EPITHELIAL RETINOIC ACID RECEPTOR BETA REGULATES SERUM AMYLOID A EXPRESSION AND VITAMIN A-DEPENDENT INTESTINAL IMMUNITY

APPROVED BY SUPERVISORY COMMITTEE

Lora V. Hooper, Ph.D.

Sebastian Winter, Ph.D.

Steven A. Kliewer, Ph.D.

Michael Shiloh, Ph.D.

DEDICATION

My gratitude goes to the innumerable mentors—in lab and in life—who have shaped the way I think and navigate the world. It has been an immense honor and privilege to be trained as a scientist in the laboratories of Dr. Cary Lai, Dr. Karen Bush, and Dr. Lora Hooper. These opportunities were made even remotely possible for me because of my parents. Rajkumar Gattu, for his nudge to higher education as a means of empowerment. Saro Gattu, for her magical ability to instill in me the greatest gift of all—a love for learning.

Special thanks go to my dear uncle, Dr. Sethuraman Subramanian, who has simultaneously supported and challenged me throughout my life. And to Vijay Ram, my number one critic, companion, and serendipitous inspiration.

In the words of former Hooperite, Dr. Xiaofei Yu, “I’m not scared of anything anymore”.

EPITHELIAL RETINOIC ACID RECEPTOR BETA REGULATES SERUM AMYLOID A EXPRESSION AND VITAMIN A-DEPENDENT INTESTINAL IMMUNITY

by

SUREKA GATTU

DISSERTATION / THESIS

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY / MASTER OF SCIENCE / MASTER OF ARTS

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

August, 2018

RETINOIC ACID RECEPTOR BETA REGULATES SERUM AMYLOID A EXPRESSION AND INTESTINAL IMMUNITY

Publication No. _____

Sureka Gattu, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2018

Supervising Professor: Lora V. Hooper, Ph.D.

Vitamin A is a dietary component that is essential for the development of intestinal immunity. Vitamin A is absorbed and converted to its bioactive derivatives retinol and retinoic acid by the intestinal epithelium, yet little is known about how epithelial cells regulate vitamin A-dependent intestinal immunity. Here I show that epithelial cell expression of the transcription factor retinoic acid receptor β (RARβ) is essential for vitamin A-dependent intestinal immunity. Epithelial RARβ activated vitamin A-dependent expression of serum amyloid A (SAA) proteins by binding directly to *Saa* promoters. In accordance with the known role of SAAs in regulating Th17 cell effector function, epithelial RARβ promoted IL-17 production by intestinal Th17 cells. More broadly, epithelial RARβ was required for the development of key vitamin A-dependent adaptive immune responses, including $CD4^+$ T cell homing to the intestine and the development of immunoglobulin A-producing intestinal B cells. My findings provide insight into how the intestinal epithelium senses dietary vitamin A status to regulate adaptive immunity and highlight the role of epithelial cells in regulating intestinal immunity in response to diet.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Gattu S, Chara AL, Harris TA, Ruhn KA, Kuang Z, Sockanathan S, Hooper LV. Epithelial retinoic acid receptor beta regulates serum amyloid A expression and vitamin A-dependent intestinal immunity. Submitted to PNAS. **2018** Jul.

Harris TA, **Gattu S**, Propheter DC, Kuang Z, Bel S, Ruhn KA, Chara AL, Edwards M, Zhang C, Jo JH, Zouboulis CC, Kong HH, Segre JA, Hooper LV. Resistin-like molecule alpha provides vitamin A-dependent antimicrobial protection of the skin. Submitted to Nature. **2018** Jan.

Derebe MG, Zlatkov CM, **Gattu S**, Ruhn KA, Vaishnava S, Diehl GE, MacMillan JB, Williams NS, Hooper LV. Serum amyloid A is a retinol binding protein that transports retinol during bacterial infection. Elife. **2014** Jul 29;3:e03206. doi: 10.7554/eLife.03206. PubMed PMID: 25073702; PubMed Central PMCID: PMC4129439.

Hayakawa K, **Gattu S**, Marchaim D, Bhargava A, Palla M, Alshabani K, Gudur UM, Pulluru H, Bathina P, Sundaragiri PR, Sarkar M, Kakarlapudi H, Ramasamy B, Nanjireddy P, Mohin S, Dasagi M, Datla S, Kuchipudi V, Reddy S, Shahani S, Upputuri V, Marrey S, Gannamani V, Madhanagopal N, Annangi S, Sudha B, Muppavarapu KS, Moshos JA, Lephart PR, Pogue JM, Bush K, Kaye KS. Epidemiology and risk factors for isolation of Escherichia coli producing CTX-M-type extended-spectrum β-lactamase in a large U.S. Medical Center. Antimicrob Agents Chemother. **2013** Aug;57(8):4010-8. doi: 10.1128/AAC.02516-12. Epub 2013 Jun 10. PubMed PMID: 23752516; PubMed Central PMCID: PMC3719715.

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

SAA – serum amyloid A

RAR – retinoic acid receptor

RA – retinoic acid

VAD – vitamin A deficient

VA+ – vitamin A sufficient

RARE – retinoic acid response element

IgA – immunoglobulin A

IL-17 – interleukin 17

Th17 – T helper 17

ALDH – aldehyde dehydrogenase

DC – dendritic cell

IEL – intraepithelial lymphocyte

HDL – high density lipoprotein

RBP – retinol binding protein

PCR – polymerase chain reaction

CEBPα,β –CCAAT/enhancer binding proteins α, β

DBD – DNA binding domain

DNA – deoxyribonucleic acid

LBD – ligand binding domain

RXR – retinoid X receptor

PPAR – peroxisome proliferator-activated receptors

FXR – farnesoid X receptor

LXR – liver X receptor

PXR – pregnane X receptor

CAR – constitutive androstane receptor

VDR – vitamin D receptor

TLR – toll-like receptor

LPS – lipopolysaccharide

GO – gene ontology

PMA – phorbol myristate acetate

APC – antigen presenting cell

CHAPTER ONE
Background and Objective

1.1 INTRODUCTION

The mammalian intestinal epithelium is a vital interface between the external environment and internal tissues. Epithelial cells interact with the environment of the gut lumen by absorbing dietary compounds and by associating with the resident bacterial communities that promote digestion. The intestinal epithelium also orchestrates development of the underlying immune system through the secretion of immunoregulatory proteins (1). Thus, epithelial cells are ideally positioned to capture information about the diet and the microbiota in order to regulate both innate and adaptive immunity. While it is known that gut epithelial cells detect intestinal microorganisms through various pathways involving pattern recognition receptors (1), little is known about how epithelial cells sense dietary components to regulate immunity.

