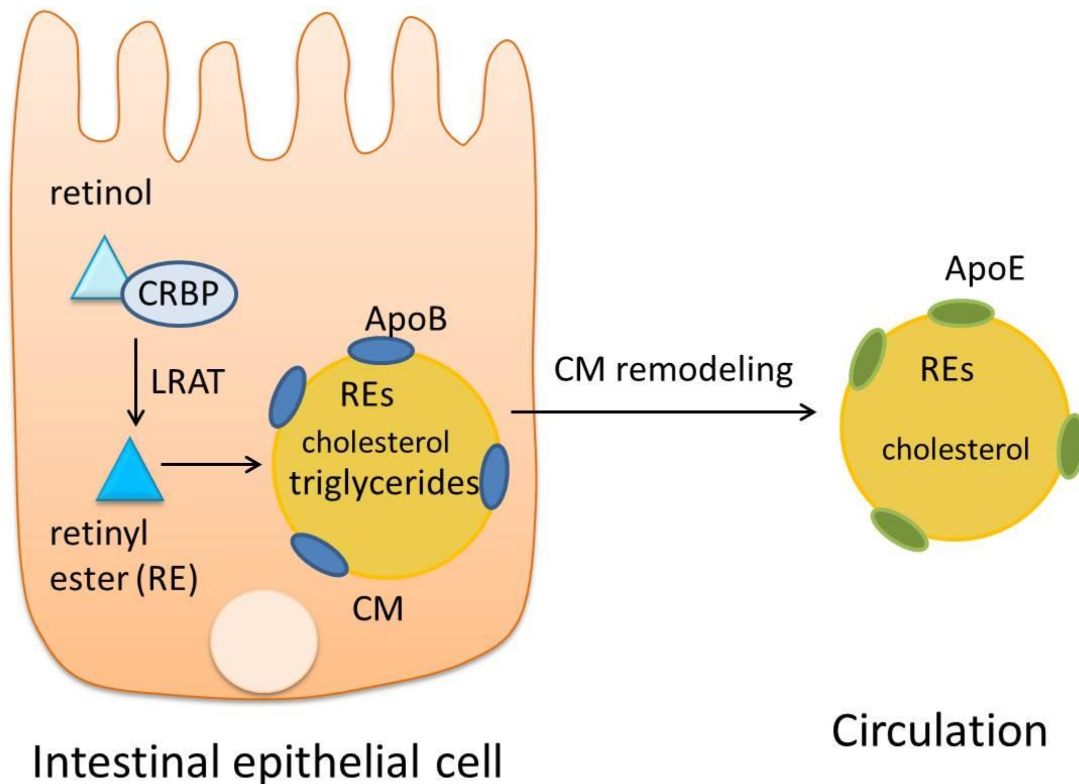


Figure 7: Retinyl esters are packaged into chylomicrons for transport. Dietary retinoids are converted into retinyl esters (REs) by lecithin retinol acyltransferase (LRAT) in intestinal epithelial cells. REs are then incorporated into chylomicrons (CM) along with cholesterol and triglycerides for transport and storage. As CMs circulate, they undergo remodeling by HDL, losing their triglyceride cargo, and replacing ApoB with ApoE in the outer protein shell, which facilitates CM clearance by hepatocytes.

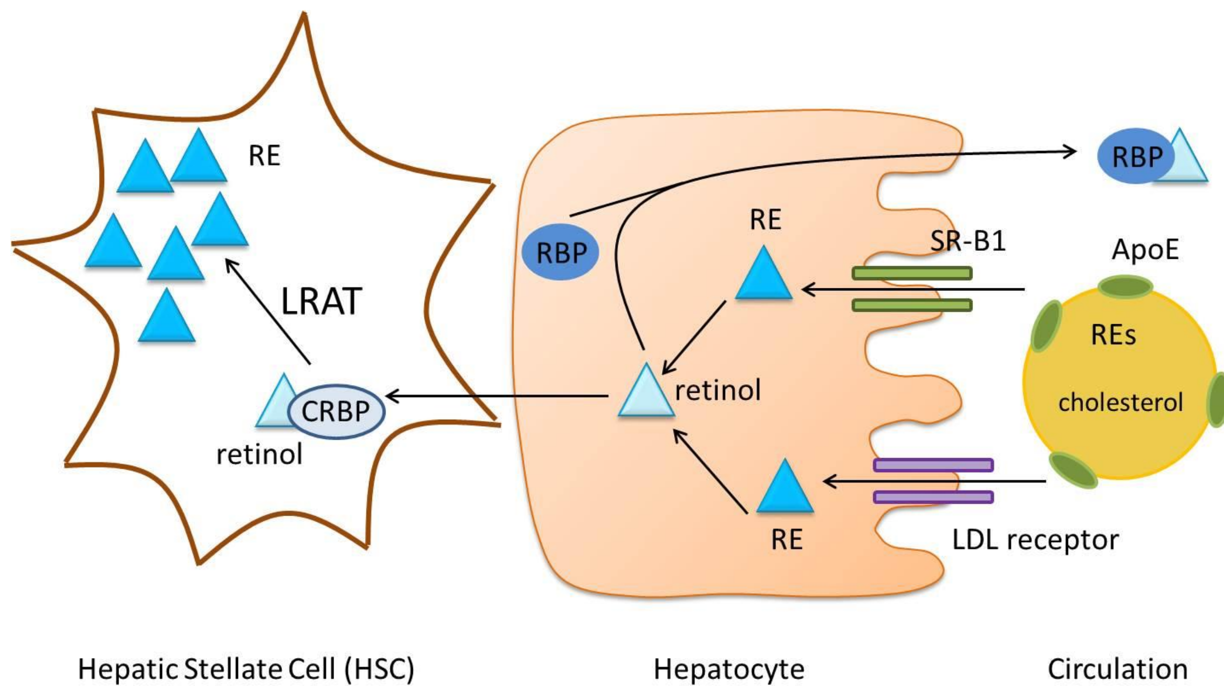


Figure 8: Clearance and storage of retinyl esters by the liver. Chylomicron remnants are cleared by hepatocytes through the LDL or SR-B1 receptors. Retinyl hydrolysis (REH) enzymes in the plasma membrane or endosomes convert retinyl esters into retinol. Retinol is then able to associate with newly folded RBP in the ER and re-enter the circulation, or be transferred to the hepatic stellate cells (HSC) for storage following esterification by LRAT. When peripheral tissues require retinol, RE stores in the HSC are transferred back to the hepatocyte and hydrolyzed to retinol, where they can join RBP in the ER for their secretion into circulation.

CHAPTER THREE

VITAMIN A EFFECTS ON HOST BIOLOGICAL RESPONSES

INTRODUCTION

A multitude of gene transcription programs require retinoic acid for their expression, making retinoids an essential compound for host physiology. So far, more than 500 genes have been identified as targets of retinoic acid directed transcription programs [192]. Retinoic acid controls transcription by acting as a ligand for a class of transcription factors known as retinoic acid receptors (RARs). RARs function as heterodimers with the retinoid X receptors (RXRs) to drive gene transcription. RARs bind to *all-trans* retinoic acid, while RXRs bind to *9-cis* retinoic acid, although the physiological relevance of *9-cis* retinoic acid is not understood [193]. RAR:RXR heterodimers bind to retinoic acid response elements (RAREs), a set of direct repeats of a consensus sequence of AGGTCA that are separated by 1-5 base pairs spacers [194]. There are 3 isoforms of RARs and RXRs present in both mice and humans, *α*, *β,* and *γ;* however, it remains unclear what specific programs or functions are controlled by the individual RAR:RXR pairings. All six RAR and RXR null mice have been generated and while single RAR*α*, *β*, and *γ* mutants have some developmental defects, RAR double null mice have major developmental problems that usually result in embryonic lethality [195-198]. This suggests that RARs share overlapping, but essential functions. The current model of RAR:RXR transcription proposes that in the absence of ligand, RAR:RXR heterodimer pairs sit already bound to promoter DNA elements [199] (Fig. 8). Without ligand, RAR:RXR heterodimers are associated with several co-repressor molecules and together, inhibit DNA transcription [200, 201]. Binding of retinoic acid to RAR results in conformational changes that strengthen the heterodimer's

affinity for DNA, allowing for dissociation of co-repressor proteins, recruitment of co-activator elements, and consequent gene transcription [202] (Fig. 8).

NON-IMMUNE EFFECTS OF RETINOIDS

Retinoids have previously been well studied for their roles in growth and development, as well as proper function of the visual system. As early as the 1930s, scientists observed the detrimental defects of vitamin A deficiency in embryonic development [203]. Since then, numerous reports have identified retinoic acid as an important morphogen required for proper growth and development of embryos. Morphogens act as signaling molecules that control pattern development by establishing concentration gradients that ultimately drive cellular processes and determine cell fate. During embryonic development, retinoic acid is specifically required for limb bud development and asymmetrical body axis patterning, as well as lung and heart organ development and vascular and nervous systems [135, 204, 205]. Retinoic acid's morphogenic capabilities are carefully directed by specific expression of RALDHs and CYP26 enzymes, which control retinoic acid concentration gradients [206]. Retinoic acid contributes to cell fate and organ development by regulating many development gene programs, including the homeobox (HOX) family members, which are transcription factors that control body axis patterning [207]. While retinoic acid is critical for proper embryo development, excess concentrations of retinoids have also proven to be equally damaging. Studies where retinoic acid is over produced, outside of its normal concentration gradient and localization, resulted in embryos with multiple developmental problems, similar to those observed in vitamin A deprivation, including defects in the heart, nervous system, limbs, and vertebrae [153, 208].

Retinal, the intermediate produced in the conversion of retinol to retinoic acid, is critical for proper vision function, night vision, and color perception. In the photoreceptor cells of the eyes, *11-cis* retinal is generated from retinol. *11-cis* retinal then complexes to G-coupled protein receptors called opsins that mediate the conversion of light photon energy into electrochemical signals [209]. Exposure to a photon isomerizes *11-cis* retinal to all *trans*-retinal, which is then regenerated back to *11-cis* retinal before the next light exposure cycle [209]. While regeneration of *11-cis* retinal does recover a large percentage of all *trans*-retinol, the eye requires steady replenishment of retinol to order to function properly [210]. Mice that lack serum RBP protein thus have poor vision due to a tissue specific insufficient supply of retinol [211].

IMMUNE EFFECTS

In 2006, the World Health Organization estimated that roughly 190 million children worldwide had low serum retinol concentrations and were at risk for vitamin A deficiency, with at least 5 million of those children already displaying disabilities that arise from vitamin A deficiency, such as night blindness [212]. Countless reports have linked vitamin A deficiency to higher occurrences of measles, malaria, and diarrheal diseases with high fatality rates. However, supplementing patients with vitamin A helped to reduce disease complications, morbidity, and mortality [213-215]. While it is clear that vitamin A plays extremely important roles in immune responses, the mechanisms behind this phenomenon have only begun to come to light in the past 20 years.

RETINOIC ACID CONTRIBUTIONS TO INNATE IMMUNITY

Intestinal epithelial cells provide a physical barrier that helps to contain the multitude of microbiota residing in the small intestine, and thus maintaining this barrier is critical to host defense. This barrier is comprised of intestinal epithelial cells, tight junction proteins, and a mucus layer composed of a collection of mucin proteins produced by goblet cells in the epithelium (Fig. 9). In studies where mice suffer from colitis-like disease, treatment by supplementation with retinoic acid prevented weight loss [216]. Retinoic acid also prevented colon shortening, a measure of intestinal damage, and bacterial translocation compared to control mice [216]. In this respect, retinoic acid treatment not only protected the epithelium, but helped the epithelial barrier regenerate following damage from inflammation.

Retinoic acid is also required for the expression of tight junction proteins by epithelial cells, another measure that helps seal the barrier against bacterial access to underlying tissues. Two varieties of tight junction proteins, occludins and claudins, are up-regulated by retinoic acid and RARα signaling [216] (Fig. 9). As mentioned, mucin proteins produced by goblet cells comprise the mucus layer and create a "de-militarized" zone that keeps bacteria partitioned away from contact with the epithelial surface [217, 218]. The high viscosity of the mucus layer limits bacterial access to the epithelial surface by reducing their ability to move and find nutrients. Vitamin A deficient animals display reduced expression of the *Muc2*, *Muc5ac*, and *Muc5* genes in the small intestine and colon [219, 220] (Fig. 9). Additionally, several studies have remarked on increased bacterial translocation or bacterial overgrowth in the intestine of vitamin A deficient mice [215, 219]. This observation is likely a consequence of compromised barrier integrity since retinoic acid induces many barrier components and aids in epithelial cell turnover, a process required to repair tissue damaged by bacterial infection or inflammation [221].

Many innate immune cells also depend on retinoic acid in order to develop and acquire effector functions. Studies utilizing RAR$\alpha/\gamma^{-/-}$ mice found high concentrations of immature hematopoietic cells that were restricted to the bone marrow and appear to be developmentally blocked, a phenotype that was not observed in either of the single knockouts [222]. This indicates that RAR expression in the bone marrow is required for proper cell differentiation and that RARα and RARγ signaling serve some redundant, but necessary functions in hematopoietic cells that promote proper development. Besides being important for early stages of hematopoietic cell development, terminally differentiated cells in the bone marrow also require RAR signaling. For example, many of the genes involved in neutrophil development and maturation require RAR dependent transcription for their expression [223].

If bone marrow derived cells can pass development checkpoints during vitamin A deficiency, they still display maturation defects in the periphery. Retinoic acid inhibits stem cell production of granulocyte-macrophage colony-stimulating factor (GM-CSF), an important hematopoietic growth factor that promotes the development of granulocytes. As a consequence, large numbers of granulocytes populate the bone marrow, blood, and spleen of vitamin A deficient mice [224] (Fig. 10). Supplementing these mice with vitamin A suppresses cellular proliferation and instead allows these cells to terminally differentiate into mature phagocytes. In the absence of retinoic acid, granulocytes become stuck in a proliferative state and cannot acquire phagocytic effector function. Instead, the cells undergo spontaneous apoptosis [225, 226] (Fig. 10). Reduced neutrophil phagocytosis and bacterial killing have also been observed in mice with vitamin A deficiency [227]. Additionally, retinoic acid was also observed to regulate dendritic cell (DC) maturation, as cells incubated with retinoic acid and inflammatory cytokines increased their expression of MHC-II and co-stimulatory molecules [228]. Together, these

observations indicate that retinoic acid not only drives terminal differentiation in innate immune cells, but also is critical for their survival and effector functions in the periphery.

Besides its roles in maintaining the physical barrier, retinoic acid also promotes IL-22 production by innate lymphocytes such as γδ T cells and innate lymphoid cells (ILC) 3 isolated from the small intestine. Further, this is dependent on RAR signaling [229]. IL-22 has been shown to play important roles in promoting epithelial barrier repair and limiting bacterial dissemination following infection or damage [230, 231]. In accordance with these findings, ILC3 numbers are decreased during vitamin A deficiency, indicating a positive role for retinoic acid in maintaining ILC3 populations in the small intestine [232]. In this way, retinoic acid contributes to mucosal immunity, which serves as another layer of protection during infection or inflammation in the gut.

Retinoic acid also plays a role in determining the subtype specification of dendritic cells (DCs), another bone marrow derived innate immune cell type. Indeed, dietary vitamin A serves as a tissue microenvironment signal for gut DCs that affects their differentiation, as vitamin A deficient mice had increased numbers of langerin-expressing DCs in the MLNs and lamina propria compared to controls fed a normal diet [233]. Langerin$^+$ DCs can also express CD103$^+$, which normally denotes the DC population responsible for generating retinoic acid signals for T or B cells; however, when langerin$^+$ CD103$^+$ DCs are generated in vitamin A deficient hosts, they display a more suppressive phenotype, indicated by the up-regulation of genes involved in suppressing innate responses, cytokines involved in inducing T_{reg} development, and co-stimulatory molecules that inhibit T cell activation [233].

Additionally, DCs also participate in critical retinoic acid related host functions involving adaptive immune cells. Co-culture experiments combining retinoic acid producing DCs and lymphocytes lead to induction of gut homing receptors on the B and T cells [148, 234]. This ability is restricted to DCs populating the mesenteric lymph nodes (MLNs) and Peyer's Patches (PPs), and not observed by spleenic DCs [148]. The ability of these DC subpopulations to produce retinoic acid is due to their ability to express RALDH enzymes, which participate in the second step of vitamin A metabolism to generate retinoic acid. Specifically, $CD103^+$ DCs are able to up-regulate RALDH2, migrate between the lamina propria and MLNs, and participate in antigen presentation to lymphocytes [235-237] (Fig. 11). Studies involving DCs incubated with RALDH inhibitors found that these DCs could no longer confer gut homing to T or B cells [238]. This ability to confer gut homing to newly activated T and B cells is necessary to stimulate adaptive responses in order to combat pathogen challenges.

RETINOIC ACID CONTRIBUTIONS TO T CELL RESPONSES

The small intestine hosts a unique and complex microenvironment that must balance interactions among the enteric microbiota and resident immune cells. Within the intestine, Th_{17} cells are responsible for providing pro-inflammatory driven protection against extracellular pathogens. Th_{17} cells are induced by TGFβ, IL-6, and IL-1 cytokines in the small intestine [239]. The inflammation generated by Th_{17} is balanced by contributions of T_{reg} cells, a T cell lineage that is responsible for anti-inflammatory responses and maintaining homeostasis throughout the body. A multitude of early *in vitro* studies observed that incubation of retinoic acid and TGFβ promoted T_{reg} induction, while blocking Th_{17} differentiation of naïve cells. TGFβ and IL-6 have been shown to direct T cells to the inflammatory Th_{17} phenotype, which is

dependent on the induction of the transcription factor RORγt [38]. However, when naïve T cells were cultured with TGFβ, IL-6, and retinoic acid, they failed to induce RORγt and therefore did not differentiate into Th_{17} cells [240]. This suggests that under inflammatory conditions, retinoic acid may repress the differentiation of Th_{17} cells and help restore homeostasis by allowing T_{reg} cells to differentiate instead. However, further *in vivo* evidence has brought this conclusion into question as experiments have begun to discover inconsistent or opposing results from the originally reported *in vitro* observations. Since then, studies utilizing vitamin A deficient mice have further elucidated retinoic acid contributions to Th_{17} and T_{reg} development.

During vitamin A deficiency, mice do not display reduced T_{reg} frequencies and mice lacking RARα signaling in their T cells appear to have normal T_{reg} numbers in the lamina propria [241]. However, this was likely observed because retinoic acid only exerts its effects on induced T_{reg} (iT_{reg}) generation [242-244]. iT_{reg}s are not thymically derived, so they require TGFβ, IL-2, and retinoic acid in order to up-regulate the T_{reg} transcription factor, FOXP3, for their development in the periphery [245-247] (Fig. 12). Interestingly, only co-culture experiments with $CD103^+$ DCs promoted the conversion of naïve $CD4^+$ T cells to $Foxp3^+$ iT_{reg}s, implicating retinoic acid generation as a necessary component for their development [248-250]. While overall T_{reg} numbers are not affected, vitamin A deficient mice have no discernible Th_{17} cells in the small intestine, an observation supported by an *in vitro* DC-T cell co-culture experiment, which demonstrated that generation of Th_{17} cells required low levels (~1 nM) of retinoic acid [251, 252] (Fig. 12). In addition to Th_{17} and T_{reg}s, retinoic acid influences the balance of Th_1 and Th_2 cells, as vitamin A deficient animals favor Th_1 development over Th_2 [244]. This most likely occurs because retinoic acid reduces IL-12 production, a Th_1 enhancing cytokine, while promoting induction of *Gata3, Maf, Stat6,* and *Il4* genes, which drive Th_2

development [215, 253, 254] (Fig. 12). Additionally, RARα$^{-/-}$ mice display less T-bet, a Th_1 transcription factor, expression after vaccination and had no detectable INFγ production following antigen recall [244]. Overall, it is clear that retinoic acid impacts T cell lineage commitment, serving as an important signal in T cell development.

Besides its role in lineage commitment, retinoic acid and RARα signaling is required for $CD4^+$ T cell activation and effector responses. A previous study demonstrated that RARα signaling, following TCR-CD3 engagement, leads to calcium mobilization and cell activation [244]. This response is RARα dependent, as RARα inhibitors and RARα$^{-/-}$ T cells were unable to mobilize calcium and failed to proliferate as effectively as wild-type counterparts [244]. Calcium mobilization is known to result in the translocation of NFAT transcription factors, which drive IL-2 production and effector functions of T cells [255]. Additionally RARα$^{-/-}$ T cells displayed reduced expression of CD69 and CD2, T cell activation markers, as well as reduced signaling through the mTOR pathway, which contribute to T helper cell responses [244]. Moreover, memory $CD4^+$ RARα$^{-/-}$ T cells were unable to respond to a mucosal challenge with *Toxoplasma gondii* and produced a smaller amount of Th1 cytokines compared to controls [244]. Recently, a new study has demonstrated that RARα signaling was also required for the survival of antigen specific $CD8^+$ T cells following *Listeria monocytogenes (L. mono.)* infection, and these cells were necessary to prevent expansion of *L. mono.* in the spleen [256]. Together, these results demonstrate that retinoic acid guides both early and continual signaling in T cells that have direct impacts on effector cell responses.

When an intestinal infection occurs, activated antigen-specific immune cells must migrate to the site of infection in order to combat the pathogen. In order to migrate, lymphocytes

up-regulate the gut homing receptors α4β7 and C-C chemokine receptor 9 (CCR9), which direct them to the small intestine [257, 258]. Co-culture experiments with retinoic acid-producing DCs induced α4β7 receptors on T cells [234, 236, 259, 260] (Fig. 11). This result was dependent on RALDH expression and RAR signaling, as DCs treated with RALDH inhibitors or RARα/β antagonists were unable to induce α4β7 expression on T cells [148, 238]. T cells also utilize retinoic acid to maintain high CCR9 expression in order to direct additional T cell recruitment and development to the small intestine [234]. The ability to up-regulate α4β7 and CCR9 in T cells is dependent on retinoic acid signaling through RARα [241, 261, 262].