Vitamin A is a fat-soluble nutrient that is essential for the development of adaptive immunity to intestinal microorganisms. It is required for immunoglobulin A (IgA) production by intestinal B cells (2), T cell homing to the intestine (3), and the production of interleukin-17 (IL-17) by T helper 17 (Th17) cells (4). Consequently, vitamin A-deficient diets result in severe immunodeficiency and increased infection rates (5).

Cells convert vitamin A into several related compounds, collectively known as retinoids. These include retinol and its derivative retinoic acid (RA). RA is a potent regulatory molecule that controls gene expression through RA receptors (RARα, β, and γ), members of the nuclear receptor family that activate the transcription of specific target genes

(6). The intestinal epithelium plays a central role in retinoid metabolism by absorbing dietary vitamin A and expressing RA-generating enzymes and RARs (7, 8). This suggests that the epithelium could help to regulate vitamin A-dependent adaptive immunity. Recent work has implicated epithelial RARα in the development of the epithelial barrier as well as lymphoid follicles that support intestinal immune cell development (8). However, a large body of the literature on vitamin A-directed immunity has focused on lymphocyte-intrinsic cell activities. It is still unclear whether epithelial cells regulate the development of specific vitamin A-dependent immunological pathways including the development of gut-homing $CD4^+$ T cells, IgA-producing B cells, and Th17 cell effector function. How the intestinal epithelial cell interfaces with the microbiota and the underlying immune system, and how vitamin A regulates this complex interface is the overarching topic of interest.

1.2 VITAMIN A-DIRECTED MUCOSAL IMMUNITY

1.2.1 Innate Immunity

Retinol is an essential diet-acquired nutrient that modulates the physiological response to microbial challenge. Retinol is derived from dietary vitamin A and can be metabolized to retinoic acid, which complexes with nuclear hormone receptors to regulate gene transcription in cells (6). Through these retinoic acid receptors (RARs), retinol aids the development of innate immune cells (9-11) and facilitates the regeneration of epithelial barriers damaged by infection (12). Consequently, a signature of vitamin A deficiency in humans is an increased susceptibility to infection (13, 14), which underscores the broad

importance of retinol for immunity. It is important to note, however, that vitamin A excess can lead to dysregulation of liver metabolism and promote toxicity (15).

To pinpoint the cell populations that play a role in vitamin A-directed immunity, I highlight those cells which contain the machinery to metabolize retinoids. The intestinal epithelium, in addition to being the site of vitamin A absorption, can metabolize vitamin A to retinoic acid based on its ability to produce the retinaldehyde dehydrogenase (ALDH), ALDH1A1 (16). A subset of lamina propria stromal cells beneath the epithelium express all isoforms of ALDH, and have been shown to influence the function of mucosal dendritic cells (17). It is also known that CD103+ dendritic cells (DCs) produce RA, and that this cell population exhibits higher ALDH activity in the proximal small intestine compared to distal small intestine or colon (18). This is likely due to increased absorption of vitamin A in the proximal small intestine. Interestingly, DCs from the proximal small intestine are also capable of inducing higher numbers of Foxp3+ T regulatory cells, and expression of markers involved in gut homing (chemokine receptor CCR9 and integrin $\alpha 4\beta 7$) on T cells (19). Lamina propria localized CD103+ DCs are known to migrate to the mesenteric lymph nodes, where they become a primary source of retinoic acid, and are critical for generating gut-tropic T cells in mice (20). A major gap in our understanding of this process, however, lies in the mechanism of how vitamin A is impacting this DC-T cell axis. How DCs transport retinoic acid to T cells remains a topic of exploration in our lab, though not without significant technical challenges.

1.2.2 Adaptive Immunity

Our understanding of vitamin A as a modulator of adaptive immunity was greatly enhanced due to the contributions of Iwata and Mora. Iwata and colleagues demonstrated that retinoic acid was necessary and sufficient to induce gut homing receptors, CCR9 and α4β7, on T cells (3). And in a key study led by Mora and colleagues, we learned that retinoic acid induces gut tropism in B cells, as well (2). These findings also revealed that vitamin A deficient diets in mice result in a severe reduction of CD4+ T cells, CD8+ T cells, and B cells in the small intestinal lamina propria. Vitamin A also affects IgA-secreting intestinal B cell populations, particularly in the intestinal lamina propria; this is attributed to both impaired induction of B cell homing and an inability to synergize with DCs producing IL-6 or IL-5 (2). In a study where RARα is specifically ablated in B cells, mice have intestinal microbiota dysbiosis (21). Intraepithelial lymphocytes (IELs) found within the intestinal epithelial layer, are also impacted by vitamin A-directed immune processes, as mice expressing a dominant negative form of RARα specifically in CD4+ T cells lack the CD4+ IEL population (22).

Despite the knowledge that dietary vitamin A is critical for the development of adaptive immunity, there is a lack of understanding regarding how this essential lipophilic nutrient is transported through an aqueous environment; how epithelial cell-intrinsic retinoids and RAR activity broadly impact immunity; and how the intestinal epithelial cell efficiently utilizes retinoids for both metabolic and immune processes. While lymphocyte-intrinsic RARα function has been studied extensively—and more recently even IEC-intrinsic RARα function—the findings presented in the upcoming chapters suggest that there is a vast territory of vitamin A-directed immune functions that remains unexplored.

1.3 SERUM AMYLOID A

1.3.1 Role in Immunity

Serum amyloid A (SAA) proteins are a family of proteins that are expressed in the intestinal epithelium *(23)* and liver (24) and circulate in the serum (25). SAA family members are encoded in the genomes of virtually all vertebrates and are highly conserved among species, suggesting essential biological functions (24). Expression of SAAs is strongly induced by microbial exposure. SAAs are induced in intestinal epithelial cells by the microbiota (23, 26-28), and have been implicated in promoting Th17 cell development in response to specific microbiota components (26). Similarly, liver and serum SAAs are markedly elevated following systemic bacterial or viral infection (29, 30).

Although it has been proposed that SAAs generally contribute to inflammation and immunity (23), the exact functions of SAAs require further elucidation. SAAs circulate in the serum associated with high-density lipoprotein (HDL), which transports lipid-bound lipoproteins amongst tissues (31), and more recently we have shown that SAAs have the capacity to bind retinol and that this complex is elevated in mouse serum post-infection by *Salmonella typhimurium* (32). It was also observed that the expression of SAA is dependent on both dietary vitamin A and the microbiota, which prompted questions regarding the transcriptional regulation of this immunoregulatory protein of various but vague functions.

1.3.2 Retinol Binding Protein

As a small lipid-soluble compound, retinol cannot freely circulate but is instead transported among cells and tissues by specialized retinol binding proteins. Serum retinol binding protein (RBP) facilitates transport of retinol among the intestine, which is the site of retinol acquisition, the liver, which is the major site of retinoid storage, and other tissues that require retinol for their physiological functions (33). Despite the increased requirement for retinol, serum RBP is markedly reduced following microbial challenge (34). The protein that we posit could be fulfilling this transportation need is serum amyloid A (SAA).

SAAs are strongly induced by microbial exposure at sites of retinol uptake (intestine) and storage (liver) and are present at high levels in the circulation following microbial challenge (26, 28, 29). We have shown that SAA expression in mice requires dietary vitamin A, that mouse and human SAAs bind tightly to retinol, and that SAA recovered from serum following bacterial infection is associated with retinol (32). These findings suggest that SAAs mediate retinol transport during microbial challenge and thus constitute a key component of the physiological response to infection (32).

1.3.3. Transcriptional Regulation

SAA expression requires dietary vitamin A

In previously published work (32), I uncovered a relationship between SAA expression and dietary vitamin A status in mice. Microarray analysis disclosed that mice fed a vitamin A-deficient diet exhibited lower abundances of serum amyloid A transcripts in the intestine as compared to mice fed a vitamin A-replete diet (**FIGURE 1.1)**.

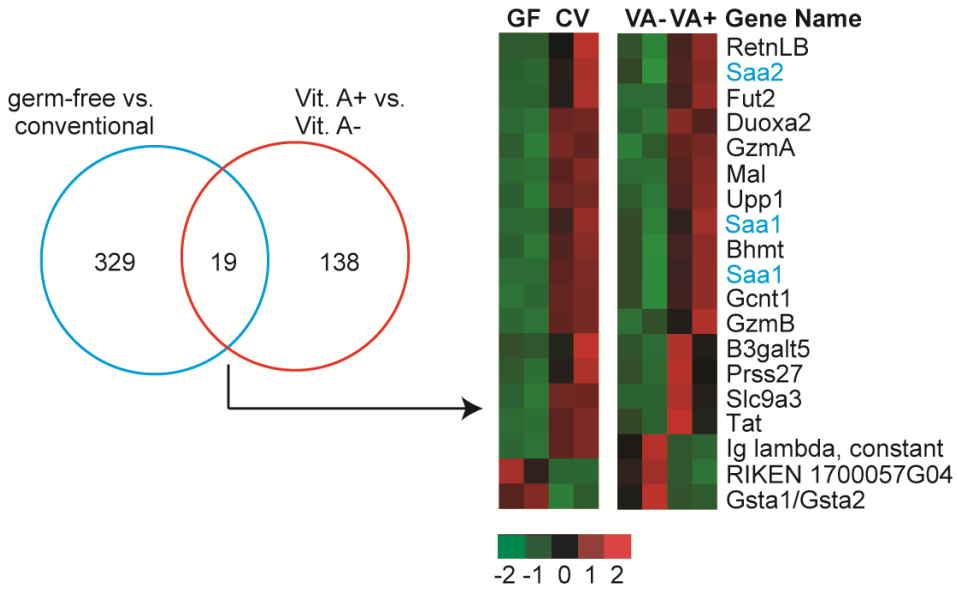

FIGURE 1.1. Intestinal genes regulated by both the microbiota and dietary vitamin A. (32)

Real-time quantitative PCR and immunofluorescence analysis verified that expression of small intestinal SAA1, 2, and 3 was reduced in mice fed a vitamin A-deficient diet (**FIGURE 1.2**). Liver expression of SAA1 and 2 was also reduced in mice fed a vitamin A-deficient diet, although the reduction in expression was less pronounced than in the intestine (**FIGURE 1.3**). This is likely because dietary vitamin A deficiency does not completely deplete stored retinoids in the liver.

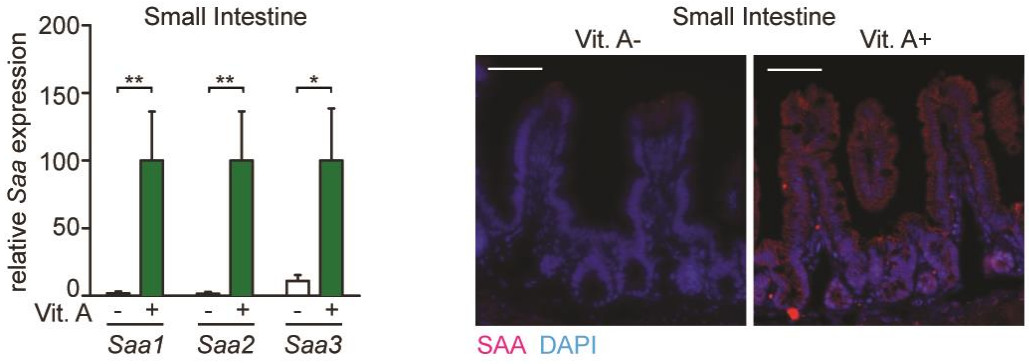

FIGURE 1.2. qPCR- and immunofluorescence-based verification of decreased SAA expression in VAD mouse ileum. (32)

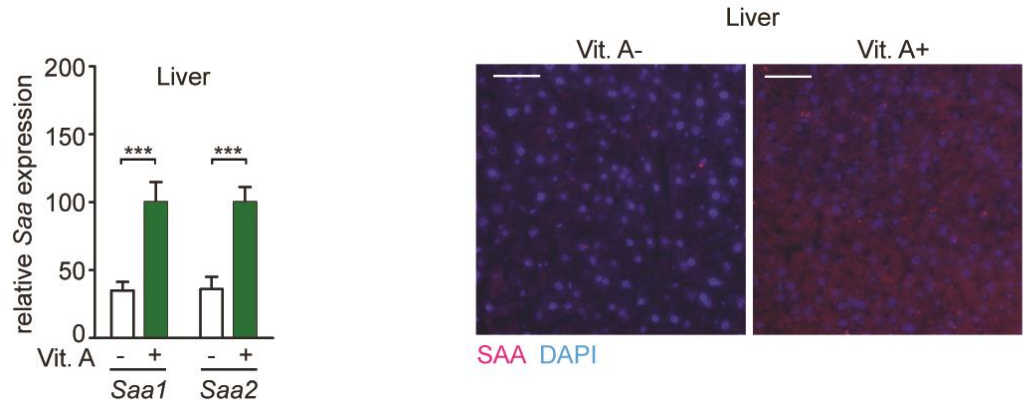

FIGURE 1.3. qPCR- and immunofluorescence-based verification of decreased SAA expression in VAD mouse liver. (32)