RETINOIC ACID INFLUENCES ON B CELL DIFFERENTIATION AND FUCNTION

As mentioned previously, retinoic acid also impacts several aspects of B cell biology including germinal centers reactions, cell proliferation and differentiation, and antibody production. During the course of an antigen challenge, B cells must migrate to germinal centers, recognize their cognate antigen, undergo somatic hypermutation, and differentiate to generate either plasma cells that produce antibody or long lived memory B cells. Germinal centers arise as follicles in secondary lymphoid organs, like spleen or lymph nodes, approximately 1 week after antigen challenge [263]. Besides B cells, germinal center follicles are populated by follicular DCs and helper T cells, who present antigen to newly activated B cells. B cells migrate to germinal centers following up-regulation of homing markers, which are induced by retinoic acid [234, 238]. In mice inoculated with a tetanus toxoid vaccine, treatment with retinoic acid increased the size and numbers of germinal centers observed per follicle [264]. An increased frequency of $IgG1^+$ B cells was also found in germinal centers, indicating that cells had

clonally expanded and differentiated into plasma cells [264]. In these ways, retinoic acid impacts germinal center formation and its responses to antigen challenge.

Depending on the B cell's developmental stage, retinoic acid appears to have opposing effects on B cell proliferation (Fig. 13). Studies on B cells isolated from peripheral blood and stimulated by either the B cell receptor (BCR) and CD38, or CD40 and IL-4, were inhibited from proliferating when cultured with retinoic acid, however, a subpopulation was expanded in $IgG1^+$ cells [265]. This expansion of the IgG^+ subpopulation likely occurred because retinoic acid also enhanced the expression of *Aid*, an enzyme required for class-switch recombination, following BCR ligation in naïve cells [266]. While retinoic acid inhibits the proliferation of normal B cell progenitors, it enhances the generation of $CD19^+sIgM^+$ B cells [265, 267] (Fig. 13). Additionally, increased proliferation of memory B cells occurs following stimulation with retinoic acid and CpG, a TLR9 ligand [265, 268]. By inhibiting mature B cell proliferation and stimulating the generation and expansion of memory cells, retinoic acid regulates the maturation of B cells at several stages during its development and helps maintain B cell pools required for memory responses.

Interestingly, memory B cell populations cultured with retinoic acid enhanced their production of IL-10, which promotes the differentiation of memory B cells into plasma cells capable of producing antigen-specific antibody [268] (Fig.14). In addition, retinoic acid affects B cell differentiation into plasma cells, by down regulating the transcription factor PAX5, which commits lymphoid progenitors to the B cell lineage early in differentiation, but is repressed during plasma cell differentiation [266, 269]. Retinoic acid also promotes the expression of CD138 on stimulated spleenic B cells as well, reflecting the cells' differentiation into plasma cells [270] (Fig. 14).

Furthermore, B cells contribute to host mucosal immunity by the class-switching, production, and secretion of IgA antibody, the most abundant antibody isotype produced at mucosal surfaces. In studies on vitamin A deficient animals, there was a noticeable absence of IgA antibody in the small intestine [234]. Since retinoic acid impacts B cell differentiation, this prompted investigators to determine if retinoic acid also influenced IgA production, especially in light of the fact that retinoic acid increased the expression of AID, an enzyme required for antibody class switching [266]. Retinoic acid can also synergize with TGFβ, in combination with IL-5 or IL-6, to induce high levels of IgA in the small intestine [271, 272] (Fig. 14). However, retinoic acid is not required for serum IgA production, as vitamin A deficient mice have equal levels of serum IgA compared to mice on a control diet [234]. Due to its role in plasma cell differentiation and IgA production, retinoic acid helps to maintain memory humoral responses at mucosal sites.

THESIS OBJECTIVE

Vitamin A and its metabolites play an important role in activating immune responses to infection. Due to their lipophilic nature, these compounds are always bound to retinoid binding proteins within cells and when transported among tissues. Serum retinol binding protein (RBP) is the key protein that transports retinol among tissues during homeostasis. As sites of immune cell differentiation and activation, cells within the spleen and lymph nodes require retinol in order to differentiate and generate functional lymphocytes. Since these tissues do not store retinyl esters, they depend on circulating retinol in order to function properly. Interestingly, serum RBP is markedly reduced during infection despite the increased requirement for retinol by

the immune system. This suggests that proteins other than RBP transport retinol during infection, but the identity of these proteins is unknown.

Here, I present evidence that demonstrates the acute phase protein serum amyloid A (SAA) is a novel retinol binding protein that transports retinol during bacterial infection. SAA proteins are induced by bacteria and additionally require retinol for their expression. I demonstrate that the retinol requirement of SAAs is not restricted to the small intestine, as mice on a vitamin A deficient diet also have reduced liver SAA expression. This requirement is likely due to intrinsic RAR signaling, as hepatocytes lose *SAA* expression when incubated with a pan-RAR inhibitor. Additionally, I demonstrate in a fluorescence-based binding assay that SAAs bind retinol at nanomolar affinities, which is comparable to the binding affinity of RBP for retinol. I also show that SAA proteins associate with retinol in the serum of mice following a bacterial challenge. In parallel with my studies, a post-doctoral fellow in the Hooper lab determined the crystal structure of mSAA3, finding that it oligomerizes to form a tetramer with a hollow hydrophobic binding pocket that can accommodate retinol. These results illustrate how SAA, as a small and mostly α-helical protein, can protect a lipophilic ligand from the aqueous environment. Altogether, these results demonstrate a new function for SAA during the acute phase and suggest a mechanism for how retinoid ligands may be delivered to immune cells to initiate downstream responses.

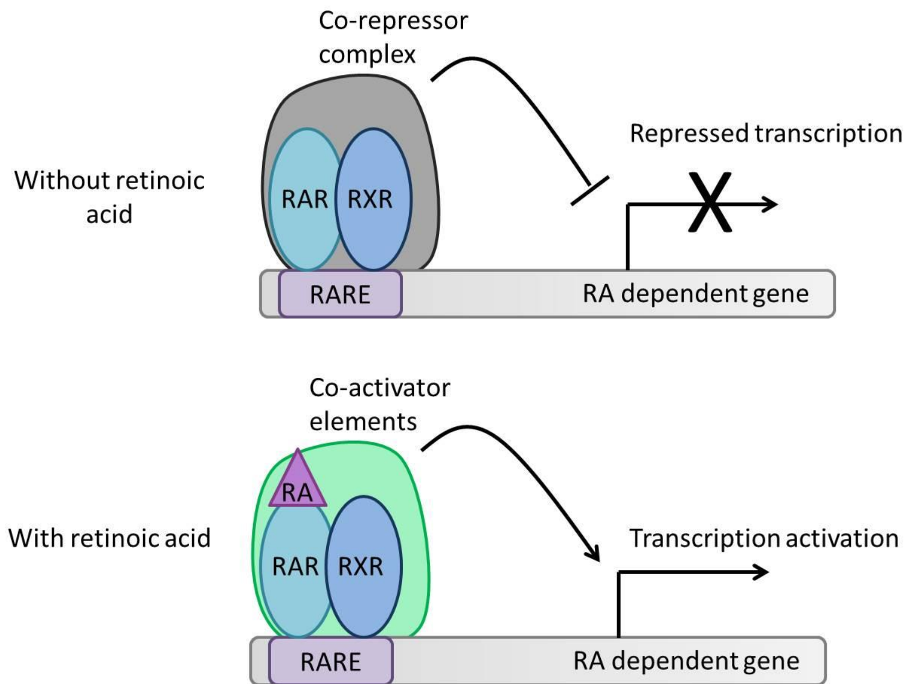


Figure 9: The binding of retinoic acid to RAR:RXR heterodimers activates gene transcription. Retinoic acid receptor (RAR) and retinoid X receptor (RXR) sit as heterodimer pairs on conserved retinoic acid response elements sequences (RARE) bound to a complex of co-repressor proteins, which prevents gene transcription. The binding of retinoic acid to RAR results in conformational changes in the heterodimer that strengthens its association with the DNA, leading to release the co-repressor proteins, recruitment of co-activator elements, and ultimately gene transcription.

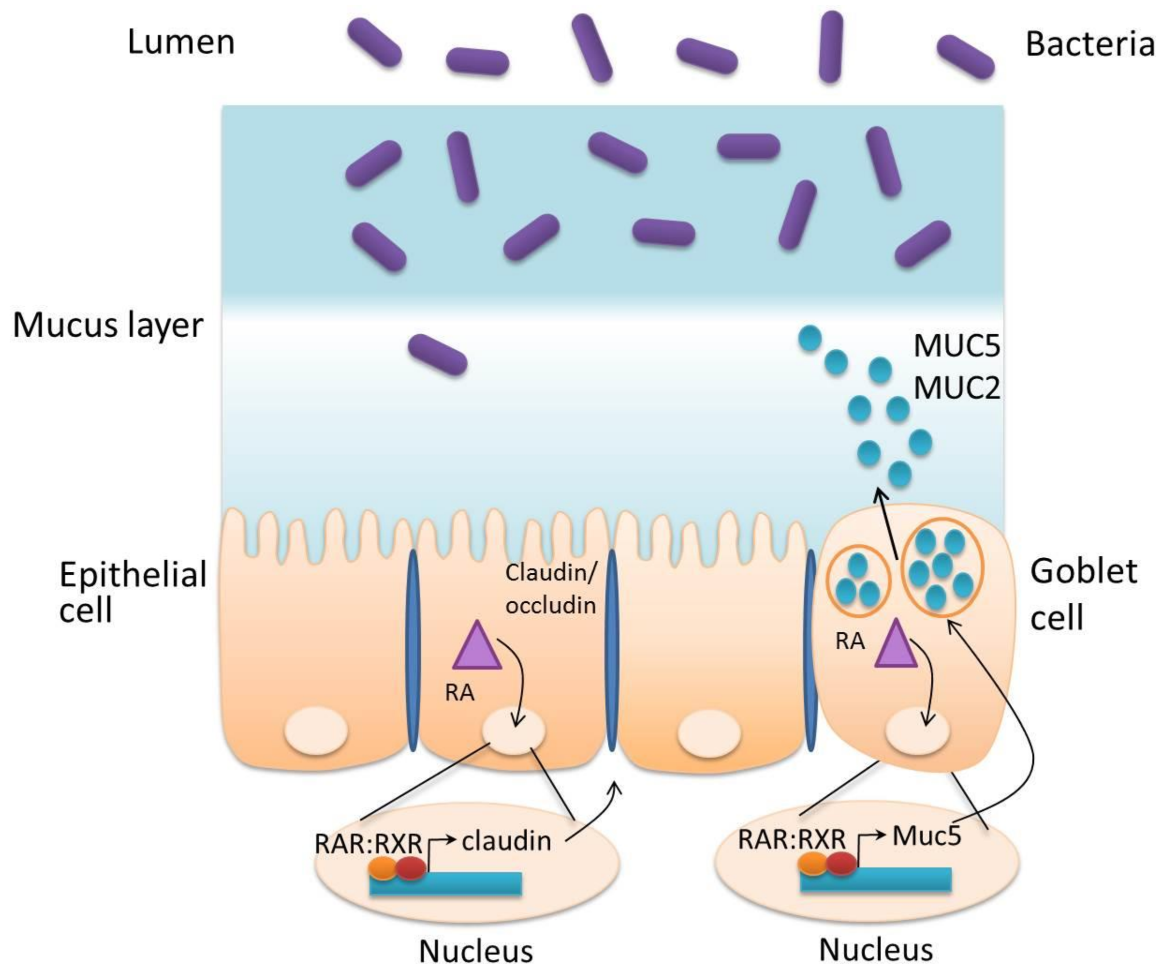


Figure 10: Retinoic acid promotes the integrity of the intestinal epithelial barrier. The intestinal barrier is comprised of gut epithelial cells, tight junction proteins, and a thick mucus layer, all of which limit the association and invasion of the microbiota. Retinoic acid promotes RAR:RXR signaling in epithelial cells to activate the transcription of claudin and occludin tight junction proteins, which seals the space between epithelial cells. Retinoic acid also stimulates the production of several mucin proteins in goblet cells, which are secreted into the lumen and contribute to the mucus barrier. In addition, retinoic acid aids in the turn-over of epithelial cells, allowing the barrier to regenerate following damage from inflammation.

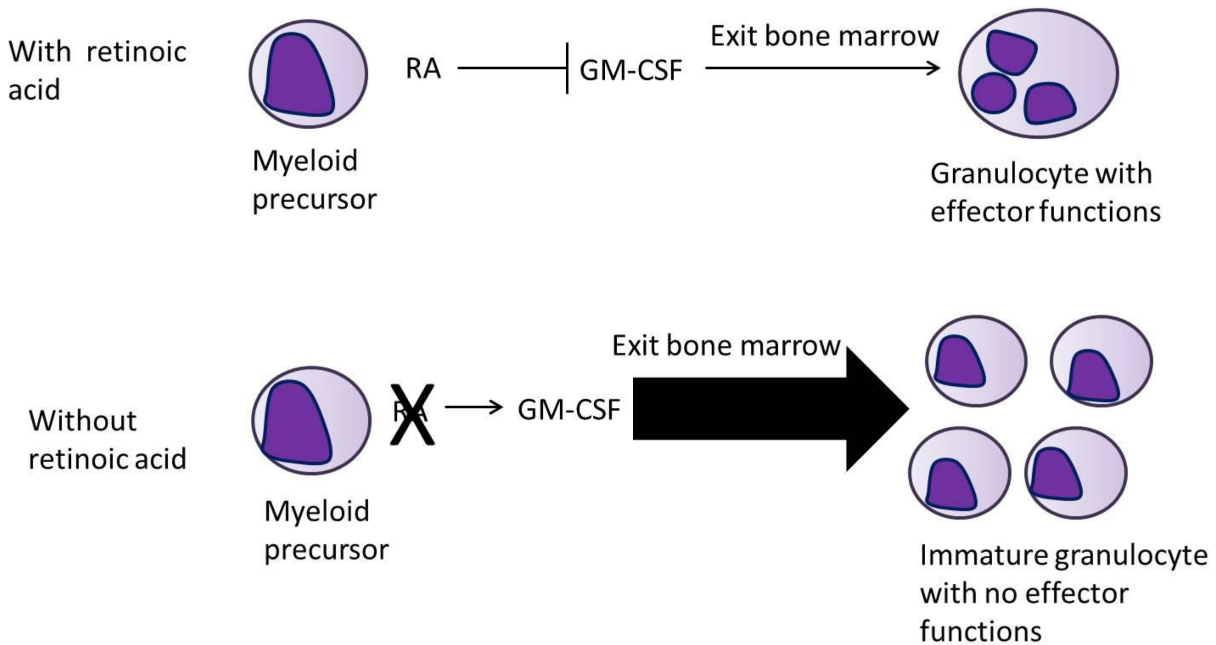


Figure 11: Retinoic acid effects on innate immune cells. Retinoic acid impacts innate immune cell differentiation and effector function. Retinoic acid promotes the terminal differentiation of granulocytes from the bone marrow by inhibiting GM-CSF, thus allowing cells to develop effector functions. In the absence of retinoic acid, excess GM-CSF leads to hyper-proliferation of immature granulocytes populating the periphery that are not capable of phagocytosis and undergo a high rate of spontaneous apoptosis.

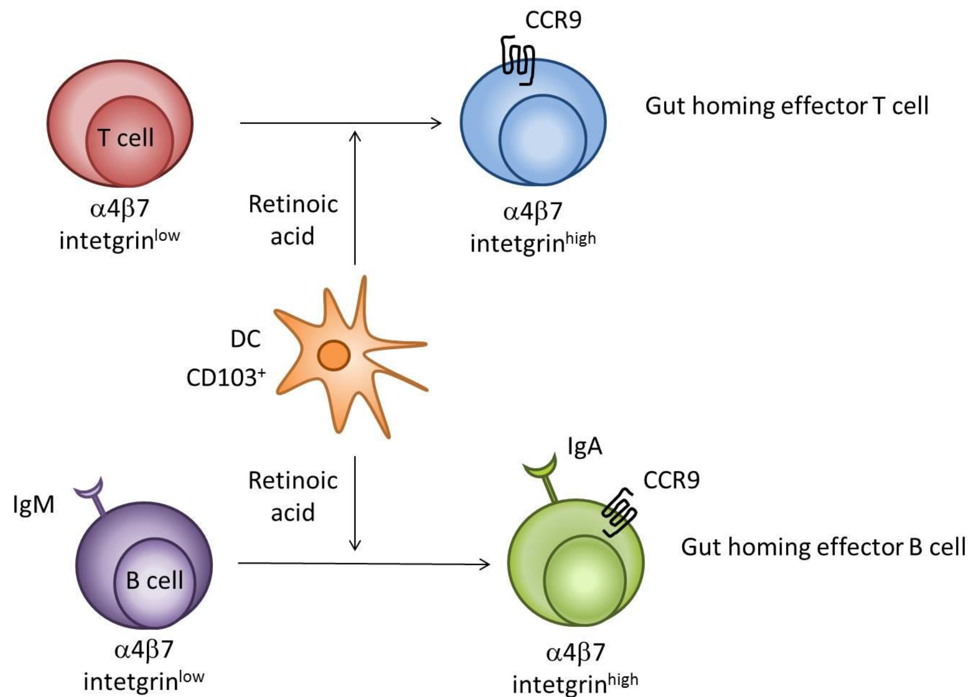


Figure 12: Retinoic acid induces gut homing of T and B cells. During an intestinal infection, activated, antigen-specific immune cells must migrate to the site of infection in order to combat the pathogen. In order to migrate to the small intestine, lymphocytes must up-regulate $\alpha4\beta7$ and CCR9, receptors which direct cells to the gut. In order to do this, lymphocytes require education from $CD103^{+}$ DCs, which express RALDH2. $CD103^{+}$ DCs produce retinoic acid, which induces $\alpha4\beta7$ and CCR9 expression on T or B cells and confers the ability of these lymphocytes to home to the gut.

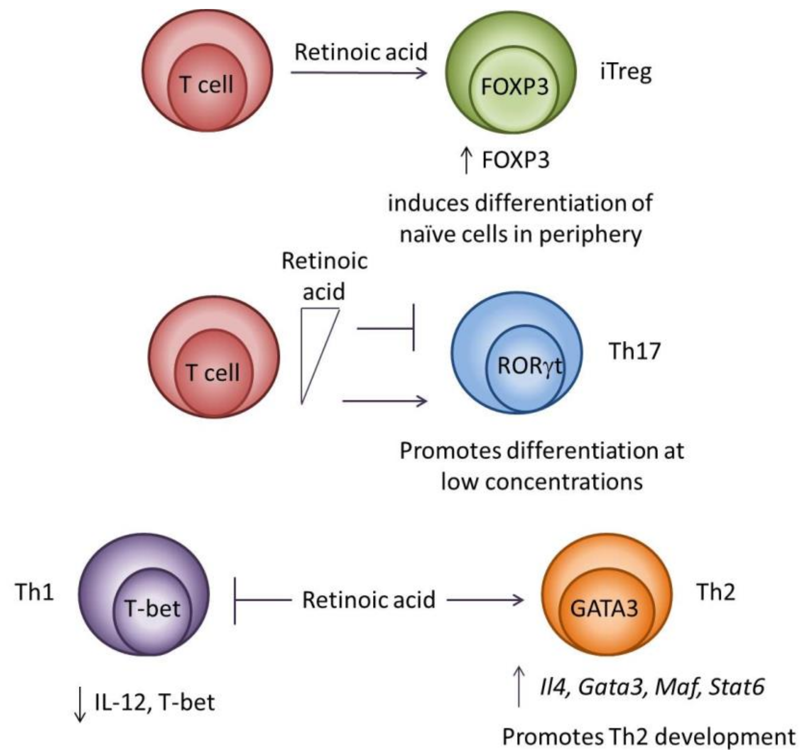


Figure 13: Retinoic acid impacts T cells lineage commitment and development. When naïve T cells in the periphery are exposed to retinoic acid, in combination with TGFβ and IL-2, up-regulate expression of the FOXP3 transcription factor, committing these cells to the iT_{reg} lineage. Retinoic acid also impacts Th17 cell development, as low concentrations of retinoic acid promote Th17 development, an outcome that is inhibited when more retinoic acid is present. Additionally, retinoic acid promotes Th2 development over Th1 by down-regulating IL-12 and up-regulating *Il4* and *Gata3*. In this way, retinoic acid impacts the differentiation of multiple T cell lineages.

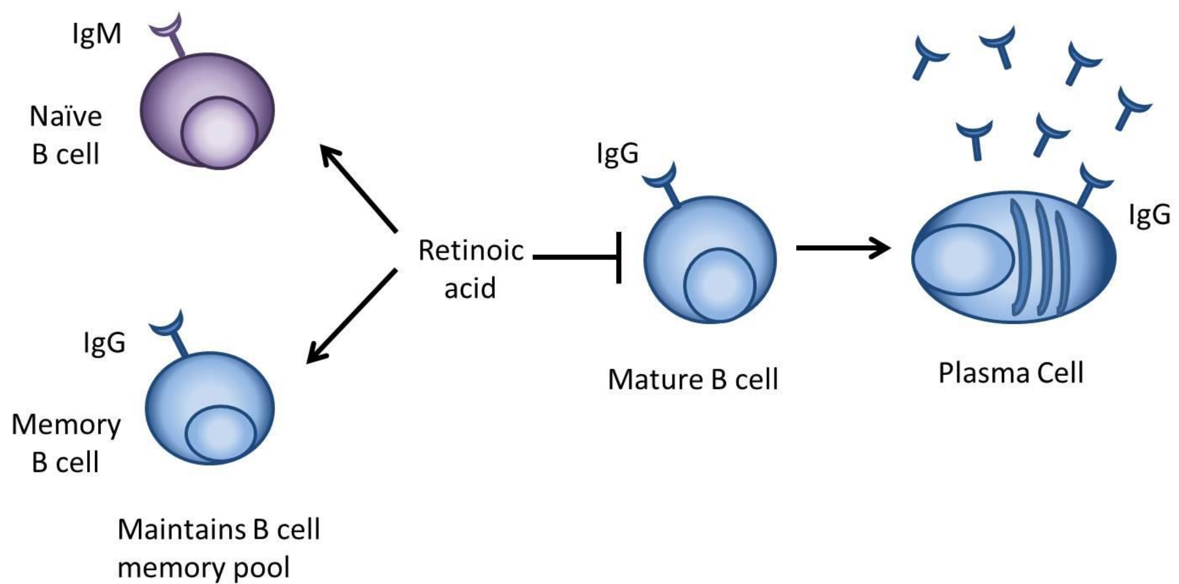


Figure 14: Retinoic acid affects proliferation at different B cell developmental stages. Retinoic acid has opposing effects on B cell proliferation depending on the B cell's developmental stage. Retinoic acid promotes proliferation of naïve (IgM^+) and memory B cells (IgG^+), while repressing the proliferation of mature B cell cells. Inhibition of mature B cell proliferation allows cells to instead differentiate and preform effector functions (like antibody production). The stimulation and expansion of memory cells by retinoic acid helps to maintain B cell pools required for memory responses.