We also observed elevated expression of intestinal *Saa1* and *Saa2* following addition of retinol directly to the epithelial surface of small intestinal explants, and of liver *Saa1* and *Saa2* after intraperitoneal supplementation with retinoic acid (32). These findings support the idea that retinoids directly impact *Saa* expression. Addition of retinol or retinoic acid to cultured HepG2 cells (a human liver cell line) enhanced expression of *SAA1* and *2* in the

presence of IL-1β and IL-6, suggesting that the impact of dietary vitamin A on SAA expression is due to cell-intrinsic effects of retinoids (**FIGURE 1.4**). Collectively, these results show that full expression of SAAs in the intestine and liver requires dietary vitamin A. As transcriptional control by retinoids is frequently observed in proteins that function in retinoid transport and metabolism (35), these results indicated to us a possibility that SAA could be a retinol transport protein. We also hypothesized that diet-acquired retinoids could directly regulate SAA expression through retinoic acid receptors (RARs).

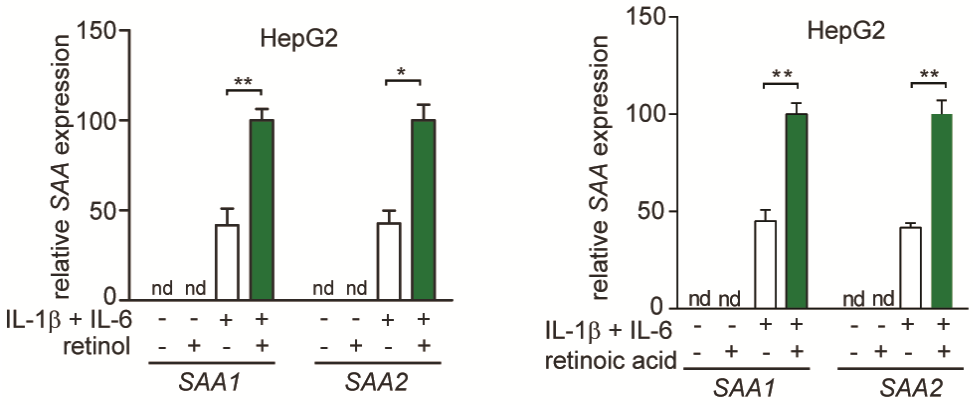

FIGURE 1.4. Retinoids enhance proinflammatory cytokine-mediated expression of *SAA* in hepatocyte model, HepG2 cells. (32)

STAT3 Regulation

It has been demonstrated that there is a STAT3 response element within the human *SAA1* gene based on studies in the human hepatocyte cell culture model, HepG2 cells (36). STAT3 and NF-KB p65 form a complex after IL-1 and IL-6 stimulation, after which STAT3 will interact with a region ~70bp downstream of the NF-KB response element within the *SAA1* promoter (36). It has also been shown more recently by the Littman group that segmented filamentous bacteria (SFB) induced production of IL-22 by type 3 innate

lymphoid cells (ILCs), which induced intestinal epithelial SAA1 and SAA2 production in a STAT3-dependent manner (37). The regulation of SAAs by STAT3 is thus supported for both a hepatic and intestinal context.

NFKB Regulation

The NFKB-dependent induction of SAA found in the hepatic HepG2 model, however, does not translate to all studied cell types. In HepG2 cells, SAA transcription is promoted by proinflammatory cytokines, LPS, glucocorticoids, as well as retinoids. In aortic smooth muscle cells, CCAAT/enhancer binding proteins α,β (CEBPα,β) were essential for glucocorticoid-induced *SAA* gene expression, though not in HepG2 cells (38). Kumon and colleagues have published extensively about the regulation and putative functions of SAAs in the context of different cell types, as well as disease states. The past connections drawn between SAAs to cardiovascular health, lipid metabolism, intestinal homeostasis, rheumatoid arthritis, and neurodegenerative diseases have generated more questions than answers thus far. This body of literature is vast but suggests a need to understand local production and regulation of SAAs, perhaps using more refined *in vivo* tools that take advantage of cell-specific genetic ablation or overexpression.

1.4 ENTERIC NUCLEAR HORMONE RECEPTORS REGULATE IMMUNITY

Nuclear hormone receptors are ligand dependent transcription factors that are essential to numerous biological processes. The nuclear receptor superfamily comprises of 48 ligand-activated transcription factors categorized by shared structural features (39). The

DNA binding domain (DBD) in most nuclear receptors binds to conserved motifs in the regulatory regions of genes. The ligand binding domain (LBD) serves to activate or inactivate transcription in response to binding of a ligand. This particular feature facilitates the ability of nuclear hormone receptors to act as sensors for endocrine hormones and dietary lipids. And it is this feature that begs the question of how we ought to think about the very nature of dietary lipids such as vitamins A and D—as lipids, as hormones; as immunoregulatory, as developmental.

Nuclear receptors bind DNA as homodimers, heterodimers, and potentially as monomers. All nuclear receptor heterodimers must include a retinoid X receptor (RXR). The nuclear receptor superfamily can be subdivided into four groups based on the type of ligand bound and the DNA binding mechanism (40). This includes the endocrine receptors that bind DNA as homodimers: estrogen, androgen, progesterone, glucocorticoid, and mineralocorticoid receptors. These all bind with nanomolar affinity to systemically-circulating steroid hormones synthesized by endocrine tissues. The second group of receptors form heterodimers with RXR and are activated by a wide range of dietary lipids (which activate peroxisome proliferator-activated receptors, PPARs), bile acids (activate FXR), xenobiotics (activate PXR and CAR), and cholesterol derivatives or oxysterols (activate liver X receptors, LXRs). These ligands exist at relatively high concentrations, particularly within enterohepatic tissues and portal circulation, where they bind their cognate receptors with micromolar affinity. The third group comprises receptors for vitamins A and D (retinoic acid receptors, RARs and vitamin D receptor, VDR respectively), and the thyroid hormone receptors (TRs). These function as RXR heterodimers, but importantly their ligands are

synthesized in the body from nutrients derived from the diet. Like other endocrine receptors, this group binds ligands with high affinity. It is now known that VDR also binds bile acids and thus satisfies the criteria of both the second and third groups (41, 42). The last group includes receptors with no known ligands and are referred to as orphan nuclear receptors.