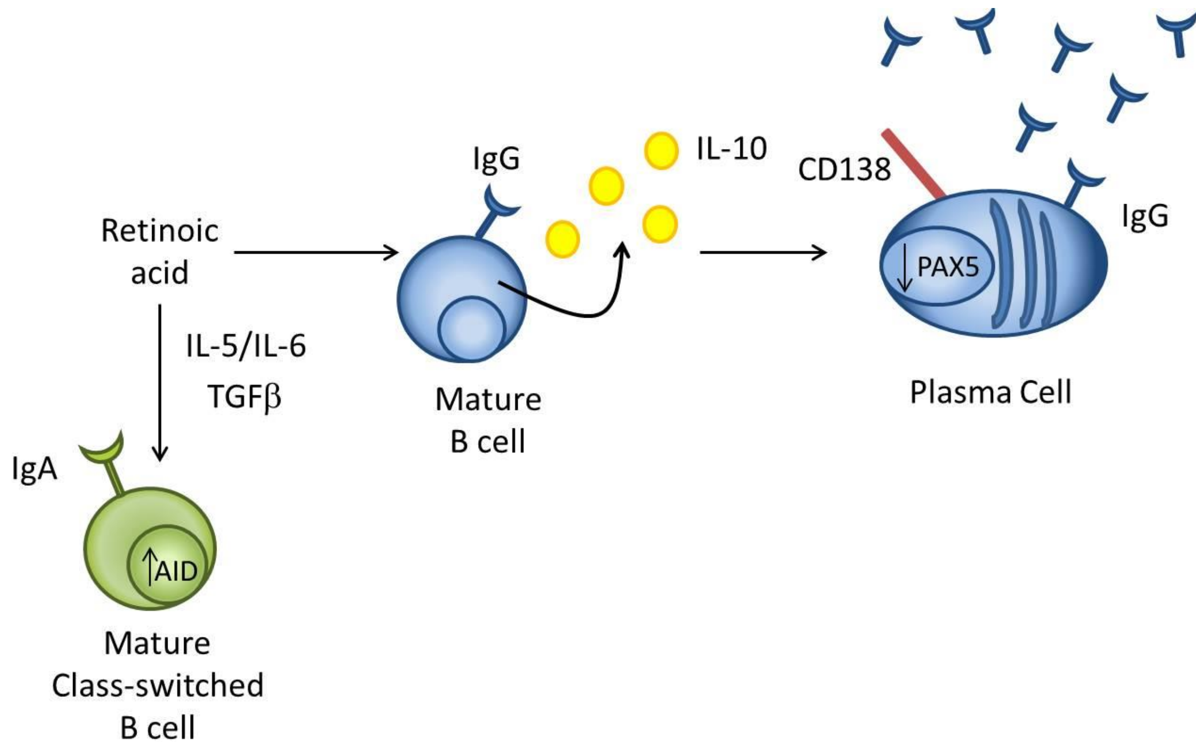


Figure 15: Retinoic acid induces differentiation and effector functions on B cells. Mature B cells undergo antibody class switching to IgA in the presence of retinoic acid and IL-5 or IL-6, which likely occurs as retinoic acid up-regulates AID, an enzyme required for antibody class-switching. In addition to antibody class-switching, retinoic acid promotes mature B cells to differentiate into memory B cells by inducing IL-10 production, which up-regulates CD138, a plasma cell differentiation marker and down-regulation of the transcription factor PAX5.

CHAPTER FOUR

METHODOLOGY

Animals. Conventionally raised C57BL/6 wild-type, *MyD88*$^{fl/fl}$, and *MyD88*$^{\Delta IEC}$ mice were maintained in the barrier at the University of Texas Southwestern Medical Center. MyD88$^{\Delta IEC}$ mice were generated by crossing mice with an intestinal epithelial cell (IEC) restricted *villin*-Cre transgene with mice carrying a loxP-flanked MyD88 allele (MyD88$^{fl/fl}$) [273-275]. Germ-free C57BL/6 mice were maintained in isolators as previously described. *Saa1/2*$^{-/-}$ mice were obtained from Dr. Frederick C. de Beer at the University of Kentucky and were maintained in the barrier at the University of Texas Southwestern Medical Center [29]. All mice were maintained on a 12 hour light cycle and fed the same autoclaved chow. 6-12 week old mice were used for all experiments. Experiments were performed using protocols approved by the Institutional Animal Care and Use Committees of the UT Southwestern Medical Center.

Vitamin A depletion. Vitamin A-deficient (TD.09838) and control (approximately 20,000 IU vitamin A/kg; TD.09839) diets were purchased from Harlan Laboratories. At day 10 of gestation, pregnant females were placed on either the control diet or the vitamin A-deficient diet. Mothers and pups were maintained on the diets until weaning, and pups stayed on the diet for 2 additional months prior to sacrifice.

Antibiotic treatment. Conventional C57BL/6 mice were given ampicillin (1 mg/mL), vancomycin (0.50 mg/mL), neomycin sulfate (1 mg/mL), and metronidazole (1 mg/mL) in

drinking water for 4 weeks. All antibiotics were from Sigma-Aldrich. Microbiota depletion was verified by aerobic and anaerobic culture of intestinal contents.

Microarray experiments. Total RNAs were isolated from mouse distal small intestines (ileum) using the Qiagen Midi-Prep RNA isolation kit. For each experimental condition, RNA was isolated from two independent groups of four mice. The RNAs in each group were pooled and used to generate biotinylated probes for microarray analysis. Probes were hybridized to Affymetrix Mouse Genome 430 2.0 Gene-Chips in the University of Texas Southwestern Microarray Core.

To identify genes that are differentially expressed between germ-free and conventional mice, I performed two-way comparisons between germ-free and conventional groups, with germ-free samples designated as baseline. Raw data were imported into GeneSpring software for analysis, to identify differentially expressed genes. The genes identified were considered significant if the following criteria were met: 1) if the probe set ID was within the top 80 percentile of genes expressed, this helped to eliminate probe sets belonging to genes that were not expressed in the samples; 2) there was a greater than 2.0 fold difference in at least one pairing of sample groups; 3) the fold difference was observed in duplicate microarray experiments. I found 329 genes passed my 2-fold criteria for my comparison between germ-free and conventional mouse groups. I then performed a similar analysis to identify genes that are differentially regulated between conventional mice fed a normal diet versus those fed a vitamin A-deficient diet. A total of 138 genes were identified by this array. I crossed-compared the list of 329 germ-free and conventional differentially expressed genes to the 138 genes I identified

that were differentially expressed by vitamin A diet. Finally, I identified 19 genes that were differentially regulated by colonization status and by dietary vitamin A content. Signal intensity data for this group of 19 genes were converted to Z-scores ($z = (x - \mu)/\sigma$, where x = signal intensity, μ = mean signal intensity for all samples, and σ = SD across all samples), which were visualized as heat maps using Java TreeView software.

Cell culture. HepG2 cells were purchased from ATCC. Cells were maintained in 1X DMEM, 10% FBS (or charcoal stripped FBS), 1X Penstrep, 1X glutamax, and 1X sodium pyruvate, and maintained at 5% CO_2. Cells were used from passage numbers 8-20. In experiments, the cells were grown overnight in DMEM containing 10% charcoal stripped FBS, to remove any retinoids present in media, before the assay was performed. For experiments, the cells were treated with 1 μM retinol or 100 nM retinoic acid, 10 ng/mL of IL-1β, and 10 ng/mL of IL-6. For experiments using pan-RAR inhibitor BMS493, cells were grown as previously stated and then treated with 1 μM retinol, 10 ng/mL of IL-1β, and 10 ng/mL of IL-6. After 3 hours, cells were washed once with 1X PBS and treated with 1μM of BMS493 for the time indicated.

Quantitative PCR. Total RNA was isolated from homogenized tissues or cells using the Qiagen RNeasy RNA isolation kit. RNAs were treated with deoxyribonuclease (Roche) prior to random primed cDNA synthesis and quantitative real-time PCR analysis. SYBR Green-based real-time PCR used *SAA*-specific primers as given in Table 1. Signals were normalized to 18S or Gapdh primers, listed in Table 1.

Immunofluorescence analysis. Zinc-fixed, paraffin embedded tissue sections were stained with anti-SAA antiserum raised against purified recombinant mSAA1 and detected using a goat anti-rabbit IgG Cy3 conjugate (Biomeda). Tissues were counterstained with DAPI and images were captured on a Zeiss AxioImager M1 Microscope.

Expression and purification of recombinant mSAA1 and 3. Genes encoding mouse SAA1 and SAA3 were cloned into the pET3a expression vectors between *XbaI* and *BamHI* restriction endonuclease sites, with an N-terminal hexa-histidine tag followed by a thrombin cleavage site as well as a C-terminal stop codon. Proteins were expressed in *E. coli* BL21-CodonPlus (DE3)-RILP cells (Stratagene) by induction with 0.4 mM isopropyl-β-D-galactoside (IPTG) for ~3 hr at 25°C for mSAA1 and at 37°C for mSAA3. Cells were harvested by centrifugation at 8,000*g* for 10 minutes at 4°C and re-suspended in lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole, 1% Triton, + proteinase inhibitors). After sonication, decyl maltopyranoside (DM) (Avanti Polar Lipids) was added to a final concentration of 40 mM and incubated for ~3 hours at 4°C. The mixture was then pelleted by centrifugation at 10,000*g* for 30 minutes and the supernatant loaded onto a Ni^{2+} metal affinity column (Qiagen) pre-equilibriated with 4 mM DM in lysis buffer. The column was washed twice with 20 mM imidazole in DM buffer (50 mM NaH_2PO_4, 500 mM NaCl, 4mM DM) to remove non-specific contaminants and the protein was eluted in DM buffer containing 500 mM imidazole. The elute was desalted with a HiTrap desalting column (GE Life Sciences) into a 25mM Tris pH8.0, 200 mM NaCl, 4mM DM buffer. Thrombin (Roche) was then added (~1 unit/1.2 mg protein) and incubated at room temperature

for 6 hours. Undigested protein was removed by passage over the Ni^{2+} affinity matrix, collecting only the flow through. The elute was concentrated in a 3K cutoff Amicon Ultra centrifugal device (Millipore) and further purified by size exclusion chromatography on either a HiLoad Superdex 75 (10/30) column (GE Life Sciences), in 20mM Tris pH 8.0, 100mM NaCl, 4mM DM. Protein was stored in aliquots at -20°C until it was used in experiments.

Binding assays. Retinoid stocks were quantified prior to each experiment with the following extinction coefficients: ε= 46,000 $M^{-1}cm^{-1}$ for retinol at 325 nm, ε=45,000 $M^{-1}cm^{-1}$ for retinoic acid at 350 nm, ε= 51,180 $M^{-1}cm^{-1}$ for retinyl acetate, and ε= 49,260 $M^{-1}cm^{-1}$ for retinyl palmitate at 325 nm. Steady state fluorescence was measured using a QuantaMaster 40 spectrofluorometer (Photon Technology International) and FelixGX software program. For retinol titrations, samples were excited at 348 nm and emissions monitored at 460 nm. For retinoic acid and other retinoids, samples were excited at 296 nm and tryptophan quenching was monitored by emissions at 334 nm. During the experiment, samples were mixed and allowed to equilibrate for each titration before fluorescence was read. For each experiment, ligands were titrated into binding buffer, and these values were used to correct fluorescence measurements to account for fluorescence of the free ligand. For experiments using mSAA1 and mSAA3, the binding buffer contained 25 mM Tris pH 8.0, 100 mM NaCl, and 4 mM decyl maltopyranoside (DM). Experiments using hSAA1, hRBP4, human transferrin (hTrf), and ApoA1 were conducted in PBS. K_d calculations were generated from 3-5 independent experiments and done as previously described using the following equations [276].

Equation 1: $K_d = \alpha/(1-\alpha)\,[R_o - nP_o(1-\alpha)]$

where α is the fraction of free binding sites on the protein for the ligand, R_o is defined as the concentration of bound ligand, P_o is the concentration of total protein in the assay, and n is the number of independent binding sites for the ligand.

Equation 1 can be rearranged to Equation 2.

Equation 2: $P_o\alpha = 1/n\ (R_o\alpha/1\text{-}\alpha)\text{-}K_d/n$

By plotting a linear least squares plot of $P_o\alpha$ verses $R_o\alpha/(1\text{-}\ \alpha)$ a straight line is observed with K_d/n equaling the y-intercept and 1/n equaling the slope of the line. The value of α was calculated for each point on the titration curve using the following equation.

Equation 3: $\alpha = (F_{max}\text{-}F)/(F_{max}\text{-}F_0)$, where F_{max} is fluorescence intensity upon saturation, F is fluorescence intensity at a certain ligand concentration (R_o), and F_0 is the initial fluorescence in the absence of ligand. When experiments measured tryptophan quenching, α was calculated using a modified Equation 3 as follows.

Equation 4: $\alpha = (F\text{-}F_{max})/(F_0\text{-}F_{max})$

Bacterial colonization. For monocolonization experiments, age-matched germ-free mice were colonized by oral gavage of stationary phase bacteria cultures of the following strains: *Bacteroides thetaiotaomicron* strain VPI-5482 or *Enterococcus faecalis* strain V583. Mice were rested for 3 days before sacrifice. For serum collection experiments, *Salmonella enterica* Serovar Typhimurium (SL1344) was grown overnight in Luria Broth at 37^oC to stationary phase. The next day, the culture was sub-cultured and grown to an OD600 of .4-.6. The bacteria were

spun down and washed twice with 1X PBS before inoculation. Mice were infected intraperitoneally with 1 x 10^4 organisms per mouse and sacrificed at the indicated time. Tissues and serum were then collected for experiments.

Purification of serum SAA. Serum was pooled from 3-5 mice after 24 hours of bacterial infection and 500 μL was separated by size-exclusion chromatography on a Superdex 75 HiLoad 16/60 column (GE Life Sciences). Peak fractions were subjected to SDS-PAGE and stained with Coomassie Blue. Duplicate samples were analyzed by Western blot with anti-SAA antibody to identify peak fractions containing SAA protein. These experiments were conducted in zero-low light conditions to help prevent retinoid degradation.

Mass spectrometry analysis of retinol and retinoic acid. Retinoid extraction was modified and scaled from a previously described procedure [277]. Unprocessed serum or SAA-containing fractions purified by size exclusion chromatography were pooled, and added to an equal volume of 1:1 1-butanol:acetonitrile, and vortexed for 60 seconds in order to precipitate the protein fraction within the serum to release retinoid ligands. 20 μL of 20.6 M K_2HPO_4 was added for each 1 mL of pooled fractions to help facilitate separation of organic and non-organic components of the serum. Samples were then vortexed 30 seconds and 5 mL of hexane per 1 mL sample was added. Samples were vortexed for another 30 seconds and centrifuged at 1,000*g* for 5 minutes to separate out the organic phase components. The top organic phase was dried in a nitrogen evaporator (Organomation Associates) before storage at -80°C. Samples were prepared the day before the assay. Standard solutions were prepared by dissolving the pure

chemical in ethanol and prepared fresh for every use. Standard curves were generated by spiking retinol or retinoic acid into 1 mL of 20 mM Tris pH 8.0, 100 mM buffer and processed exactly as serum samples. Samples were re-suspended in 200 μL of acetonitrile before injection. Compound levels were monitored by LC-MS/MS on an AB/Sciex (Framingham, MA) 4000 Qtrap mass spectrometer coupled to a Shimadzu (Columbia, MD) Prominence LC after a 20 μL injection. The compounds were detected using electrospray ionization (ESI) with the mass spectrometer in MRM (multiple reaction monitoring) mode by following the precursor to fragment ion transition 269.2 -> 93.1 and 269.2 -> 119 for retinol (pos. mode; $M+H^+-H_20$) and 301.2 -> 123.1 for retinoic acid (pos. mode; $M+H^+$). An Agilent (Santa Clara, CA) Eclipse XDB C18 column (150 X 4.6 mm, 5 micron packing) was used for chromatography with the following conditions: Mobile phase A: acetonitrile:methanol:H_2O:formic acid (55:33:12:.01); mobile phase B: acetonitrile:formic acid (100:0.01). Over a total run time of 18 min, the following gradient was applied: 0 to 3 min 50% B; 3 to 10 min gradient to 100% B; 10 to 17 min 100% B; 17 to 18 min gradient to 50% B. Stoichiometry of the SAA-retinol association were determined by quantifying serum retinol and serum SAA. Total serum retinol was calculated based on peak areas from the mass spectrometer analysis in samples compared to a retinol standard curve. Serum SAA was quantified by Western blot analysis with the anti-SAA antiserum and densitometry. All solvent used in these experiments were HPLC grade. Experiments were performed in the dark or under red light whenever possible to reduce retinoid degradation.

Experiments using LC/MS were conducted using samples prepared as I stated above. Compound levels were monitored by LC/MS on an Agilent Technologies 6130 Quadruple LC/MS after a 60 μL injection. The compounds were detected using electrospray ionization (ESI) with the mass spectrometer using an Agilent (Santa Clara, CA) Eclipse XDB C18 column (150 X 4.6 mm, 5

micron packing) using the same chromatography conditions listed previously. Positive compound identification was verified by monitoring retention time and UV wavelength of eluted peaks in both retinol standard and serum samples. Retinol displays a characteristic UV wavelength peak at 325 nm.

Statistical analysis. Statistical analysis was performed using Graphpad Prism software (Graphpad; San Diego, CA, USA), and all p values were calculated using the unpaired two-tailed Student's t-test or Mann Whitney test. Results are expressed as the mean ± standard error of the mean (SEM).

Table 1:

Real-time PCR primers

mouse SAA1 F	CATTTGTTCACGAGGCTTTCC
mouse SAA1 R	GTTTTTCCAGTTAGCTTCCTTCATGT
mouse SAA2 F	TGTGTATCCCACAAGGTTTCAGA
mouse SAA2 R	TTATTACCCTCTCCTCCTCAAGCA
mouse SAA3 F	CGCAGCACGAGCAGGAT
mouse SAA3 R	CCAGGATCAAGATGCAAAGAATG
human SAA1 F	GGCATACAGCCATACCATTC
human SAA1 R	CCTTTTGGCAGCATCATAGT
human SAA2 F	GCTTCCTCTTCACTCTGCTCT
human SAA2 R	TGCCATATCTCAGCTTCTCTG
mouse 18S F	CATTCGAACGTCTGCCCTATC
mouse 18S R	CCTGCTGCCTTCCTTGGA
mouse Gapdh F	CACTGCCACCCAGAAGACTGT
mouse Gapdh R	GGAAGGCCATGCCAGTGA
human Gapdh F	CCTGGTCACCAGGGCTGCTTTTAAC
human Gapdh R	GTCGTTGAGGGCAATGCCAGCC

CHAPTER 5

SERUM AMYLOID A IS A RETINOL BINDING PROTEIN THAT TRANSPORTS RETINOL DURING BACTERIAL INFECTION

INTRODUCTION

Retinol and its derivative retinoic acid play vital roles in the physiological response to infection. As a metabolite of dietary vitamin A, retinol is enzymatically converted to retinoic acid, a ligand for nuclear receptors that regulate gene transcription programs in cells. In this way, retinoic acid promotes the maturation of innate immune cells, governs the differentiation of adaptive immune cells, and facilitates the regeneration of epithelial barriers damaged by infection [216]. As small lipophilic compounds, retinoids do not freely circulate but are instead transported among cells and tissues by retinoid binding proteins. At steady state, retinol binding protein (RBP) facilitates the transport of retinol from the liver to peripheral tissues that require retinol for their physiological functions [278]. However, serum RBP is markedly reduced during infection despite the increased requirement for retinol by the immune system. This suggests that proteins other than RBP transport retinol during infection, but the identity of these proteins is unknown.

Here, I identify SAA proteins as new retinol binding proteins, capable of binding retinoids *in vitro*. I show that dietary vitamin A is required for SAA expression in the small intestine and liver. Additionally, I demonstrate that *Saa* expression in the small intestine is dependent on the microbiota and epithelia-expressed MyD88. I further establish that SAA protein is found in the serum with retinol in wild-type mice, a phenotype that is absent in *SAA1/2*$^{-/-}$ mice. Complementing my studies, the recently solved crystal structure of mSAA3

explains how a small, alpha-helical SAA can oligermize to form a ligand binding pocket to protect retinol from an aqueous environment. Together these results provide insight into the biological function of SAAs and reveal new information about the physiological response to infection.

RETINOL TRANSPORT DURING INFECTION

Dietary vitamin A plays essential roles in host response to infection; however, most studies on proteins involved in retinol transport and metabolism have been conducted during homeostasis. At steady state, retinyl ester stores in the liver are liberated when peripheral tissues need additional retinol for normal function. While some tissues contain small stores of retinyl esters that can be used and replenished by circulating RBP:retinol complexes, cells populating the immune system do not store retinyl esters, and thus rely completely on retinol binding proteins as their source of retinol [184]. However, during infection RBP serum levels drop dramatically, in spite of the host tissues' increased need for retinol during an immune response. Despite the key roles retinoids play in the host physiological response, very little is known about these pathways during infection. This supports the idea that the immune system would need access to more retinol early during the acute response in order to initiate the required immune functions necessary to clear the pathogen. However, previous reports have observed a decrease in serum retinol concentrations following infection [279-282]. In these reports, the earliest time point noted began at least 24 hours to several days past infection or were observations made in hospitalized human cases, where the time of infection was unknown [279, 281].