1.4.1 Retinoic X Receptors and Their Heterodimer Partners

An additional layer of regulation associated with nuclear receptors that function as RXR heterodimers is that RXR itself is activated by the endogenous vitamin A derivative, 9-cis retinoic acid. This implies that these RXR heterodimers could be activated by two ligands, that of RXR and that of its partner. The reality is complex. RXR heterodimers could operate under three modes of activation: permissive, conditional, and non-permissive (43). Permissive heterodimers can be activated by either receptor's ligand, as is seen with the dietary lipid-sensing receptors (LXRs). Conditional heterodimers can only be activated by RXR agonist when the partner ligand is also present (RARs). Non-permissive heterodimers are endocrine receptors in which the RXR agonist has no effect on the activity of the heterodimer (TRs). RXR binding partners relevant to intestinal homeostasis are discussed below.

Vitamin D Receptors

In the intestine, calcium is absorbed in the proximal end and VDR is important for regulating calcium transport within the enterocyte. It regulates calcium influx across the apical membrane, intracellular trafficking, and basolateral efflux (44). VDR also regulates

the cytosolic calcium binding protein, calbindin, thought to transport calcium from the apical to basolateral membrane and prevent toxicity (45)

VDR has also been shown to prevent autoimmune disease and regulate immunity (46). Vitamin D can inhibit T cell activation and promote tolerance in dendritic cells (47, 48) and has also been implicated in toll-like receptor (TLR) signaling and macrophage function (49). VDR regulation of the cathelicidin gene, which encodes an antimicrobial peptide, is a key example of how a nutrient can directly modulate an innate immune response within the intestinal epithelium (50). It is possible that VDR also regulates other antimicrobial factors within the intestinal epithelium, and this is a topic of exploration in the lab that mirrors our interest in vitamin A.

Peroxisome Proliferator-Activated Receptors

PPARγ is a key nuclear hormone receptor within the colonocyte, and thematically represents an interesting link between metabolism, inflammation, and the microbiota. This nuclear receptor's ligands include free fatty acids, which consequently has a profound impact on lipid storage in adipose tissues (through intestinal ANGPTL4) as well as colonocyte-intrinsic metabolic and immune pathways (51, 52). PPARγ activation is additionally linked to the presence of butyrate and propionate, which are intestinal microbe metabolites. Many of the underlying mechanisms of how the microbiota contribute to immune and metabolic status are still unknown, but recent years have highlighted the importance of short chain fatty acids for intestinal homeostasis. Short chain fatty acids are produced in the colon by bacterial

fermentation of dietary fiber. Butyrate especially plays an important role in regulating intestinal epithelial cell genes.

Farnesoid X Receptors

FXRs regulates the reabsorption of bile acids produced in the liver, in the distal small intestine. How the enterocyte regulates this process is multifold. First, the ileal bile acid transporter (IBAT) facilitates transport of the conjugated bile acids across the brush border of the intestine. Next, at the basolateral membrane, bile acid efflux occurs using OSTalpha/beta. And finally, the ileal bile acid binding protein Fabp6 binds to bile acids in the cytosol.

The connection between bile acids and the microbiota in the gut has garnered attention in recent years, by elucidating how FXR activation in the intestine could mediate the antimicrobial activity of bile acids. In a model for cholestasis by bile duct ligation, bacterial overgrowth and mucosal invasion were prevented by an FXR agonist (53). The extent to which FXR plays a role in normal intestinal immunity, however, requires further investigation.

1.4.2 Retinoic Acid Receptors

RARs, of which there are three subtypes (RAR-α, -β, and -γ) and their heterodimer partner, RXR (also of three subtypes, RXR-α, -β, and -γ) include additional isoforms that are alternative splice variants that have the capacity to utilize different promoters to regulate transcription. This structurally results in unique N-termini (54). RARs and RXRs are variably expressed across different cell and tissue types, which owes to the complexity of retinoid

signaling. RA response elements, or RAREs, are found in promoters, introns, and intergenic regions that are distal to the promoter. They are typically composed of two direct repeats or core motifs that are separated by either 1-, 2-, or 5- bp spacers; these RAREs are referred to as DR1, DR2, or DR5. There are a number of noncanonical RAREs, in addition, and each RARE may preferentially bind to specific RAR/RXR heterodimers, furthering the complexity of RA-mediated transcriptional programming.

Retinoic acid receptors are generally found in the nucleus, though under certain conditions, they can be found in the cytoplasm and are thought to display non-genomic activities in ligand-dependent or -independent manners. While these extranuclear signaling pathways were not assessed in these studies, it is important to consider non-transcriptional effects while drawing conclusions about the impact of RAR knockdown in our mouse models. One line of thinking to consider is that RARs may act as phosphoproteins that maintain crosstalk with kinase cascades.

Retinoic Acid Receptor β

While RARα has fairly ubiquitous expression, RARβ and RARγ have more complex tissue-specific expression (55). RARγ has been shown to have critical roles in stem cell maintenance, inflammatory cytokine production, and skin immunity. Specifically, this subtype could play an important role in psoriasis and other skin conditions, as it represents 90% of the RARs in both human and mice epidermis (56). I posit that intestinal epithelial cell-intrinsic RARβ plays as significant a role in modulating intestinal homeostasis, which I explore here.

The loss of RARβ and RARγ has been observed in cancers, suggesting that these subtypes may play a role as tumor suppressors. The loss of RARβ expression is associated with tumorigenesis in lung, breast, and cervix tissues. In these cancers, the RARβ gene is either deleted or its promoter is silenced in response to DNA methylation or histone modifications; subsequent restoration of the promoter activity by demethylation of HDAC inhibition can decrease the tumorigenicity of the cancer cells (57, 58).

1.5 STATEMENT OF PURPOSE

My findings provide insight into how the intestinal epithelium senses dietary vitamin A status to control vitamin A-dependent adaptive immunity. I explore here the role of a critical immunoregulatory protein, serum amyloid A (SAA), which requires both dietary vitamin A and microbial colonization for its expression. I delineate how vitamin A regulates SAA expression through retinoic acid receptor β (RARβ) and explore how RARβ more broadly regulates vitamin A-dependent adaptive immune responses. I provide preliminary insight into the physiological role of SAA, as a transporter of retinol. Lastly, I expand my study of RAR-driven immunity within the intestinal epithelium to an exploration of vitamin A-dependent immunity in the skin.