In order to gain an understanding of early events in retinol transportation during the acute phase, I intraperitoneally infected wild-type mice with *S. typhimurium,* collected serum across a time course, and quantified retinol by performing LC-MS/MS. At steady state, I calculated serum retinol concentrations to be 300 ng/mL and observed a decrease in retinol concentrations after 24 and 48 hours, which agreed with reported values [279-281]. Interestingly, starting at 4 hours following infection, retinol concentrations significantly increased in the serum and were maintained at elevated levels until 12 hours after infection (Fig. 16). By increasing the amount of retinol in the serum, the immune system would gain access to the additional retinol needed to activate and amplify an immune response, which could have a significant impact on the host's ability to fight infection.

COMPARATIVE MICROARRAY OF THE SMALL INTESTINE

The epithelial cells populating the small intestine serve as a component of the physical barrier separating host tissues from the microbiota colonizing the gut lumen. The microbiota is a complex population of multiple bacterial species, which maintains a beneficial relationship with the host. In this way, the microbiota serves two purposes: to aid in nutrient breakdown and to prime the immune system for its proper development and function. While the presence of a microbiota is necessary for a healthy host, it still presents a large immunological challenge in order for the host to contain the commensal microbiota within the gut lumen.

As the small intestine also serves as the site of vitamin A absorption, this complex environment presents a unique setting in which to find potentially novel retinoid binding proteins that may play important roles in the transportation and delivery of retinoids necessary for the

immunological responses operating within the small intestine. In order to better understand how retinol transport and metabolism are affected by the intestinal microbiota, I performed several whole genome DNA microarrays on the mouse small intestine. I analyzed the results of these microarrays by limiting the gene hits to those that change by at least 2 fold, and also change in the same direction on duplicate microarrays. The first microarray compared gene expression between germ-free and conventionally raised mice. This analysis generated a list of 329 genes that were responsive to the intestinal microbiota (Fig. 17).

However, since I was also interested in how vitamin A affected host responses, I performed a second microarray on mice fed either a vitamin A deficient diet or a vitamin A replete diet. Since genes involved in retinol transport and metabolism require retinoids for their expression, removing vitamin A from the diet allowed me to identify genes dependent on retinoids for their expression. Because retinoids are critical for host survival, pregnant females were placed on the vitamin A deficient diet and their pups were kept on this diet for at least 2 months, in order to render them vitamin A deficient. Analysis of the vitamin A microarray data generated 138 genes that were differentially regulated by vitamin A in the diet (Fig. 17). By comparing genes that were differentially regulated on both the germ-free and conventional, and the vitamin A deficient and vitamin A replete microarrays, I was able to generate a list of 19 genes that were regulated by both the microbiota and vitamin A (Fig. 17). This list contained 16 genes that were down-regulated and 3 genes that were up-regulated during vitamin A deficiency. Within this list were several genes associated with immune responses including: resistin like-beta, granzymes A and B, Duoxa2, and SAA (Fig. 17). The microarray results identified two isoforms of SAA: SAA1 and SAA2. SAA proteins are classically categorized as acute phase proteins, whose serum concentration can exceed 1 mg/mL during inflammation or infection.

Despite this massive protein induction in the serum, there has previously been little functional information available on SAAs. Since SAAs are secreted proteins that are induced during an infection response, I choose to further follow up with them to determine if they serve an unknown function in vitamin A transport.

SAA EXPRESSION REQUIRES BACTERIA IN THE SMALL INTESTINE

To validate the initial microarray findings, I performed quantitative real-time PCR (Q-PCR) on amplified RNAs from individual mice for each condition used in the microarray analysis. My Q-PCR analysis revealed that *Saa1*, *Saa2*, and *Saa3* (not identified on the vitamin A microarray) were all up-regulated in conventional-raised mice compared to germ-free controls (Fig. 18). This was not surprising, as SAAs have previously been demonstrated to be induced in enterocytes by the intestinal microbiota [29, 37, 38]. Past studies have implicated a requirement for MyD88 signaling to induce *Saa* expression [37]. In order to determine if MyD88 signaling within epithelial cells was required for *Saa* expression, I analyzed MyD88$^{\Delta IEC}$ mice, which harbor an epithelial-specific deletion of MyD88 [218]. MyD88$^{\Delta IEC}$ mice were generated by crossing mice with an intestinal epithelial cell (IEC) restricted *villin*-Cre transgene with mice carrying a loxP-flanked MyD88 allele (MyD88$^{fl/fl}$) [273-275]. Epithelial-specific MyD88-deficient mice were compared to floxed MyD88 controls and analyzed by Q-PCR for *Saa* expression. As expected, the MyD88$^{fl/fl}$ mice had robust *Saa* expression, however, this was reduced in mice lacking epithelial-specific MyD88, indicating a requirement for this signaling adapter (Fig. 18). As NF-κB binding sites have been reported in the promoter regions of *SAA* genes, this leads to two possibilities for this signaling cascade: either intestinal epithelial cells are

directly sensing the microbiota, or they are responding to IL-1 cytokines up-regulated by other cells in the environment, which relay signals through both the IL-1 receptor and MyD88 complex.

As the presence of a conventional microbiota drives *Saa* expression, I wanted to determine if a specific species of bacteria could also stimulate *Saa* expression. To test this, I orally gavaged germ-free mice with either the Gram negative bacteria *B. thetaiotaomicron* or the Gram positive bacteria *E. faecalis* and assayed for *Saa* expression by Q-PCR. Compared to germ-free controls, mice colonized with either species up-regulated *Saa* transcription (Fig. 19). I observed more robust *Saa* expression from mice colonized with *E. faecalis*; however, this is likely due to the fact that *E. faecalis* is capable of associating closely with the epithelial cell surface, while *B. thetaiotaomicron* is largely restricted to the lumen of the intestine.

SAA EXPERSSION IS DEPENDENT ON VITAMIN A

As mentioned previously, the vitamin A diet microarray identified *Saa1* and *2* as vitamin A dependent genes. I validated these results by Q-PCR and found that small intestinal *Saa1, 2*, and *3* expression were dramatically reduced in mice fed a vitamin A deficient diet compared to vitamin A replete diet controls (Fig. 20a). I further confirmed this result by immunohistochemistry (IHC) staining of the small intestine that showed the protein levels of SAA closely mimic the transcript levels I observed (Fig. 20b).

As part of the acute phase response, SAA proteins are known to be produced by the liver. In order to determine if vitamin A dependent *Saa* expression was tissue specific, I performed Q-PCR on liver tissue from mice fed a vitamin A deficient or a vitamin A replete diet. As I

observed in the small intestine, liver *Saa* expression is dependent on vitamin A, as vitamin A deficient mice had lower levels of *Saa* expression, although the reduction in expression was less pronounced than in the intestine (Fig. 20c). Additionally, I performed immunohistochemistry on zinc-fixed paraffin embedded liver tissue to study SAA protein expression in the liver. In accordance with my Q-PCR results, I saw reduced, but not absent, levels of SAA protein present in vitamin A deficient mice (Fig. 20d). The diminished levels of SAA expression in the liver is likely due to the fact that dietary vitamin A deficiency does not completely deplete stored retinoids in the liver [283], even after mice have been on a vitamin A deficient diet for at least 2 months.

SAA EXPRESSION IN CELL CULTURE

As mentioned previously, all known genes involved in retinol transport or metabolism rely on retinoic acid for their transcription. In this way, they control their own metabolism, only expressing binding proteins and enzymes when retinol is present. Since my *in vivo* diet studies demonstrated a requirement for dietary vitamin A for *Saa* expression, I wanted to set up a cell culture system that would allow me to ask more direct questions about how retinoids drive *Saa* expression. I began by moving my studies into a mouse epithelial-like cell culture model system – MODE-Ks, which have been used previously to study intestinal cell responses [284]. However, MODE-K cells did not reliably up-regulate *Saa* upon either LPS or cytokine stimulation and therefore did not serve as a useful cell culture system to explore how vitamin A regulates *Saa* expression. However, since *Saa* expression is not limited to the intestine, I decided to pursue these studies in a human hepatoma cell line, HepG2, in collaboration with graduate

student Sureka Gattu. HepG2 cells have previously been shown to up-regulate SAA expression upon cytokine stimulation [36]. In order to reliably measure retinoid impact on gene expression, any traces of retinoids present in the cell culture media had to be removed prior to the experiment. To do this, HepG2 cells were grown overnight in charcoal-stripped fetal bovine serum (FBS) media the day before the assay in order to remove all lipid ligands from the media and therefore have no trace amounts of retinoids that could confuse my experimental study. The following day, HepG2 cells were incubated with retinol, IL-1β and IL-6, or the combination of both retinol and cytokines. IL-1β and IL-6 have been shown to synergize and up-regulate robust *SAA* expression, and I observed this in my experiment as well [36] (Fig. 21a). However, cells incubated with only retinol did not up-regulate *SAA* expression. This result was not surprising, since *in vivo*, *Saa* induction requires either a bacterial or cytokine signal in addition to retinol, to produce *Saa*. Interestingly, cells given both the cytokine signals and retinol, up-regulated *SAA* to a greater degree compared to the cytokine alone condition, indicating these two signals synergized to induce *SAA* expression (Fig. 21a). Since retinol, in addition to the cytokines, was able to drive *SAA* expression, it's likely that the hepatocytes enzymatically converted retinol to retinoic acid to generate this response. As the liver serves as a major storage location for retinoids, hepatocytes are equipped with the necessary retinoid metabolism genes for the enzymatic conversion of retinol to retinoic acid.

In order to test if retinoic acid could also stimulate *SAA* expression, HepG2 cells were grown overnight in charcoal stripped FBS, and then incubated with retinoic acid alone, IL-1β and IL-6, or the combination of retinoic acid and cytokines. As with retinol, retinoic acid and cytokines synergized to induce *SAA* expression above the cytokine alone condition (Fig. 21b). These results indicate that both retinol and retinoic acid can synergize and stimulate *SAA*

production when provided with an inflammatory signal, like cytokines. As retinoic acid is known to control gene transcription through its interaction with the RAR transcription factors, these results suggest that the RARs could govern the up-regulation of *SAA* during an inflammatory response.

RAR CONTRIBUTIONS TO SAA INDUCTION IN HEPG2 CELLS

To address if RARs contribute to *SAA* induction, HepG2 cells were incubated with the IL-1β and IL-6 cytokines, in order to activate *SAA* expression. Initial studies indicated that *SAA* expression was induced after three hours of stimulation, in which cells were then washed with PBS and incubated with 1 μM of BMS493, a pan-RAR inhibitor. BMS493 inhibits all three RAR isoforms by physically competing for the binding pocket with retinoic acid, and in this way, it blocks other enhancers from binding to the DNA complex and activating gene transcription. After 6 hours, I observed a decrease in *SAA* expression; however, this effect is more dramatic after 24 hours incubation with BMS493, as cells completely lost all previously induced *SAA* expression (Fig. 22a,b). Analysis of *HOXA4*, a known RAR dependent gene, indicates that the BMS493 blocks RAR transcription, as its expression was also reduced in cells treated with the inhibitor (Fig. 22c,d).

SAA PROTEINS BIND TO RETINOIDS *IN VITRO*

As transcriptional control by retinoids is a hallmark of proteins involved in retinoid transport and metabolism, this motivated me to test for retinol binding activity of SAA proteins.

As massively up-regulated serum proteins, SAAs would be positively poised to function as a retinoid delivery system during the acute phase response. To investigate this idea, I developed a fluorometric retinol binding assay, which exploits the unique spectral properties of retinol, where the intrinsic fluorescence of retinol is enhanced upon binding to proteins through energy transfer from tryptophan residues, and this fluorescence change can be used to quantify binding [276] (Fig.24a). In this respect, retinol binding can be quantified by observing increases of fluorescence emission, following ligand titration into the system.

In my binding assays I used a variety of proteins including: recombinant human SAA1, a commercially available consensus SAA molecule corresponding to human apo-SAA1α except for the presence of an N-terminal methionine and substitution of asparagine for aspartic acid at position 60 and arginine for histidine at position 71, recombinant mouse SAA1 and recombinant mSAA3. I originally cloned mSAA1 and mSAA3 from mouse small intestinal cDNA and generated the proteins in an *E. coli* bacterial expression system, which allowed me to isolate large quantities of pure SAA protein in high concentrations. Unfortunately, I was not able to express and purify mSAA2 in this system. Using my fluorometric binding assay, I performed titrations with hSAA1, mSAA1, and mSAA3 proteins with the all-*trans* isomer of retinol (Fig. 25b). The titration curves I generated from these assays allowed me to produce linear least squares plots for each experiment and extract the apparent dissociation constants (K_ds) for each protein interaction with retinol (Fig. 25a) (a detailed description of the math equations used to generate these plots and K_ds can be found in Chapter 4). hSAA1 bound to retinol with a K_d of 259 nM, while mSAA1 and mSAA3 generated K_ds of 169 and 145 nM respectively (Fig. 25b) (Table 2). These values are similar to the 165 nM Kd I previously calculated for human RBP and retinol (Fig 27a), which functions to transport retinol in the serum at steady state and served

as a positive control in my assay. I additionally performed titrations of all-*trans* retinol with human ApoA1 and human transferrin (Trf), which are both known serum proteins that served as negative controls in my binding assays. Transferrin is an iron binding transport protein and has no affinity for retinol, while ApoA1 is the main component of HDL that SAA displaces during an acute phase response. As expected, hTrf and ApoA1 displayed no affinity for retinol (Fig 25b). Thus, these experiments demonstrated that SAAs bind retinol tightly with similar affinities as a known retinol binding protein.

All retinoids share a β-ionone ring with a polyunsaturated side chain, followed by an alcohol, carboxylic acid, or ester group, depending on the retinoid (Figure 23). Due to their similar structures, I wanted to investigate if other retinoid metabolites could also bind to SAAs with high affinity. As retinoic acid lacks intrinsic fluorescence, binding can be observed by monitoring the quenching of the inherent protein fluorescence due to energy transfer from tryptophan residues [276] (Fig 24b). This change in fluorescence can be quantified to determine the K_d of the binding interaction. I therefore measured retinoic acid binding using a modified fluorescence assay that monitored quenching of protein fluorescence (Fig. 26b). Titration of all-*trans* retinoic acid yielded K_ds of 268 and 224 nM for retinoic acid binding to hSAA1 and mSAA3, respectively (Fig. 26b) (Table 2), which are similar to binding affinities calculated for human RBP binding to retinoic acid [276] (Fig. 27b). There was weak binding of retinoic acid to mSAA1 and I was unable to calculate a K_d for the interaction (Fig. 26b) (Table 2). Thus, while hSAA1 and mSAA3 bind both retinol and retinoic acid, mSAA1 selectively binds retinol. This difference I observed between retinol and retinoic acid binding to mSAA1 gave some insight as to the flexibility of the SAA binding pocket and could possibly be due to electrostatic charge differences between retinol and retinoic acid.

To further investigate the ligand binding specificity within the SAA family, I chose to also test other retinoid ligands such as β-carotene, 4-keto retinol, retinyl acetate, and retinyl palmitate. Each of these ligands allowed me to determine if changes to the all-*trans* retinol structure dramatically impacted the ability of SAAs to bind, and allowed me to further evaluate the flexibility of the SAA binding pocket to determine how well it could accommodate more bulky retinoid ligands.

β-carotene is a predominant dietary form of pro-vitamin A that is taken up in the intestine following food in-take. β-carotene consists of two β-ionone rings connected by a polyunsaturated hydrocarbon chain that is split into two molecules of retinal within intestinal epithelial cells [92]. Structurally, β-carotene very closely resembles retinol, so I wanted to determine if SAAs could bind this pro-carotenoid as well. To address this, I utilized the tryptophan quenching assay I developed to test for retinoic acid binding. I tested hSAA1 and mSAA3 for binding and calculated K_ds of 347 nM and 159 nM respectfully, while mSAA1 demonstrated no binding to β-carotene (Fig. 28a) (Table 3).

4-keto retinol contains a double bond to oxygen on the β-ionone ring, making the head group on this retinoid more bulky than the all-*trans* isomer of retinol. This ligand allowed me to test the flexibility of the SAA binding pocket to accommodate more bulky ligands. hSAA1 showed tight binding to 4-keto retinol with a calculated K_d of 104 nM, indicating that this more bulky head group did not interfere with binding (Fig. 28b) (Table 3). mSAA3 displayed a very weak binding affinity at 2526 nM and mSAA1 displayed no binding affinity for 4-keto retinol (Fig. 28b) (Table 3).

Both retinyl acetate and retinyl palmitate are forms of vitamin A found in food products. As esterified versions of vitamin A, these ligands allowed me to determine if SAAs are capable of interacting with stored forms of retinol. Structurally, retinyl acetate is a natural form of vitamin A containing an acetate ester. As retinyl acetate closely resembles the structure of retinol, it was not surprising that all SAA proteins bound retinyl acetate to some degree (Fig. 28c). I calculated apparent K_ds of 497 nM, 769 nM, and 390 nM, for hSAA1, mSAA1, and mSAA3 respectively (Table 3). Retinyl palmitate is also an esterified form of vitamin A with a 14 carbon saturated fatty acid palmitate chain and is the main source of retinol found in vitamin A supplements. All SAA isoforms bound weakly to retinyl palmitate (Fig. 28d), however, I was not able to calculate K_ds for these interactions (Table 3). As long chain retinyl esters (such as retinyl palmitate) are the major form of stored retinoid in the liver [189], this suggests that SAAs are not involved in transporting the stored form of retinol. Collectively, these results expand our understanding of the flexibility of the SAA binding pocket and provide further insight as to the physiological ligand of SAAs.

CHOLESTEROL DOES NOT COMPETE WITH RETINOL FOR SAA BINDING

As SAA proteins concentrations increase in the serum during the acute phase, they replace ApoA1 as the major lipoprotein component on HDL. Because serum SAAs have been observed to circulate in association with HDL, a role for SAAs in cholesterol transport and metabolism has been previously proposed [189]. Therefore I wanted to determine if cholesterol competes with retinol for the same binding site on SAAs. To do this, I modified the tryptophan quenching assay I previously described. In this experiment, I incubated a saturating amount of

retinol with SAA and then added an excess concentration of cholesterol into the solution. As retinol binds SAA to saturation, I observed maximal quenching as expected. If cholesterol competed for the same binding site as retinol on SAA, excessive amounts of cholesterol should replace retinol in the binding site and result in a loss of quenching. However, cholesterol was unable to competitively inhibit retinol binding to SAAs (Fig. 29), suggesting that cholesterol is not an SAA ligand and arguing that retinoid binding to SAAs is not due to a non-specific affinity for hydrophobic ligands.

SAA IS ASSOCIATED WITH RETINOL *IN VIVO*

As my *in vitro* binding studies suggested that SAAs might associate with retinol *in vivo*, I sought to purify SAAs from tissues and assay for the presence of associated retinol. I began by trying to purify SAAs from small intestine tissue, however, this proved to be very difficult due to the large amounts of contaminating proteins, even under conditions where expression of SAAs was maximally induced. However, under inflammatory conditions, plasma SAA levels can reach concentrations of 1 mg/mL, constituting a high proportion of serum protein that I could use [285]. Thus, I was able to use size exclusion chromatography to recover a SAA-enriched fraction from the sera of mice infected intraperitoneally with *S. typhimurium* for 24 hours (Fig. 30).

I observed two distinct UV peaks from wild-type mouse serum from the size exclusion chromatography column (Fig. 30a). I analyzed fractions from each peak by SDS-PAGE and observed the banding patterns by Commassie staining. Fractions from peak 1, but not peak 2, contained bands that aligned with recombinant SAA protein (Fig. 30b). In parallel, I performed

Western blotting on the same fractions to identify SAA within each peak. As seen in Fig. 30c, most of the higher molecular weight bands observed by Commassie staining were positive for SAA by Western blot, indicating that SAA constitutes a majority of the protein found in peak 1. Additionally, I isolated non-SAA bands from peak 1 of wild-type mouse serum and performed mass spectrometry analysis that revealed the SAA-enriched protein fraction was devoid of other known retinol binding proteins (Fig. 30d). Therefore, any retinol I identify is likely associated with SAA protein in the peak.

After determining peak 1 to be the SAA-enriched peak, I combined fractions from peak 1 together and proceeded to isolate retinoids. To accomplish this, I used 1:1 ratio of acetonitrile:butanol solvent to precipitate out the protein fraction of my sample, allowing any organic compounds free access to the solution. I then proceeded to extract the organic layer of this solution using hexane, and dried down any extracted retinoids under nitrogen gas, which concentrated the sample. The sample was then re-suspended in 200 µL of acetonitrile and injected into the liquid chromatography tandem mass spectrometry (LC-MS/MS). LC-MS/MS is a highly sensitive technique that allowed me to detect picomolar amounts of retinoids from a complex sample. This is accomplished by ionizing the sample and passing it through a series of tandem mass spectrometry chambers separated by a collision chamber, where a specific mass can be selected and enriched; this is called the parent or precursor ion (Fig. 31). Due to its hydrophobic nature, ionization of retinol leads to a loss of water; hence for retinol the parent ion decreases from 286 to 269. Once the parent ion is selected, the ionized particles are accelerated into the collision chamber where they fragment due to sheer collision force. Each compound has a specific fragmentation pattern which acts as a molecular fingerprint to positively identify a compound. These fragments, referenced as daughter ions, are passed into the second mass

spectrometry chamber where they can be identified by the detector. As observed for retinol, the parent molecule of 269 fragments into daughter ions of 93, and this enabled me to track retinol within my complex serum samples.