CHAPTER TWO
Transcriptional Regulation of Serum Amyloid A

2.1 INTRODUCTION

Serum amyloid A proteins are a family of immunoregulatory proteins that highlight the integration of dietary and microbiota signals by the intestinal epithelium. SAAs are retinol-binding proteins that are expressed at the site of retinoid uptake (intestinal epithelium) and retinoid storage (liver) and circulate retinol following systemic bacterial exposure (32). In the intestine, SAAs stimulate IL-17 expression by Th17 cells (37), thus shaping their effector functions. Expression of SAAs in the intestine and the liver requires both a microbial signal (microbiota colonization in the intestine and systemic bacterial challenge in the liver) (32, 37) and dietary vitamin A (32). The microbiota triggers intestinal epithelial SAA expression through a multicellular signaling circuit involving dendritic cells, innate lymphoid cells, and epithelial STAT3 (37). However, the mechanisms by which vitamin A regulates SAA expression are unknown. Here I show that RARβ activates *Saa* expression through direct binding to retinoic acid response elements (RAREs) in *Saa* promoters.

2.2 METHODS

2.2.1 Cell Culture

The MODE-K cell line was provided by Dominique Kaiserlian (59). HepG2 cells were purchased from the ATCC. Cells were cultured in 1X DMEM with GlutaMAX, 10% FBS, 1X Penstrep, and 1X sodium pyruvate. Cells were maintained in 5% CO_2 at 37^{o}C. Prior to addition of retinol and LPS or cytokines, cells were cultured in a serum-free medium for

48 hours. MODE-K cells were treated for 3 hours with retinol (100 nM; Sigma) and lipopolysaccharide (100 ng/ml; Sigma) before BMS493 (100nM) was added. HepG2 cells were treated for 3 hours with retinol (100 nM), IL-1β (50 pg/ml; R&D Systems), and IL-6 (100 pg/ml; R&D Systems) before BMS493 (100 nM) was added. Cells were collected 24 hours post-stimulation.

2.2.2 Quantitative RT-PCR

Total RNA was isolated from cells or homogenized tissues using the RNeasy Lipid Tissue Kit (Qiagen). cDNAs were synthesized using MMLV reverse transcriptase (ThermoFisher) and were analyzed using Taqman gene expression qPCR assays (Invitrogen) or by using SYBR Green qPCR reagents with specific primers. Relative expression values were determined using the comparative C_t ($\Delta\Delta C_t$) method, and transcript abundances were normalized to *Gapdh* transcript abundance.

2.2.3 Western Blot

Total protein was isolated from cells or homogenized tissues as previously described (60). Membranes were detected with antibodies against SAA (32), RARα (Affinity Bioreagents), RARβ (Invitrogen), RARγ (ThermoFisher), and actin (ThermoFisher). The anti-SAA antibody was generated by Cocalico and recognizes all isoforms (32).

2.2.4 Retinoid Quantification

Retinol and retinoic acid (Sigma) were freshly reconstituted in ethanol and quantified under red or amber lighting. Quantification based on absorbance at 325 nm (retinol) and 350 nm (retinoic acid) is in accordance with previously published molar extinction coefficients (61). Retinoids for use in cell culture treatments were utilized in a vehicle of 0.1% ethanol in cell culture medium.

2.2.5 Specific Knockdown of RAR Isoforms

MODE-K or HepG2 cells cultured in a serum-free medium for 48 hours were trypsinized and plated at 5 x 10^4 cells per well of a 12-well cell culture plate. OptiMEM transfection reagent (Invitrogen) was combined with diluted siRNA pools (sequences are listed in Appendix B) then added to freshly split cells. After gentle mixing, additional experimental treatments were added. MODE-K cells were treated for 24 hours with retinol (100 nM; Sigma) and lipopolysaccharide (100 ng/ml; Sigma). HepG2 cells were treated for 24 hours with retinol (100 nM), IL-1β (50 pg/ml; R&D Systems), and IL-6 (100 pg/ml; R&D Systems). Cells were harvested 24 hours post-treatment.

2.2.6 Chromatin Immunoprecipitation of RAR Targets

MODE-K cells were crosslinked in PBS with 1% formaldehyde for 3 minutes at room temperature and quenched in 125 mM glycine at 4ºC for 10 min. Nuclei from fixed cells

were pelleted and used for chromatin immunoprecipitation per manufacturer's instructions (Diagenode). Each immunoprecipitation reaction included chromatin from 5 x 10^6 cells, 5 μg of goat anti-RARβ (Santa Cruz) or total goat IgG (Millipore), and 20 μl of Magna protein A beads (Millipore). Bound *Saa3* promoter sequences were quantified using SYBR Green-based real-time PCR. Relative enrichment of the *Saa3* promoter was calculated as the ratio of specific antibody pull-down to input DNA.

2.2.7 Dual Luciferase Reporter Assay for SAA3 Promoter

A 4103bp (-4000 to +103) fragment of the *Saa3* promoter was cloned into the pEZX-G04 vector containing two reporter genes (Genecopoieia) to generate the wild-type reporter construct. The putative RAR binding site (DR5) in the wild-type reporter construct was mutated from TGCCTTctttcTGCCCC to TGCCATctttcAGCCCC to generate the mutant reporter construct (Invitrogen GeneArt Site-Directed Mutagenesis Plus). MODE-K cells were transfected in 12-well plates (5 x 10^4 cells/well) with 400 ng of wild-type or mutant reporter plasmid or 400 ng of empty vector. Cells were treated with retinol (100 nM) and LPS (100 ng/ml) for 24 hours. Luciferase activity was quantified using the Secrete-Pair Luminescence Assay Kit (Genecopoieia) and measured with a SpectraMax M5e plate reader (Molecular Devices). Gaussia luciferase (GLuc) activity was normalized against secreted alkaline phosphatase (SEAP) activity and then compared to the activity of cells transfected with pEZX-G04 alone.

2.2.8 Animals and Animal Husbandry

dnRAR$^{\text{Villin-Cre}}$ mice were generated by crossing dnRAR mice (62) with *Villin-Cre* mice. *Saa1/2*$^{-/-}$ mice were obtained from F. de Beer (23); RARa$^{fl/fl}$ mice were from P. Chambon (63) through Y. Belkaid (National Institutes of Health). 6-14 week-old mice were used for all experiments. Because microbiota composition is known to impact intestinal immune cell frequencies, I used age- and sex-matched littermates that were cocaged to minimize microbiota differences in all experiments. Experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of the University of Texas Southwestern Medical Center.