Tracking daughter ion 93 by liquid chromatography tandem mass spectrometry (LC-MS/MS) indicated the presence of retinol in the SAA-enriched fraction from wild-type mice, which matched the elution time of the retinol standard (Fig. 32a). I further validated these findings by confirming retinol in the SAA-enriched serum by spectroscopy, as retinol has a characteristic display maximum of 325 nm (Fig. 33a). In order to confirm the presence of retinol in these mouse serum samples, I additionally extracted retinoids from unprocessed serum or the SAA-enriched serum fraction of wild-type mice infected with *S. typhimurium*. Again, I confirmed that retinol was detected in both samples by analyzing two daughter ions (93 and 119) (Fig. 33b,c). Daughter ion 119 is a less abundant fragment of retinol than daughter 93, but demonstrated that I was reliably detecting retinol in the samples. Interestingly, no retinoic acid was detected in these analyses, a further indication that retinol is the physiological ligand for SAA (Fig. 32b). I also did not detect retinol or retinoic acid after analyzing the equivalent serum fraction from SAA*1/2*$^{-/-}$ mice (Fig. 32a). I preformed stoichiometry calculations by quantifying retinol in the wild-type SAA-enriched serum samples and performed densitometry calculations of SAA concentrations from the same samples, revealing a molar ratio of ~1 mol retinol/4 mol SAA (Fig. 34). Together, these results support the idea that serum SAAs circulate with bound retinol during acute infection.

THE CRYSTAL STRUCTURE OF MOUSE SAA3 IS A TETRAMER WITH A CENTRAL PORE

Since my binding studies demonstrate that SAA proteins bind retinoids, this led to the question of how a small 12 kDa protein could protect a hydrophobic ligand from the aqueous environment it would encounter in the serum. While known retinol binding proteins have diverse primary sequences, they share conserved secondary fold features, including large β-barrels that come together to form pore-like channels [109, 286]. There is extensive structural data available for RBP, as it has been crystalized from a variety of species, and demonstrates a conserved β-barrel structure where retinol is believed to bind [109, 286]. However, as SAAs lack sequence homology to the two known families of retinol binding proteins: cellular retinol binding proteins (CRBP) and serum retinol binding proteins (RBP), the three-dimensional structures of these proteins provide no direct insight into the structural basis for retinol binding by SAAs [94, 109, 278, 286].

Previous analysis of SAA primary sequences revealed that SAAs are predicted to form amphipathic helices with a hydrophobic face that could interact with non-polar molecules [287]. The predicted, mostly alpha-helical, fold of SAA proteins is in contrast to the β-sheets prevalent in most retinol binding proteins. While the data generated from my fluorescence binding assays supported the idea that SAAs function as retinoid binding proteins, early secondary structural predictions suggested that SAAs were likely binding retinol in a novel way, unlike what had been previously described for other retinoid binding proteins.

To address this, a post-doc fellow in the Hooper Lab, Mehabaw Derebe, determined the three-dimensional structure of recombinant mSAA3 by X-ray crystallography and the structure

was determined to a resolution of 2 Å by single-wavelength anomalous dispersion (SAD) phasing. The crystal structure revealed that mSAA3 is highly α-helical (Fig. 35a), as predicted on the basis of its primary sequence, consisting of four α-helices forming two sets of antiparallel helices. He observed an extensive network of hydrogen bonding interactions among conserved residues as well as to water molecules in the interior of the monomer, which resulted in a stable, compact monomer.

As all SAA isoforms in both human and mouse share significant homology and have similar predicted secondary structures, suggesting that the three-dimensional structure of SAA3 would offer general insight into the SAA family. He analyzed the mSAA3 crystal structure using the Protein Interfaces, Surfaces and Assemblies (PDBePISA) server (http://www.ebi.ac.uk/msd-srv/prot_int/), which indicated a tetrameric quaternary structure. This was supported by size exclusion chromatography and cross-linking experiments that showed a tetrameric species in solution. This is in contrast to the recent hexameric structure derived for human SAA1.1 [288]. There are several potential reasons for the discrepancy in the oligomeric structures. First, it has been suggested that different SAAs can adopt different oligomeric states, as SAA1.1 was also observed to produce a ~43 kDa species in solution, suggesting that hSAA1.1 may in part adopt a tetrameric state [288-290]. Second, hSAA1.1 has a more hydrophobic N-terminus than mSAA3, which is thought to be a determinant of amyloidogenicity [288, 291]. Third, the crystallized hSAA1.1 protein retained the hexa-histidine tag, which may have contributed to the difference in oligomeric state [288].

Retinol is a highly apolar molecule consisting of a β-ionone ring, an isoprenoid tail, and a hydroxyl group. Structures of known retinol binding proteins, including serum RBP, indicate that these proteins consist mainly of β-sheet secondary structures forming a β-barrel tertiary

structure, with the retinol molecule held in an interior non-polar binding pocket [109]. In contrast, the mSAA3 tetramer is composed of α helices forming a hollow pocket lined with mostly hydrophobic amino acids that could serve as a binding pocket for a non-polar small molecule (Fig. 35b). A ligand docking analysis suggests that retinol can be docked in this hydrophobic pocket with favorable free energy. Consistent with this prediction, introduction of a Trp71Ala (W71A) mutation in the mSAA3 hydrophobic core reduced the affinity of mSAA3 for retinol (Fig. 36). Thus, the mSAA3 structure supports my biochemical data showing a retinol binding function for SAAs and explains how mSAA3 could bind retinol.

SAA CONTRIBUTES TO HOST IMMUNITY

As part of the acute phase response, SAA concentrations rise in the serum to over 1 mg/mL within 24 hours [15]. SAA proteins are also highly conserved in a large and diverse collection of vertebrates from mice, birds, fish, turtles, and even the invertebrate sea cucumber [18, 20, 21, 292]. Together, these observations imply that SAAs play an important and necessary role in host defense against infection. Collectively, the data I present here demonstrates that SAA proteins are novel retinol binding proteins and supports the idea that SAAs circulate with bound retinol, functioning as a transport mechanism for retinol during an acute infection. However, the physiological consequences of SAA transporting retinol during the immune response are unknown. In order to address this, I intraperitoneally infected wild-type or SAA*1/2*$^{-/-}$ mice with *S. typhimurium* and determined the bacterial counts in the liver and spleen. After 24 hours, SAA*1/2*$^{-/-}$ mice carried higher loads of bacteria in both their liver and spleen, compared to wild-type controls (Fig. 37a,b). This result supports the idea that SAA function contributes to some aspect of host immunity during infection. This also supports the previous observation that

SAA*1/2*$^{-/-}$ mice exhibit increased susceptibility to chemically-induced colitis [29], indicating that SAA may contribute to intestinal immunity as well.

DISCUSSION

Retinol is derived from dietary vitamin A and is essential for the physiological response to infection. By regulating gene transcription programs in cells, retinoids are broadly required for many immune responses to infectious challenge [127, 293]. As a small lipophilic compound, retinol does not freely circulate, but is instead transported among cells and tissues by retinol binding proteins. However, little is known about the mechanisms by which retinol is transported and delivered to sites of immune cell differentiation and development during infection.

Based on my research, I propose that SAAs function in part to transport retinol following systemic infection or bacterial colonization of mucosal sites. Because expression of SAAs is strongly induced by microbial exposure, this suggests that SAAs may function to deliver retinol from sites of uptake (intestine) and storage (liver) to sites of immune cell differentiation, although, further work will be required to identify the tissue targets of circulating retinol-bound SAAs. In support of this idea, there is evidence that SAAs contribute to host immunity. First, *Saa1/2*$^{-/-}$ mice exhibit increased susceptibility to chemically-induced colitis in mice, suggesting that SAAs contribute to intestinal immunity [29]. Second, studies in zebra fish show that commensal microbiota stimulate neutrophil migration through induction of SAA [294]. Third, my experiment observing bacterial loads following intraperitoneal infection of *Saa1/2*$^{-/-}$ mice with *S. typhimurium* results in higher bacterial loads in liver and spleen as compared to wild-type mice (Fig. 36a,b), suggesting that SAAs also contribute to systemic immunity.

Altogether, these results provide insight into the biological function of SAAs, uncover a unique protein architecture involved in retinol binding, and reveal new information about the physiological response to infection. These findings may help in designing new strategies for enhancing immunity to infection and/or controlling inflammation during disease.

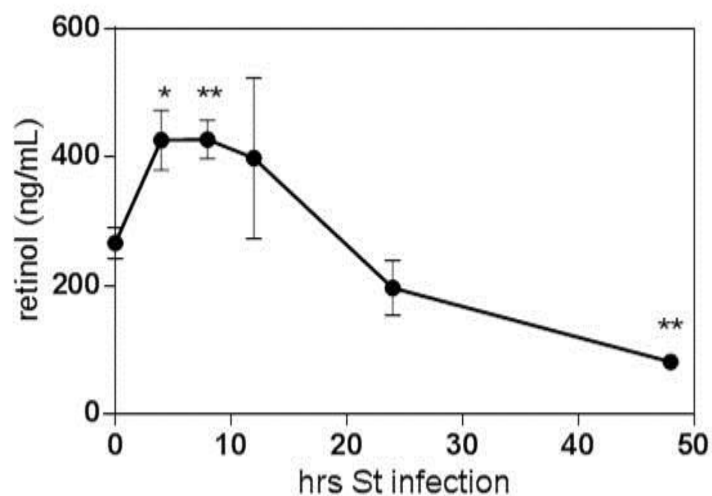


Figure 16: Serum retinol quantification time course in wild-type mice. Serum was analyzed from mice at varying times following intraperitoneal *S. typhimurium* infection and processed as described in Chapter 4. Retinol was quantified by tracking daughter ion 93 by LC-MS/MS. Data are graphed as SEM with n=3-6 mice per time point. * <.05, ** <.01 by a two-tailed Student's *t* test (compared to the un-infected starting time point).

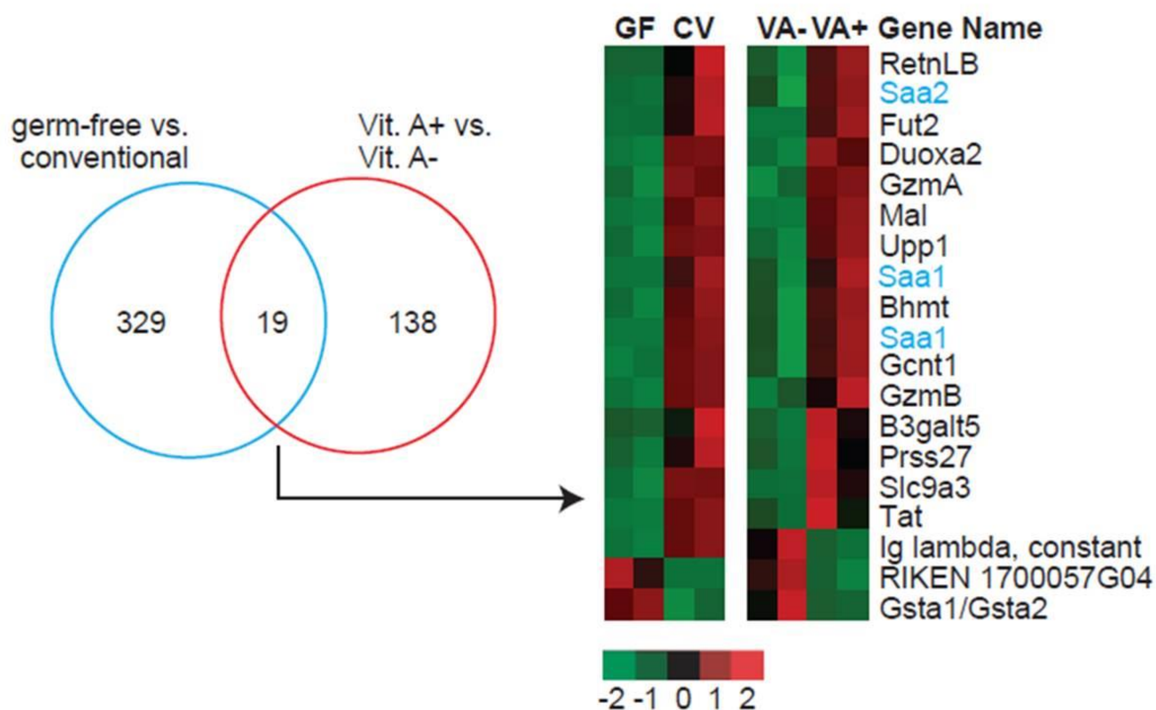


Figure 17: Intestinal Saa1 and Saa2 are differentially regulated by the microbiota and dietary vitamin A. Affymetrix Mouse Genome 430 2.0 arrays were used to compare transcript abundance in small intestines from germ-free (gf) and conventional (cv) mice, and from mice fed a normal diet versus a vitamin A-deficient diet. Differentially expressed transcripts were identified as outlined in Methods, revealing 329 differentially-expressed genes between the germ-free and conventional groups, and 138 differentially-expressed genes between the vitamin A+ and vitamin A- groups. A Venn diagram representation of the experimental results is shown at left. 19 genes were differentially expressed in both comparisons and are displayed as a heat map in which expression level is defined by Z-score (defined in Chapter 4). Saa1 and 2 are highlighted in blue.

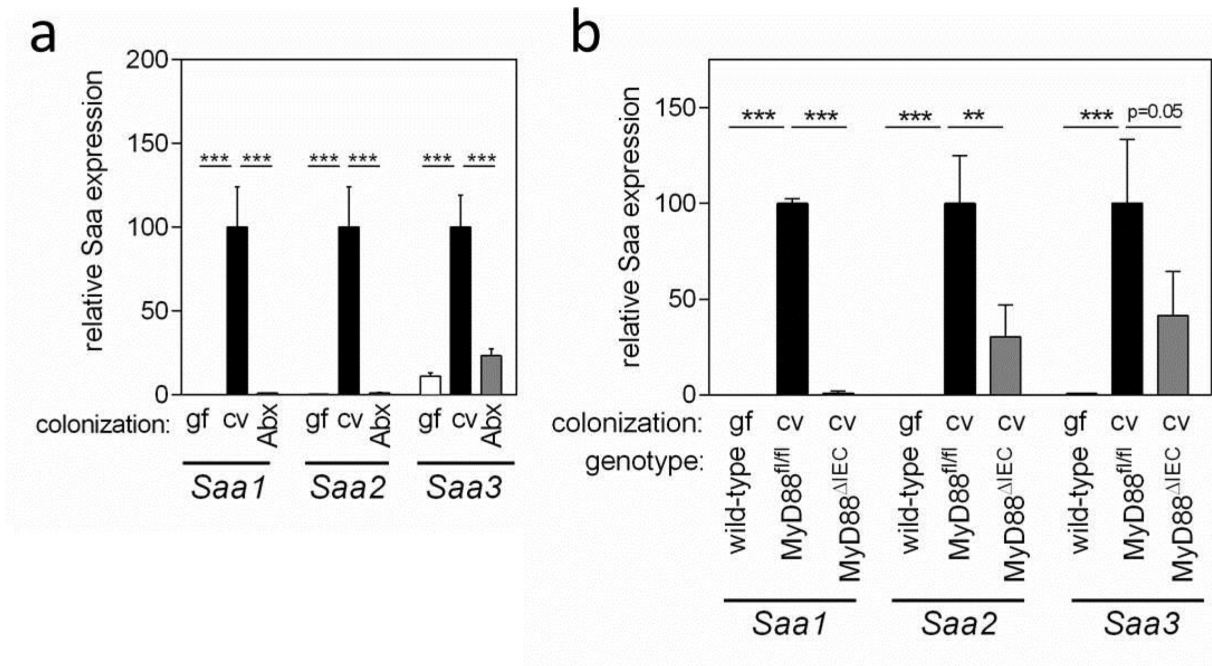


Figure 18: SAA induction requires bacteria and epithelial cell MyD88. **(a)** Q-PCR analysis of ileal *Saa* expression in germ-free (gf), conventional (cv), or antibiotic-treated (Abx) mice. N=5 mice per condition. **(b)** Q-PCR determination of ileal *Saa* expression in germ-free wild-type, conventional $MyD88^{fl/fl}$, or conventional $MyD88^{\Delta IEC}$ mice. N=3-5 mice/genotype. Mean±SEM is plotted. nd, not detected. *, p<0.05; **, p<0.01; ***, p<0.001. *P* values were determined by two-tailed Student's *t*-test.

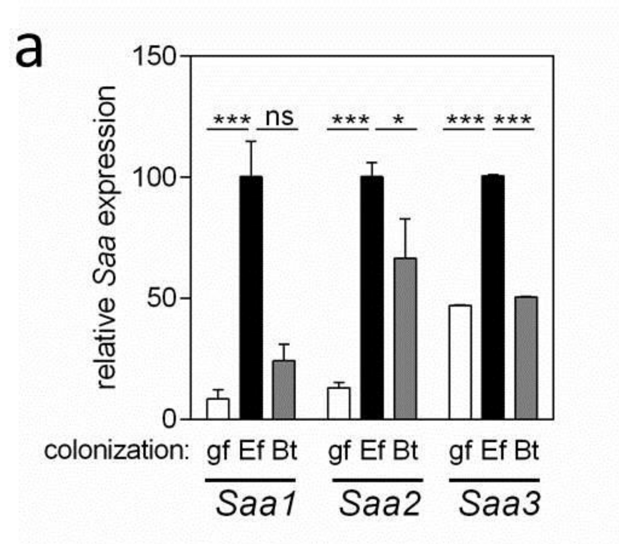


Figure 19: Gram negative and Gram positive bacteria induce SAA expression in the small intestine. **(a)** Q-PCR analysis of ileal *Saa* expression in germ-free (gf), or germ-free mice mono-colonized with *B. theta* or *E. faecalis*. Mean±SEM is plotted. ns, not significant. *, p<0.05; ***, p<0.001. *P* values were determined by two-tailed Student's *t*-test.

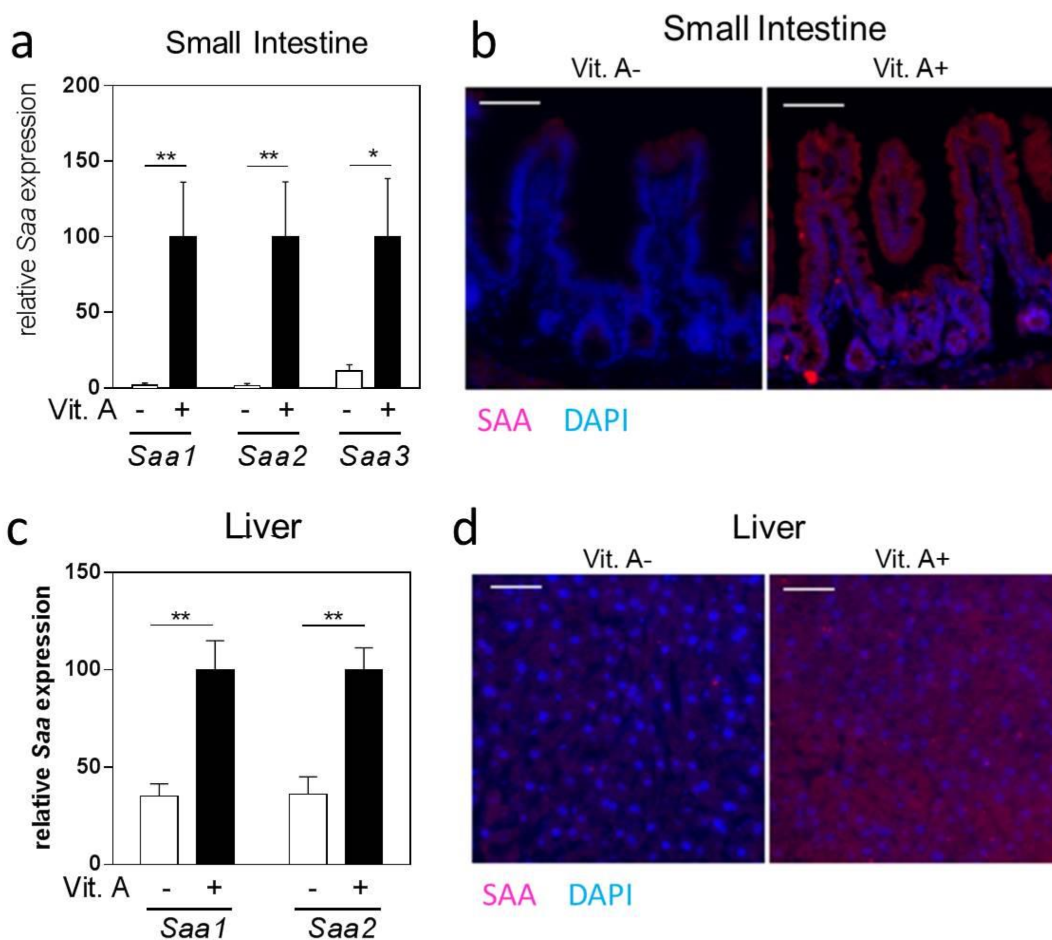


Figure 20: SAA expression requires dietary vitamin A. **(a)** Mice were maintained on a normal (Vit. A+) diet or a vitamin A-deficient (Vit. A-) diet. Ileal *Saa* expression was quantified by Q-PCR. N=3-5 mice per condition. **(b)** Ileal sections were stained with anti-SAA antibody (see Methods) and anti-rabbit IgG-Cy3 (red). Tissues were counterstained with DAPI (blue). Scale bar=50 µm. **(c)** Q-PCR determination of *Saa* expression levels in livers of mice on a normal or vitamin A-deficient diet. N=5 mice/condition. **(d)** Liver sections were stained with anti-SAA antibody and anti-rabbit IgG-Cy3 (red), and counterstained with DAPI (blue). Scale bars=50 µm. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. *P* values were determined by two-tailed Student's *t*-test.