2.2.9 Salmonella Infection

Salmonella enterica serovar *typhimurium* (SL1344) was grown in Luria-Bertani broth with ampicillin (100 μg/ml) at 37°C. Mice were infected intragastrically by gavage with *S. typhimurium* at 10^{10} colony-forming units (cfu) per mouse. cfu in the small intestine, liver, and spleen were determined by dilution plating on Luria broth plates containing ampicillin (100 μg/ml).

2.2.10 Statistical Analysis

All statistical analyses were performed using two-tailed Student's *t* test. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; and ns, $P > 0.05$.

2.3 RESULTS

2.3.1 SAA Expression is Dependent on RAR Activity

Dietary vitamin A is required for SAA expression in mouse intestine and liver, and the vitamin A derivatives retinol and RA stimulate *SAA1* and *SAA2* expression in the human liver cell line HepG2 ((32); **FIGURE 2.1**). To determine if retinoids also stimulate SAA expression in intestinal epithelial cells, I studied the mouse intestinal epithelial cell line MODE-K. MODE-K cells do not express SAA1 and SAA2; however, they do express SAA3, which is expressed in the intestine but is absent in the liver. SAA3 expression increased with the addition of bacterial lipopolysaccharide (LPS) and retinol (**FIGURE 2.2**), showing that SAA3 expression in MODE-K cells is highest in the presence of both a bacterial signal and a retinoid.

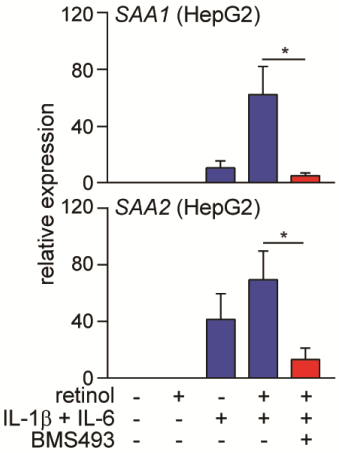

FIGURE 2.1. SAA1/2 expression is dependent on RAR activity. qPCR analysis of *Saa* expression in HepG2 cells, a hepatic cell culture model. *Saa1* and *Saa2* expression in HepG2 cells treated for 24 hours with retinol, IL-1β, IL-6, and the RAR inhibitor BMS493. *N*=4 replicates/group; data represent two independent experiments. Means±SEM are plotted. *P<0.05, **P<0.01, ***P<0.001 as determined by Student's t-test.

To further our mechanistic understanding of how retinoids stimulate SAA expression, I tested whether RARs are required. I added the RAR inhibitor BMS493 to HepG2 cells in the presence of retinol and the cytokines IL-1β and IL-6, which are generated during systemic infection (64). I chose to use retinol instead of retinoic acid, as retinol is more stable than retinoic acid (65), freely diffuses across membranes (66), and both HepG2 and MODE-K cells convert retinol to retinoic acid (67). BMS493 inhibited retinol-dependent *SAA1* and *SAA2* expression in HepG2 cells (**FIGURE 2.1**) and *Saa3* expression in MODE-K cells (**FIGURE 2.2**), suggesting that RARs are required for retinoid-induced SAA expression in cells.

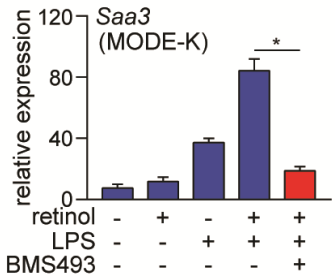

FIGURE 2.2. SAA3 expression is dependent on RAR activity. *Saa3* expression in MODE-K cells treated for 24 hours with retinol, LPS, and BMS493. *N*=4 replicates/group; data represent two independent experiments. Means±SEM are plotted. *P<0.05, **P<0.01, ***P<0.001 as determined by Student's t-test.

2.3.2 SAA Expression is Dependent on RARβ Expression

The mouse genome encodes three RAR isoforms (RARα, β, and γ), and I therefore sought to identify which isoform governs *Saa* expression. I used small interfering (si) RNAs to target individual *Rar* isoforms in HepG2 (**FIGURE 2.3**) and MODE-K cells (**FIGURE 2.4**). siRNA knockdown of *Rarb* suppressed *SAA1* and *SAA2* expression in HepG2 cells (**FIGURE 2.3**) and *Saa3* expression in MODE-K cells (**FIGURE 2.4**), while knockdown of *Rara* and *Rarg* had little effect. Thus, RARβ is uniquely required for retinol-dependent *Saa* expression in cells.

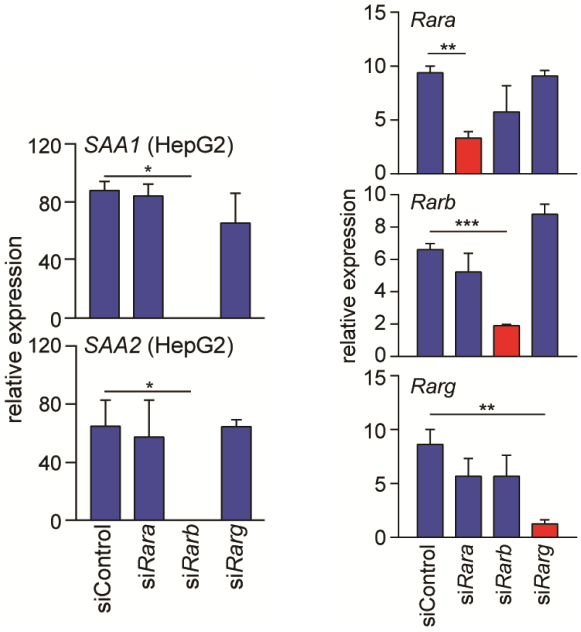

FIGURE 2.3. RARβ directs retinoid-dependent SAA1/2 expression. qPCR analysis of *Saa* expression after siRNA knockdown of specific *Rar* isoforms (left), with validation of specific knockdown by qPCR (right). *Saa1* and *Saa2* were analyzed in HepG2 cells treated with retinol, IL-1β and IL-6 as in Figure 2. *N*=3 replicates/group; data represent two independent experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01, ****P*<0.001 as determined by Student's *t*-test.