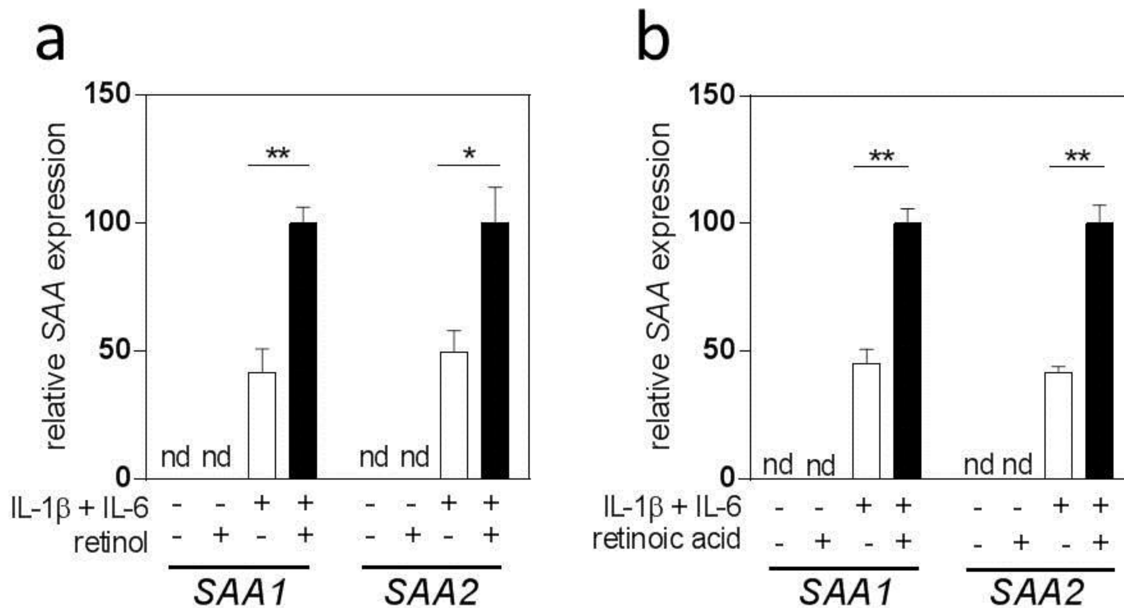


Figure 21: Analysis of SAA expression in HepG2 cells. HepG2 cells were analyzed for their ability to up-regulate SAAs following cytokine stimulation. Cells were cultured in retinoid-free medium and then treated with IL-1β and IL-6 and/or 1 μM retinol **(a)** or 100 nM retinoic acid **(b)**. *SAA* expression was determined by Q-PCR. A representative experiment is plotted with n=3 independent experiments. Mean±SEM is plotted. nd, not detected. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$. *P* values were determined by two-tailed Student's *t*-test.

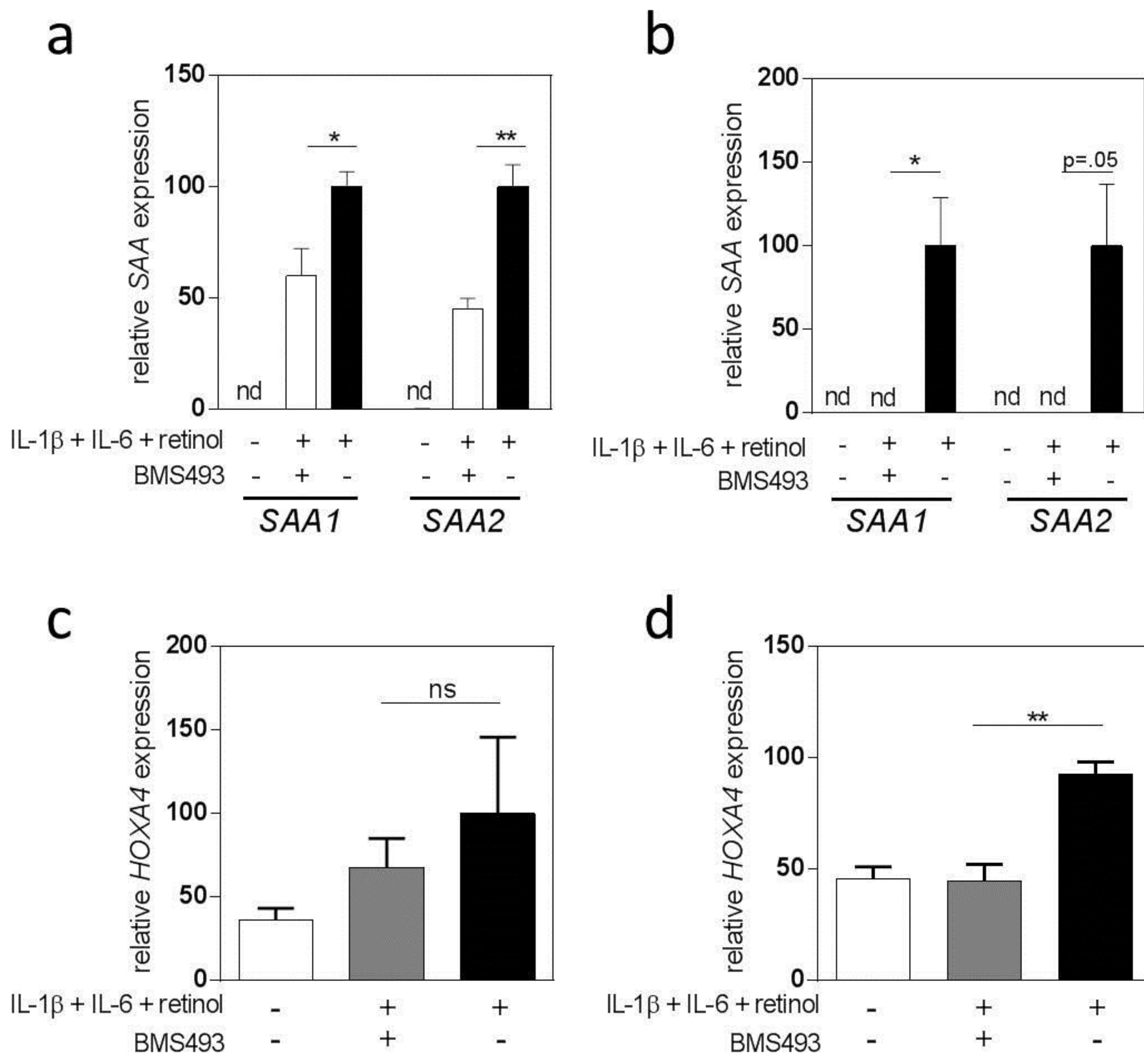


Figure 22: An RAR inhibitor suppresses SAA expression in HepG2 cells. Analysis of SAA expression in HepG2 cells. Cells were cultured in retinoid-free medium and then treated with IL-1β and IL-6 and 1 μM retinol for 3 hours before incubation with 1 uM BMS493, an RAR inhibitor for 6 **(a,c)** or 24 hours **(b,d)**. *SAA* **(a,b)** or *HOXA4* **(c,d)** expression was determined by Q-PCR. A representative experiment is plotted, with n=2 independent experiments. Mean±SEM is plotted. nd, not detected. *, $p<0.05$; **, $p<0.01$. *P* values were determined by a two-tailed Student's *t*-test.

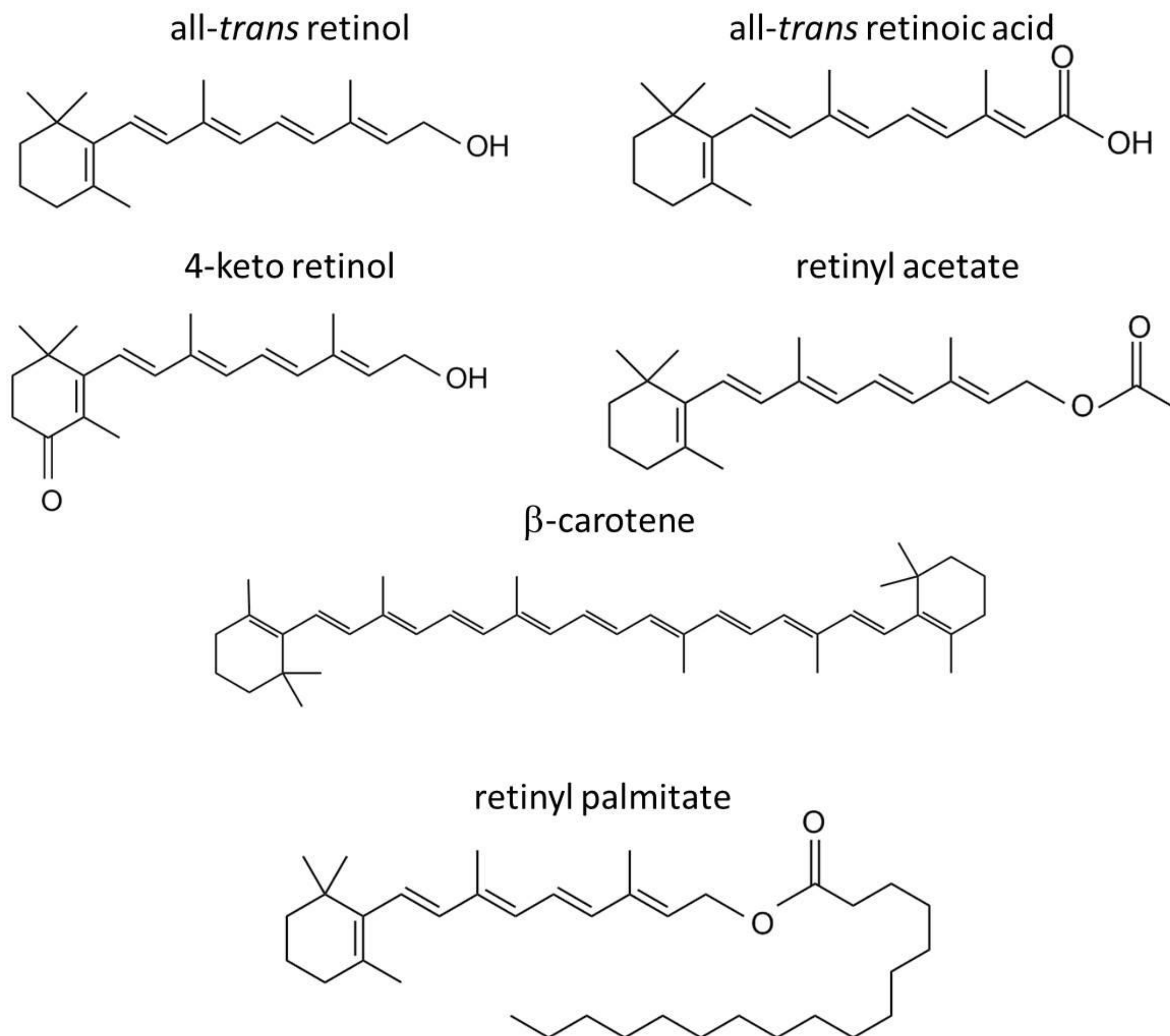


Figure 23: Structures of Retinoids. Structures of retinol, retinoic acid, 4-keto retinol, retinyl acetate, β-carotene, and retinyl palmitate are pictured above.

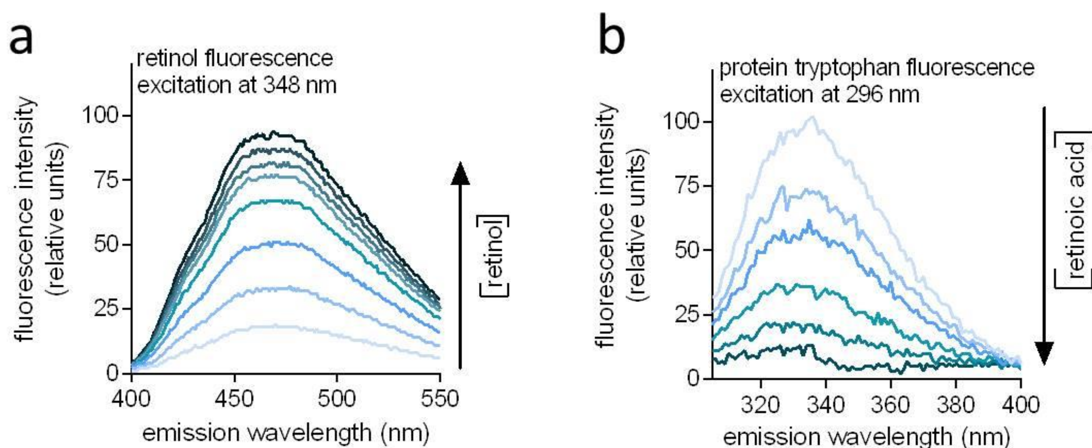


Figure 24: Fluorescence emission output following protein binding to retinol and retinoic acid. **(a)** Retinol exhibits intrinsic fluorescence that is enhanced upon binding to proteins through energy transfer from tryptophan residues, and this fluorescence change can be used to quantify binding. All-*trans*-retinol was titrated into mSAA3 and fluorescence emission was monitored following excitation at 348 nm. **(b)** Retinoic acid lacks intrinsic fluorescence but can quench intrinsic protein fluorescence due to energy transfer from tryptophan residues [276]. All-*trans*-retinoic acid was titrated into mSAA3 and fluorescence quenching was monitored following excitation at 296 nm.

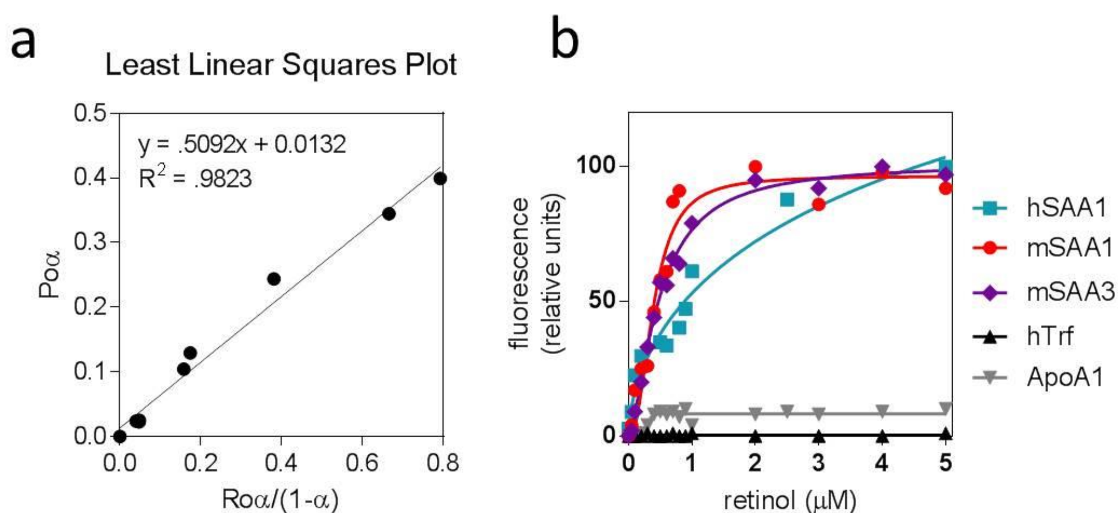


Figure 25: Human and mouse SAAs bind retinol. **(a)** Representative least linear squares plot of mSAA1 binding to retinol. The slope of this line is used to calculate the apparent dissociation constant for each experiment. **(b)** All-*trans*-retinol was titrated into hSAA1, mSAA1, mSAA3, human transferrin (hTfr; negative control), and apolipoprotein A1 (ApoA1; negative control). Binding was quantified by monitoring retinol fluorescence at 460 nm following excitation at 348 nm as in Figure 24a. Plots are representative of 5 independent experiments.

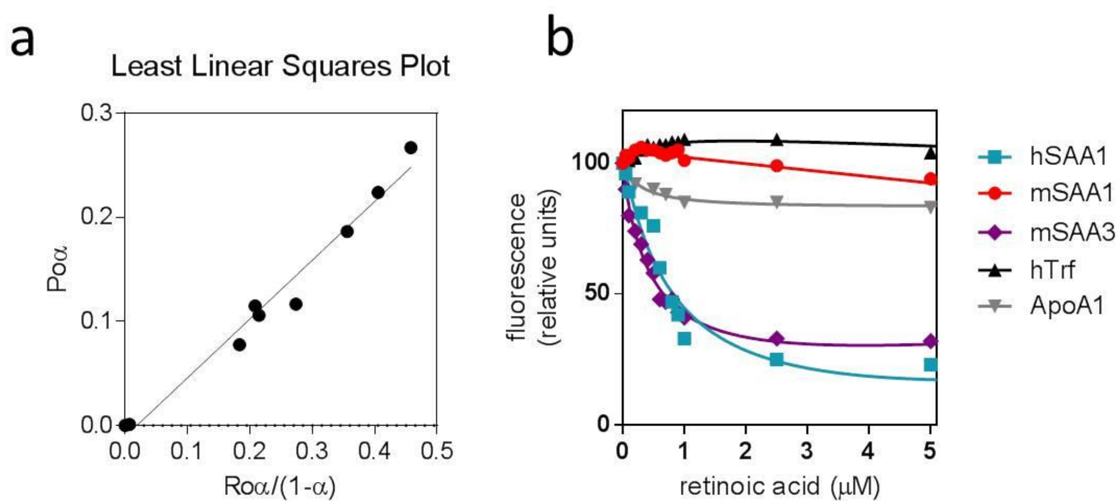


Figure 26: Human and mouse SAAs bind retinoic acid. (a) Representative least linear squares plot of mSAA3 binding to retinoic acid. **(b)** The slope of this line is used to calculate the apparent dissociation constant for each experiment. All-*trans*-retinoic acid was titrated into hSAA1, mSAA1, mSAA3, hTfr, and ApoA1. Fluorescence emission was monitored at 334 nm with excitation at 296 nm as in Figure 24b. Plots are representative of 3 independent experiments.

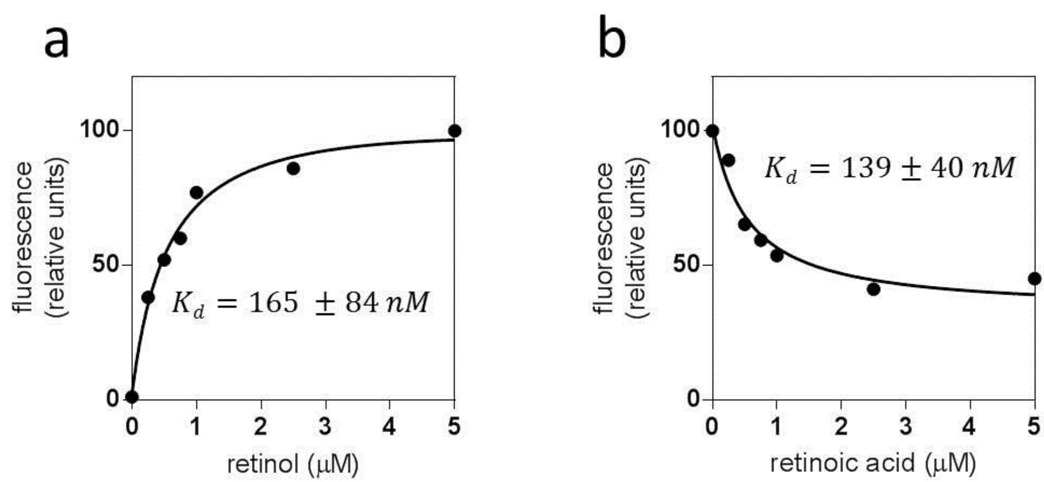


Figure 27: Retinol and retinoic acid binding to human retinol binding protein 4 (hRBP4). For comparison, I calculated binding affinities of retinol and retinoic acid to hRBP4 using the fluorescence binding assays described in Figure 24. The results are consistent with published values [276] and are similar to the binding affinities calculated for human and mouse SAAs in Figure 25 and Figure 26.

protein	K_d for Retinol binding (nM)	K_d for Retinoic acid binding (nM)
hSAA1	259 ± 132	220 ± 69
mSAA1	169 ± 42	nd
mSAA3	145 ± 73	269 ± 128

Table 2: SAA apparent dissociation constants for retinol and retinoic acid. K_ds were calculated from the binding assay data plotted in Figure 25 and Figure 26 and were derived from 3-5 independent experiments. nd, not determined.

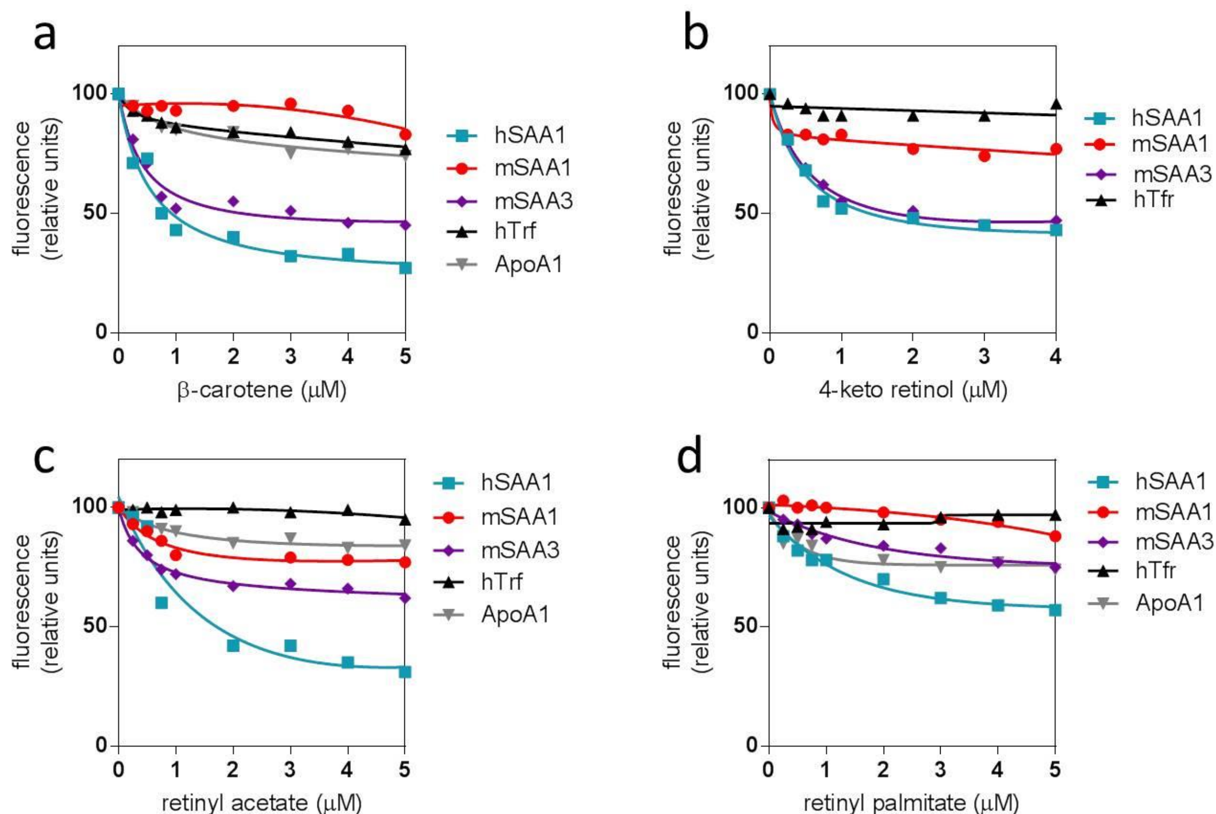


Figure 28: Ligand binding studies on human and mouse SAAs. (a-d) β-carotene **(a)**, 4-keto retinol **(b)**, retinyl acetate **(c)**, and retinyl palmitate **(d)** were titrated into hSAA1, mSAA1, mSAA3, hTfr (negative control) and ApoA1 (negative control), and fluorescence quenching was monitored at 334 nm with excitation at 296 nm. Plots are representative of 3 independent experiments. The K_ds are averages of the values derived from the 3 experiments.

protein	K_d for β-carotene (nM)	K_d for 4-keto retinol (nM)	K_d for retinyl acetate (nM)	K_d for retinyl palmitate (nM)
hSAA1	347 $\pm$ 214	104 $\pm$ 49	497 $\pm$ 264	nd
mSAA1	nd	nd	769 $\pm$ 166	nd
mSAA3	159 $\pm$ 38	2526 $\pm$ 2003	390 $\pm$ 299	nd

Table 3: Apparent dissociation constants for SAA proteins binding with retinoid ligands. The K_ds were calculated from the binding assay data plotted in Figure 27 a,b,c,d and are averages of the values derived from the 3 experiments. nd, not determined.

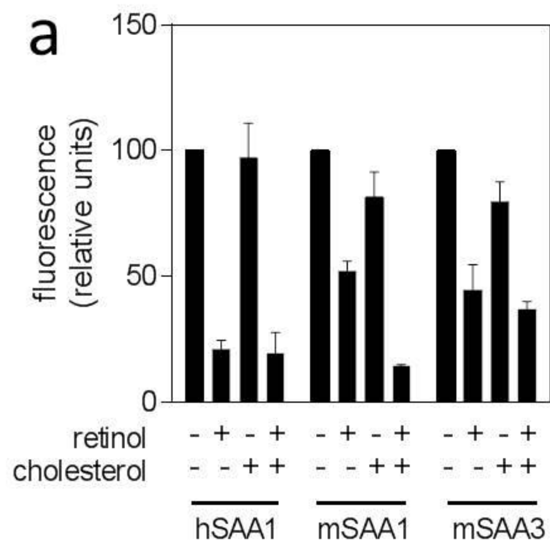
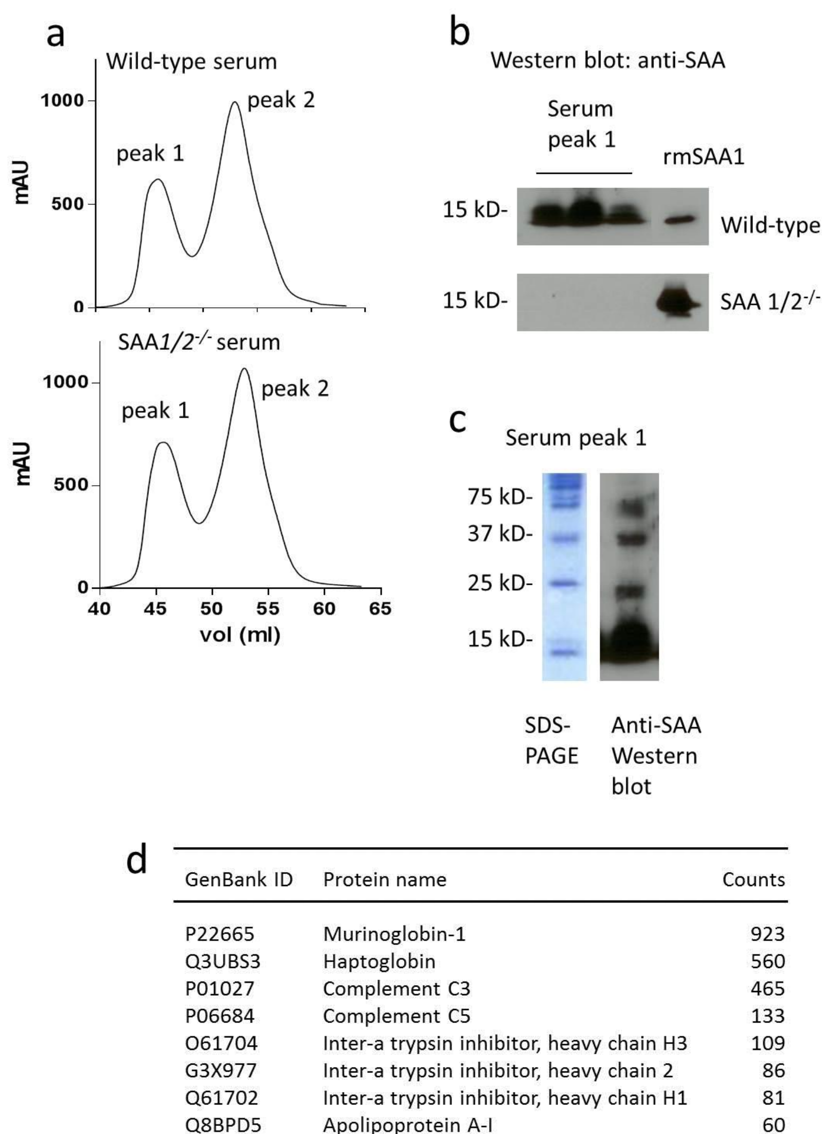


Figure 29: Cholesterol does not compete with retinol for SAA binding. **(a)** Cholesterol binding was assessed by monitoring competitive inhibition of retinol binding. Saturating concentrations of retinol were added to hSAA1, mSAA1, and mSAA3, and fluorescence quenching was monitored as in Figure 24b. 10 μM cholesterol was added into the assay and inhibition of fluorescence quenching by retinol was monitored. Values are the average±SEM of triplicate experiments.



GenBank ID	Protein name	Counts
P22665	Murinoglobin-1	923
Q3UBS3	Haptoglobin	560
P01027	Complement C3	465
P06684	Complement C5	133
O61704	Inter-a trypsin inhibitor, heavy chain H3	109
G3X977	Inter-a trypsin inhibitor, heavy chain 2	86
Q61702	Inter-a trypsin inhibitor, heavy chain H1	81
Q8BPD5	Apolipoprotein A-I	60

Figure 30: Size-exclusion chromatography and mass spectrometry analysis of SAA-containing serum fractions. (a) Wild-type and *Saa1/2*$^{-/-}$ mice were challenged intraperitoneally with *S. typhimurium* and serum was collected 24 hours later. The serum was concentrated and the proteins were fractionated into two major peaks by size exclusion chromatography on a Superdex 75 HiLoad 16/60 column. **(b)** SAA was detected in peak 1 by Western blot. **(c)** SDS-PAGE and Western blot analysis of serum peak 1. SDS-PAGE reveals four major protein bands that correspond to SAA bands as determined by Western blot. **(d)** Mass spectrometry analysis was performed to identify other proteins in serum peak 1. No other retinoid binding proteins were detected in the peak.

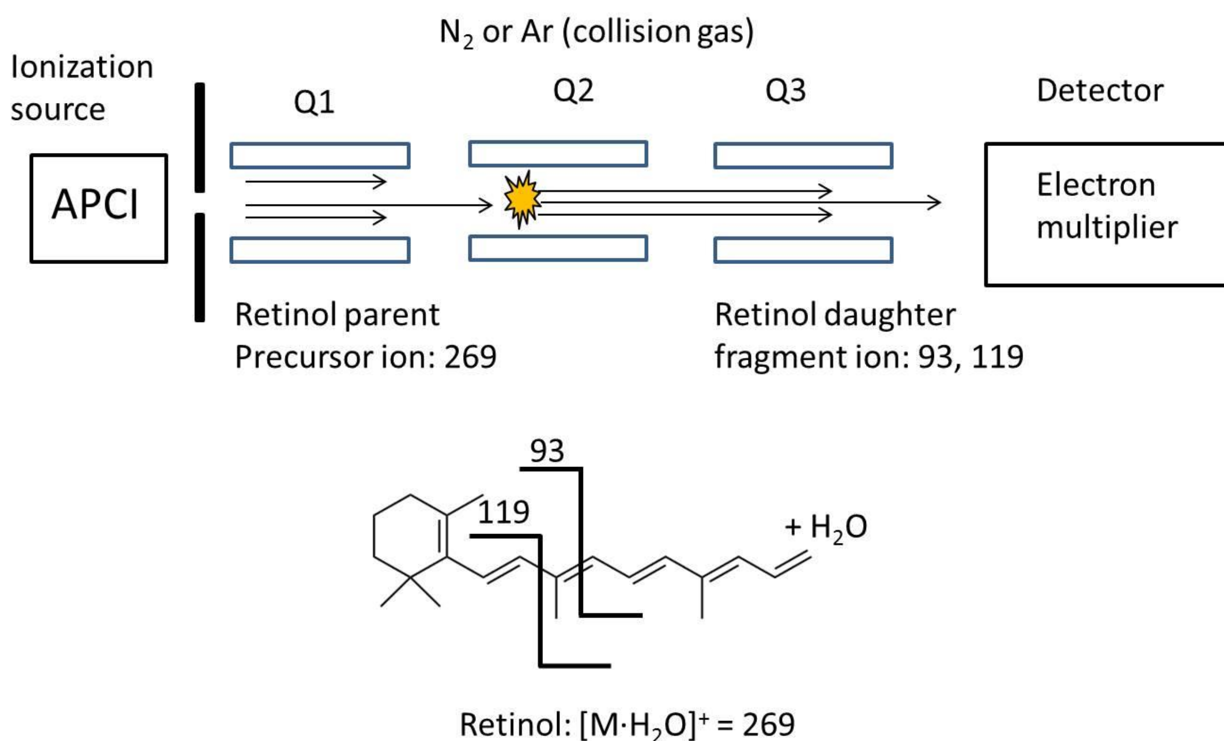


Figure 31: Schematic of LC-MS/MS and retinol fragmentation. Parent compounds are identified in the Q1 chamber and accelerated in the Q2 collision chamber. Within the Q2 chamber, molecules are fragmented in specific patterns, which can be reliably tracked to identify a compound within a complex sample. The parent compound of retinol has a molecular mass of 286, however, the initial ionization of retinol leads to the loss of a single water molecule, rendering a molecular mass of 269, which is designated as the precursor ion. The two highest abundant fragment ions of retinol have molecular masses of 93 and 119, pictured above, and are used to identify retinol within serum samples.

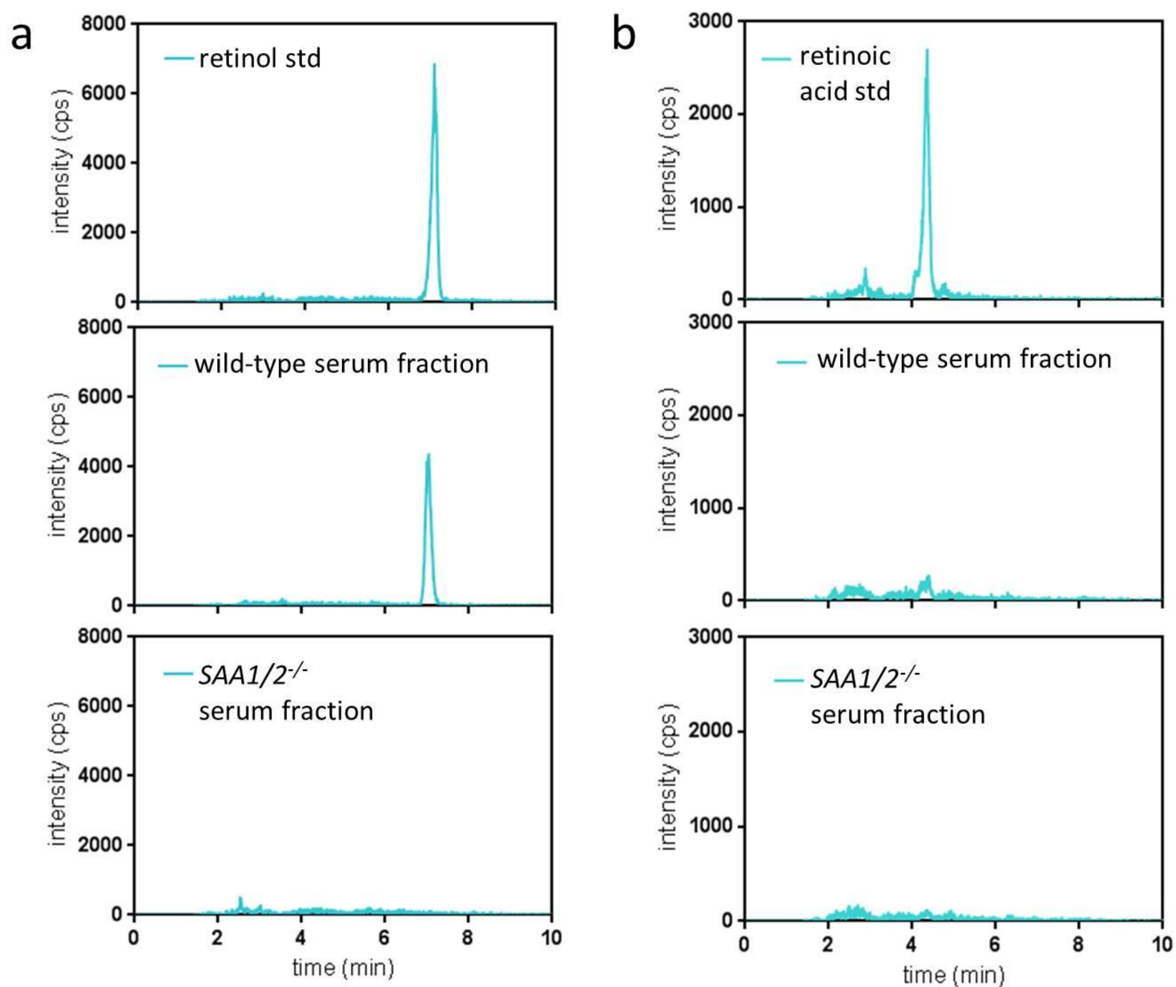
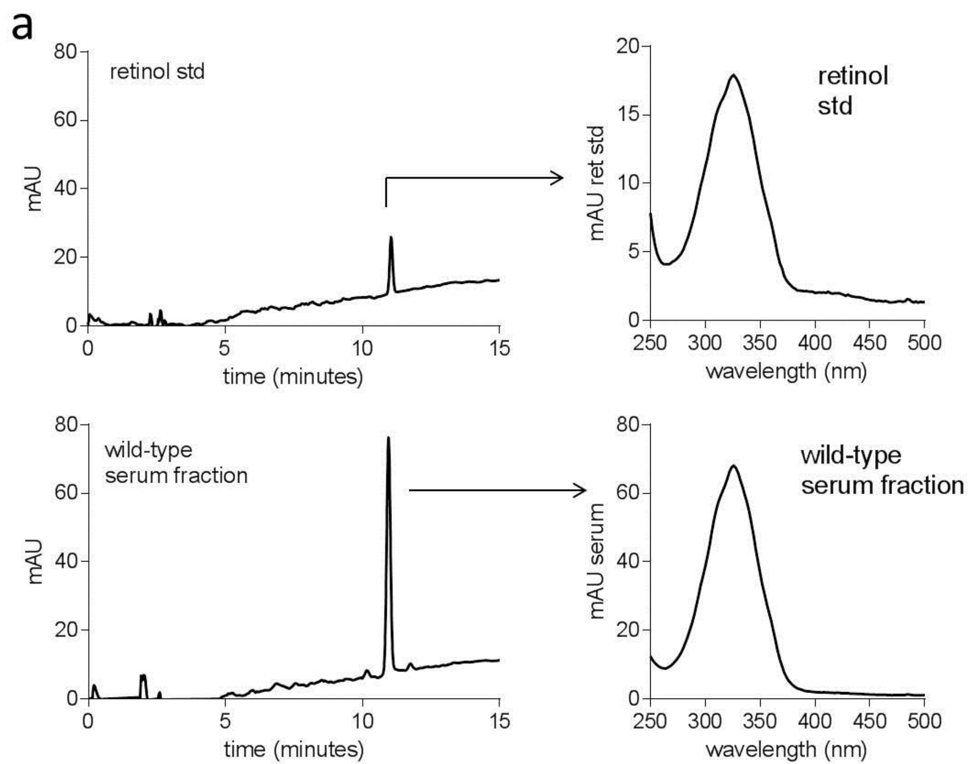


Figure 32: Serum SAA is associated with retinol *in vivo*. Wild-type or *Saa1/2*$^{-/-}$ mice were infected intraperitoneally with *S. typhimurium* and serum was collected 24 hours later. The serum was fractionated by size exclusion chromatography and a major SAA-containing fraction from wild-type mice was identified by Western blot (Figure 30). The SAA-containing fraction and the equivalent serum fraction from *Saa1/2*$^{-/-}$ mice were hexane-extracted and analyzed by LC-MS/MS against retinol (a) and retinoic acid (b) standards. The LC-MS/MS chromatograms of daughter ion 93 are shown.


a
retinol std
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time (minutes)
mAU ret std
retinol
std
wavelength (nm)
wild-type
serum fraction
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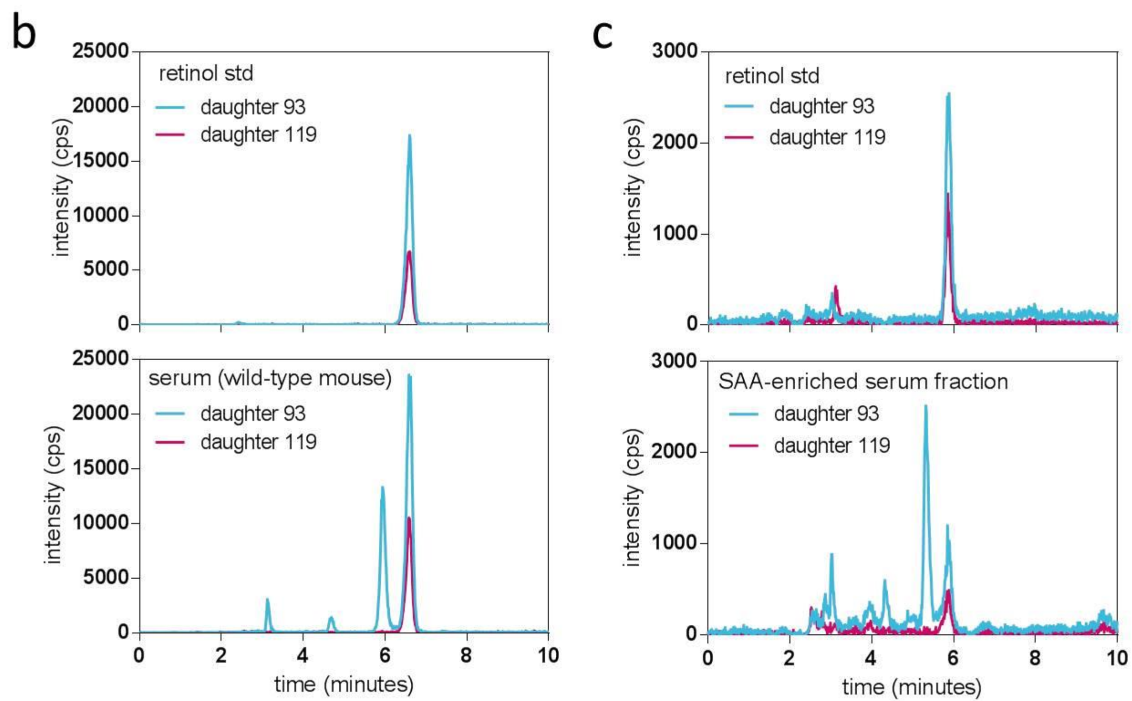
b
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SAA-enriched serum fraction
time (minutes)

Figure 33: Mouse SAA is associated with retinol in the serum following infection. (a-c) Wild-type mice were infected by intraperitoneal delivery of *S. typhimurium* and serum was collected and processed for retinoids as described in Figure 31. **(a)** LC-MS profiles of retinol standard and the SAA-enriched fraction from wild-type mouse serum, prepared as described in Figure 29. The retinol peaks were further analyzed by spectroscopy and display maxima at 325 nm, which is characteristic of retinol. **(b,c)** Retinoids were extracted from unprocessed serum **(b)** or from the SAA-enriched serum fraction **(c)** of *S. typhimurium* infected wild-type mice and analyzed by LC-MS/MS. Retinol was detected by analyzing two daughter ions (93, 119). I noticed a modest decrease in the elution time of retinol between the experiments shown in (b) and (c); however, this difference was observed in both the experimental sample and the retinol standard.

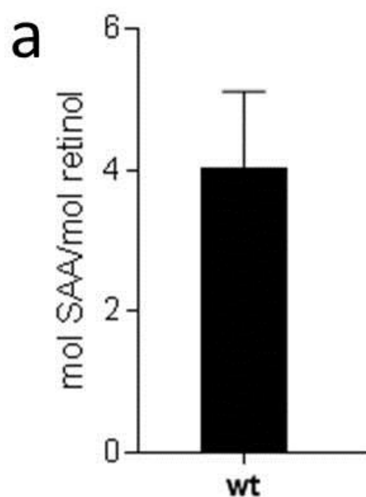

Figure 34: Stoichiometry of SAA and retinol in SAA-enriched serum fraction. **(a)** Total serum retinol was calculated based on peak areas from the mass spectrometer analysis in wild-type SAA-enriched samples compared to a retinol standard curve. Serum SAA was quantified by Western blot analysis with anti-SAA antiserum and densitometry. mol SAA/mol retinol is shown above. Data are representative of duplicate experiments with triplicate samples in each experiment.

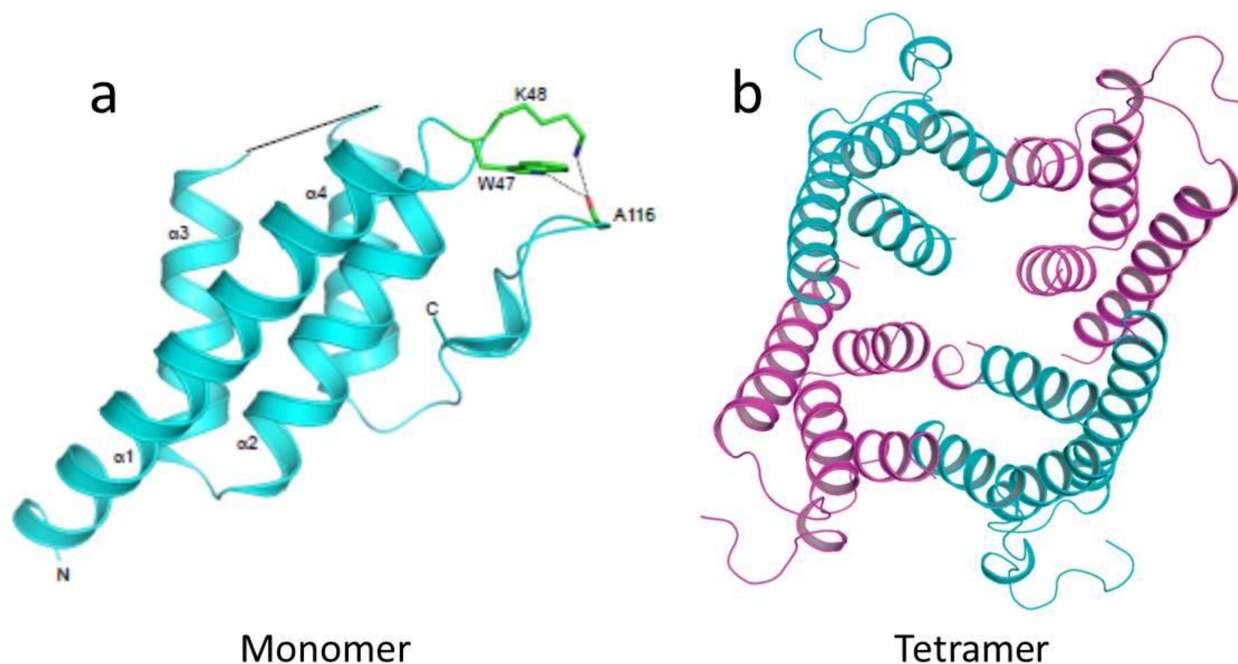

Figure 35: mSAA3 crystal structure reveals a tetramer. **(a)** Structure of the mSAA3 monomer (side view), with helices and termini labeled. **(b)** Top view of the tetrameric mSAA3 structure. Chains forming dimer pairs are colored cyan and magenta. Helices pointing inward are lined with hydrophobic residues, forming a hollow pocket that can accommodate a single molecule of retinol. These data were generated by Mehabaw Derebe in the Hooper Lab.

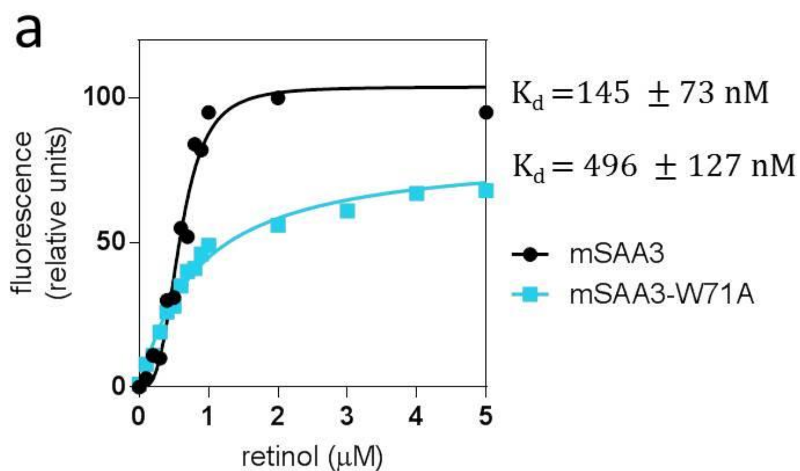

Figure 36: Mutation within the hydrophobic core reduces retinol binding for mSAA3. (a) Wild-type or Trp71Ala (W71A) mutant mSAA3 was assayed for retinol binding as described in Figure 24a. Representative plots and K_ds were calculated from the binding assay data and were derived from 5 independent experiments.

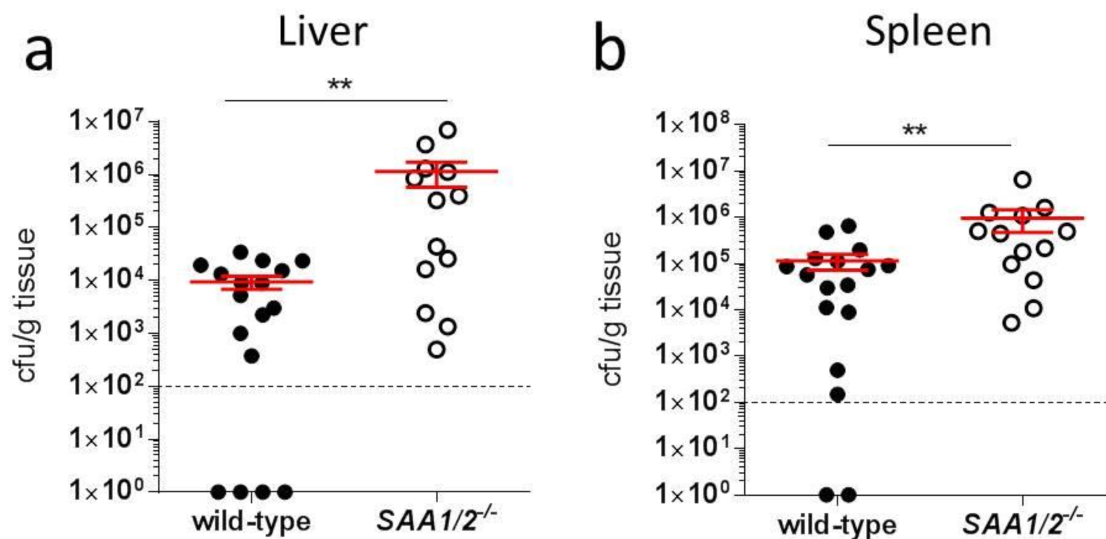

Figure 37: *Saa1/2*$^{-/-}$ mice have higher bacterial burdens following *S. typhimurium* infection. 10 week old wild-type and *Saa1/2*$^{-/-}$ mice were inoculated with 10,000 cfu of *S. typhimurium*. Livers **(a)** and spleens **(b)** were collected after 24 hours and analyzed for bacterial counts by dilution plating. Combined results from two independent experiments are shown. Means±SEM are plotted. Dotted line indicates limit of detection. **, p<0.01 using the Mann-Whitney test.

CHAPTER SIX

CONCLUSIONS

INTRODUCTION

Dietary metabolites, such as vitamin A, are essential for maintaining proper host health and immune responses to infection. Studies on vitamin A from the 1970-1980s identified and characterized the enzymatic reactions and key retinoid binding proteins that are necessary for host survival. After a decade of research, interest in vitamin A dwindled, as retinol was thought to only be important for vision and embryonic development. However, within the last decade, vitamin A has experienced a revival as a significant research topic as scientists began to discover its importance to both innate and adaptive immunity.

Despite a renewed interest in vitamin A and its links to immunity, very little is known about its metabolism and transport during an immune response. This is especially interesting in the light of the requirement for vitamin A in activating and maintaining proper immune functions. Previous observations have noted a down-regulation of RBP, the serum vitamin A transport protein, during the acute phase response. This has major downstream consequences, as cells populating the immune system do not contain retinyl ester stores and therefore completely rely on delivery of serum retinol for proper function. If RBP is unavailable to transport retinol to vital tissues fighting infection, the question remains: how do these tissues acquire their vitamin A? As RBP concentrations become limiting during the acute phase response, it is possible that unknown retinoid binding proteins act to transport retinol during the acute phase.

The overarching goal of this thesis was to identify a potentially new retinoid binding protein responsible for transporting retinol during infection. After identifying a potential candidate I wanted to determine the signals necessary for its induction, determine if it could

bind retinoids *in vitro* and *in vivo*, and finally to begin understanding its impact on host immunity. Here, I have presented evidence that SAA functions as a novel retinol binding protein induced during an immune challenge.

SAA REQUIRES BACTERIA AND VITAMIN A FOR EXPRESSION

SAAs have long been classified as acute phase proteins produced by hepatocytes following infection or inflammation. However, expression of SAAs is not limited to the liver, as the colon, small intestine, and adipose tissue have all been shown to express *Saa* transcripts as well [15, 29, 37]. Prior studies in the small intestine demonstrated that *Saa* induction required bacteria, and my results support an epithelial cell intrinsic MyD88-dependent mechanism for *Saa* expression. The requirement for bacteria is not dependent on the presence of an individual bacteria species, as both *B. thetaiotaomicron* and *E. faecalis* were capable of inducing *Saa* expression in the small intestine. In this way, SAA is another component of the MyD88 gene transcription program that is induced by bacterial signals in the gut.

Interestingly, my studies utilizing microarray data on mice fed vitamin A replete or deficient diet demonstrated that SAA expression is also controlled by dietary vitamin A. I further confirmed that vitamin A is required for *Saa* expression in the small intestine by Q-PCR and immunofluorescence analysis of mice fed a vitamin A replete diet compared to mice fed a vitamin A deficient diet. In addition to the small intestine, liver expression of *Saa* is also dependent on vitamin A, as livers of vitamin A deficient mice have reduced *Saa* expression. In this way, *Saa* expression in multiple tissues requires vitamin A. Furthermore, I helped to establish a HepG2 cell culture system that verified my Q-PCR results that retinol, as well as

retinoic acid, induces *SAA* expression in presence of cytokine signals. As both retinol and retinoic acid induced *SAA* expression in HepG2 cells (Fig. 21), it is likely that retinol is being converted into retinoic acid for gene transcription, as epithelial cells and hepatocytes both contain the enzymatic machinery for retinoic acid conversion. While this result suggests that RARs participate in *SAA* induction, it is still unclear if this is a direct effect of retinoids on the *SAA* promoter, or an indirect effect, in which another RAR-dependent gene in turn induces *SAA* expression. In accordance with this idea, my *in vivo* results demonstrating that *Saa* expression requires vitamin A also cannot determine if this observation is due to a direct effect from retinoids. During vitamin A deficiency, many innate lymphoid cells have compromised or non-functioning effector functions in the periphery. As cytokines are known to up-regulate *Saa* expression in other tissues, like the liver, I cannot rule out the possibility that cytokines may also play a role in regulating *Saa* expression in the small intestine. To my knowledge, there have been no reports discussing if cytokine levels are altered during vitamin A deficiency. Given that many aspects of the immune response are compromised during vitamin A deficiency, it stands to reason that cytokine levels may also be compromised or reduced. If that is the case, this idea could provide an alternative explanation as to why *Saa* expression in the small intestine is reduced during vitamin A deficiency. Collectively, these findings reveal that SAAs are microbe-induced proteins that are a novel set of vitamin A dependent proteins that likely regulate host immune responses.

SAA BINDS RETINOIDS *IN VITRO*

Previous studies have suggested many different functions for SAA, including modulation of cholesterol transport and amplification of inflammatory responses. Despite having identified SAA proteins over 40 years ago, these proposed explanations fail to unify all the observed characteristics of SAAs. My studies present evidence that SAAs belong to a novel class of retinoid binding proteins that function during the acute phase response. In fluorescence based binding assays, I demonstrated that human and mouse isoforms of SAA bind to all-*trans* retinol and all-*trans* retinoic acid, with mSAA1 selectively only binding retinol. The slight differences I observed in the apparent dissociation constants for retinol and retinoic acid binding are potentially due to differences in electrostatic charges between the retinoids, as well as difference in the amino acid sequences between the protein isoforms, even though they share a high amino acid identity. Differences in the amino acid sequence could have consequences on the accessibility of ligands to access the binding pocket, resulting is differences in binding constants. This idea was supported by performing site-specific mutagenesis, as replacement of a tryptophan with an alanine at position 71 within the hydrophobic core of mSAA3 resulted in reduced binding with retinol. As hSAA1, mSAA1, and mSAA3 bound β-carotene, retinyl acetate, retinyl palmitate, and 4-keto retinol to varying degrees, they illustrate the flexibility and accessibility of the SAA binding pocket to these structurally different retinoid ligands. Based on the biochemical and structural insight Dr. Derebe and I generated, we can speculate that the β-ionone ring is buried in the SAA binding pocket, which is potentially why there is some flexibility in binding with some of the other ligands, as many of the retinoid ligands share an identical β-ionone ring with polyunsaturated carbon side chain. Together, these results reveal

novel retinol binding functions for SAAs, as well as, a new architecture for retinol binding that differs from the previously identified retinoid binding proteins.

RETINOL TRANSPORT DURING INFECTION

The transport of retinol is critical for immune responses, as retinoids serve as key signals in the development of innate and adaptive immunity, and also promote maintenance and repair of epithelial barriers. [216, 223, 232, 240, 293]. Indeed, a prominent response to acute infection is the marked decline in serum retinol. This decline was originally correlated to decreases in RBP concentrations in the serum [281, 295, 296]. This paradoxically occurs at a time of increased demand for retinol in order to support the development of immunity and barrier defense. Thus, it has been unclear how retinol is transported among tissues following an acute microbial challenge. As my studies demonstrated, serum retinol concentrations are reduced, compared to steady state levels, 24 hours post infection. However, this occurs after a marked spike in retinol in the serum, beginning as early as 4 hours post infection, which is maintained for an additional 8 hours before decreasing. This early increase in serum retinol is likely used to prime immune responses required to resolve the infection. As this increase in serum retinol corresponds to increases in serum SAA concentration, this suggests that SAAs may be responsible for delivery of retinol early during infection.

Based on my studies, I propose that SAA serves to transport retinol during the first few hours following infection. Supporting this idea, I found SAA associated with retinol in the sera of wild-type mice following infection in a ratio of 1:4 moles of retinol to SAA, which is consistent with the tetrameric mSAA3 crystal structure. In agreement with this finding, I did not

detect retinol in the similar serum protein fraction of SAA*1/2*$^{-/-}$ mice, thus further supporting the role of SAA in retinol transport.

SAA IMPACTS IMMUNE RESPONSES

During the acute phase, the liver secretes high concentrations of SAA into the serum following infection. Supporting a role for SAA in host defense and immunity, several studies have linked NF-κB signaling to SAA induction in a variety of cell culture models. Indeed, MyD88 signaling in intestinal epithelial cells also contributes to SAA induction in the intestine in response to the commensal microbiota or infection. Additionally, SAA orthologs have been identified in multiple vertebrate and even invertebrate species. This high degree of conservation off SAA indicates it plays a key role in host survival and coupled with its increase in expression during infection, likely plays a role in host defense and immunity.

Indeed, several recent observations support the idea that SAAs promote immunity to infection. First, *Saa1/2*$^{-/-}$ mice exhibit increased susceptibility to chemically-induced colitis in mice [29], suggesting that SAAs contribute to intestinal immunity. Other studies have also suggested that SAAs promote Th_{17} cell development in response to specific components of the microbiota, such as segmented filamentous bacteria [38]. Consistent with a function for SAAs in retinol binding and transport, retinoids are required to elicit Th_{17} cell responses to infection and mucosal vaccination [293]. Second, studies in zebra fish show that commensal microbiota stimulate neutrophil migration through induction of SAA [294]. Third, I found that intraperitoneal infection of *Saa1/2*$^{-/-}$ mice with *S. typhimurium* resulted in higher bacterial loads

in liver and spleen as compared to wild-type mice, suggesting that SAAs also contribute to systemic immunity.

RETINOIC ACID AND IBD

Inflammatory bowel disease (IBD) is a condition that includes Crohn's disease and ulcerative colitis and is marked by chronic inflammation in the intestine. While the cause of IBD is not known, genetics and environmental conditions are thought to play a role in the disease course. Indeed, genome-wide association studies (GWAS) have identified a large number of genetic risk loci associated with IBD. Many of these genes are involved in innate or adaptive immune functions, a further indication that the chronic inflammation observed in IBD patients is due to inappropriate immune responses, possibly to the commensal microbiota population. Dysregulation of cells within the immune system is thought to produce excessive concentrations of the inflammatory cytokines TNFα and IL-1β, which lead to uncontrolled inflammation in the gut. Additionally, IL-17 producing Th_{17} cells populating the gut are also thought to contribute to the total inflammation observed. Interestingly, a polymorphism within the *CYP26B1* gene, important in the catabolism of retinoic acid, was found at higher frequency in patients with Crohn's disease, indicating that homozygous carriers of the affected allele have a higher likelihood to develop Crohn's disease [297]. This suggests that control of the tissue concentrations of retinoic acid may be important in the prevention of unnecessary inflammation.

Another external factor that may contribute to IBD pathology is the diet, as it has been observed that some patients with active IBD have low concentrations of serum retinol [298]. This could be problematic as retinoic acid is important in maintenance of the mucosal immune

system and important in the induction of T_{reg}s, which function to control inflammation. In a mouse model of chemically induced colitis, vitamin A deficiency led to an exacerbation of the disease, whereas treatment with retinoic acid alleviated disease symptoms including lower concentrations of inflammatory cytokines, increased colon length, and a decrease in the total inflammatory score [299]. The opposite effect was observed when mice were treated with a RAR antagonist, indicating that in this model system, retinoic acid signaling was protective. Even mice fed a vitamin A replete diet seem to benefit from additional retinoic acid, which is protective during chemically induced colitis, and results in reduced colon shortening, reversed weight loss, and lower inflammation following bacterial infection [229]. Another colitis model using mice is induced by the transfer of $CD4^{+}CD45RB^{high}$ T cells into immune deficient hosts (*RAG-/-* mice), which generates high levels of inflammation. This inflammation can be abrogated by an additional transfer of T_{reg} cells into the hosts. However, the ability of transferred T_{reg}s to confirm protection from colitis depended on the expression of CD103 by host cells [235]. The expression of CD103 by innate immune cells in this model, suggests that the generation of retinoic acid may be an important factor in T_{reg} mediated suppression, especially in light of the fact that retinoic acid is important in the differentiation of T_{reg}s in the periphery.

A model of IBD has also been established in zebrafish larvae, which is induced following exposure to dextran sodium sulfate (DSS), and is characterized by microbe-dependent neutrophil-induced inflammation. DSS treatment results in large accumulation of mucus proteins, which were protective when zebrafish larvae were later challenged with another form of chemically induced colitis. However, mucin production is suppressed when larvae are treated with exogenous 1 μM retinoic acid, and this exacerbated later colitis challenges [300]. As this is a much higher concentration of retinoic acid than is found at physiological conditions, it remains

difficult to determine if retinoic acid is naturally suppressive during uncontrolled inflammation. However, there have also been reports that treatment of mice chronically infected with *Trichuris muris* (a helimith parasite) with a RAR agonist adversely increased the inflammation generated to control the infection, as seen by increased cellular infiltrates, and this was dependent on IL-6 [301]. As retinoic acid is known to skew T cells to a Th2 phenotype, which is necessary to combat parasitic infections, it is surprising that additional RAR signaling is harmful and not beneficial in resolution of the infection. This implies that perhaps the source of the inflammation seen in IBD is important, as retinoic acid appears to be either protective or harmful in different scenarios.

DISCUSSION

SAAs are markedly induced in the intestinal epithelium by the microbiota, and thus my findings may provide insight into how the intestinal microbiota regulates host immunity and inflammation [29, 37, 38]. Collectively, my results describe SAAs as a microbe-induced, novel class of retinol binding proteins that function early during the infection response. While my thesis supports a new function for SAA, there are still many questions to address concerning SAA and the established retinol transport pathways. For example, how is SAA loaded with its retinol cargo? Does this happen via a mechanism similar to that used by RBP? Which tissues are targeted by SAAs for retinol delivery? How does SAA identify its target cell and transfer its retinol cargo? It is possible that this involves protein-mediated endocytosis process through the SR-B1 receptor or could even occur through passive diffusion.

The structural information generated from the mSAA3 crystal provide new insights into retinol transport during the acute phase and suggest a completely novel mechanism for retinol binding that can expand our understanding of their function. However, there are still unresolved questions as to how tetrameric SAA associates with HDL and if retinol transport is tied to cholesterol transport during the acute phase response. It's also possible that the majority of tetrameric SAA:retinol complexes circulate completely free of HDL. These are questions that will need to be addressed in future studies.

Considering the importance of retinol in activating effector and memory immune responses, further studies will need to continue to explore the consequences of early retinol spikes in the serum following infections and the impact of this on host immune responses. Additionally, as SAA*1/2*$^{-/-}$ mice continue to be analyzed, future experiments will further our understanding of how SAAs contributes to host immune processes. Another key question that remains to be addressed is whether there are tissue-specific effects of intestinal epithelial SAAs, or whether the intestinal SAAs also enter the circulation with bound retinol acquired directly from the diet. It is possible that intestinal epithelial SAAs could be involved in the direct delivery of retinol from epithelial cells to underlying immune cells in the lamina propria, or from epithelial cells to mucosal lymphoid tissues. Altogether, my research supports the idea that transport of retinol early during infection by SAA likely impacts host immune responses, thereby further linking the immune system responses to intestinal epithelial cells, the microbiota, and the diet.

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