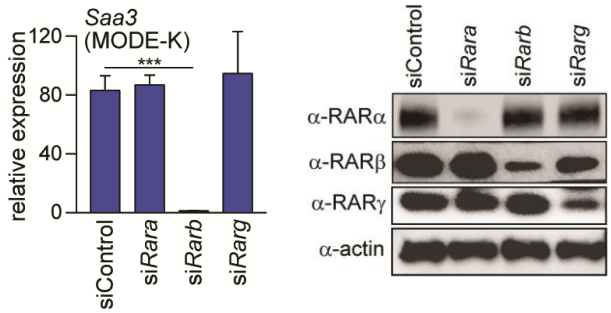

FIGURE 2.4. RARβ directs retinoid-dependent SAA3 expression. qPCR analysis of *Saa* expression after siRNA knockdown of specific *Rar* isoforms (left), with validation of specific knockdown by Western blot (right). *Saa3* expression was analyzed in MODE-K cells treated with retinol and LPS as in Figure 3. *N*=3 replicates/group; data represent two independent experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01, ****P*<0.001 as determined by Student's *t*-test.

2.3.3 RARβ Binds Directly to the SAA3 Promoter

I next asked whether RARβ regulates SAA expression through direct binding to *Saa* promoters. RARs bind to canonical promoter sequences called retinoic acid response elements (RAREs) that consist of the direct repetition of two core motifs. Most RAREs are composed of two hexameric motifs, 5′-(A/G)G(G/T)TCA-3′, arranged as palindromes, direct repeats, or inverted repeats (68).

In silico analysis of the ~4.1 kb mouse *Saa3* promoter region using NUBIScan (69) identified multiple potential RAREs. I analyzed the *Saa1* and *Saa2* promoters similarly, for comparison (**FIGURE 2.5**). I selected the 30 RAREs identified by NUBIScan as having the highest statistical chance of being functional RAREs in the *Saa3* promoter and performed

chromatin immunoprecipitation (ChIP) assays for RARβ binding at each (**FIGURE 2.6**; **TABLE 2.1**).

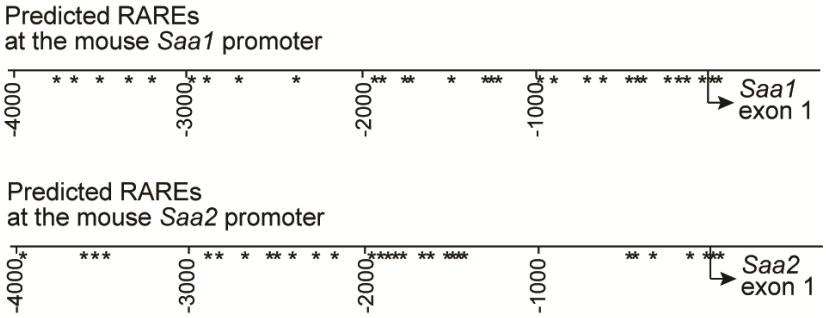

FIGURE 2.5. Predicted RARE sequences in the mouse *Saa1* and *Saa2* promoters. RARβ binding targets in the Saa1 and Saa2 promoters were predicted by NubiScan (Podvinec et al) and detailed in Appendices D and E. The most frequent DRs with 1, 2, or 5 nucleotides spacing are termed DR1, DR2, and DR5 elements, respectively. Predicted DR2 and DR5 elements are highlighted here, and have the highest statistically significant likelihood of being RARβ binding targets.

RARβ bound to the *Saa3* promoter in MODE-K cells at multiple RAREs, including those located at -224, -327, and -1740 (**FIGURE 2.6**). I further verified binding to RARE -224 (**FIGURE 2.6**).

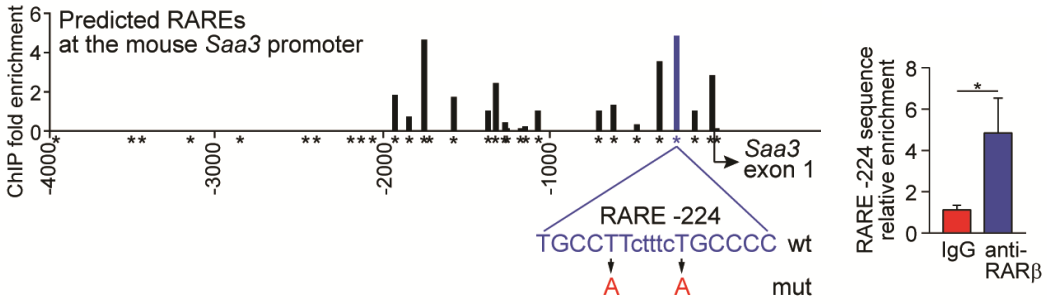

FIGURE 2.6. RARβ binds directly to the *Saa3* promoter. RARβ binding to the *Saa3* promoter was measured by chromatin immunoprecipitation (ChIP) assay with an anti-RARβ antibody. Bound promoter sequences were detected by qPCR with primers flanking each predicted retinoic acid response element (RARE, indicated by *). The RARE located 224nt upstream of the *Saa3* start site (RARE -224) was further validated by ChIP. *N*=3 replicates/group; data represent two independent experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01 as determined by Student's *t*-test.

TABLE 2.1. Predicted RARE sequences in the mouse *Saa3* promoter.

Predicted RARE sequence	RARE type[1]	RARE location[2]	RARβ ChIP fold enrichment
TGAGTGgcttcTGTCCT	DR5	-3895	ND
TAAGCTaaTGCCCT	DR2	-3556	ND
AGCTCAgttcaGGTTTA	DR5	-3537	ND
TGGTCTtcTCTCCT	DR2	-3142	ND
GAGGCAatAGCTCA	DR2	-2949	ND
TTGGCTcacgtTGTCCT	DR5	-2490	ND
TGTCCTagcagTAAGCA	DR5	-2479	ND
GGTGCCtgtagAGGTCA	DR5	-2230	ND
AGGTCAgaAAATGA	DR2	-2116	ND
TTAACTgcTGACCC	DR2	-2007	ND
TGGACTttttcTTTACT	DR5	-1915	1.9
AGTGCAcacccAAGTCC	DR5	-1832	0.8
ACAACAacaacAGAGCA	DR5	-1740	4.7
ACAACAacAGAGCA	DR2	-1737	ND
TATCCTgttgcTGCCCT	DR5	-1563	1.8
AGGACAcaggaGGGACA	DR5	-1357	1.1
AGACAAgtgaaGGGTCA	DR5	-1310	2.5
TGCACAaggccTGTTCT	DR5	-1255	0.5
TGTTCTccTGAATG	DR2	-1244	0.2
CGCACAgccccTGGACA	DR5	-1160	0.2
TCCTCTaaggaTGCTCT	DR5	-1134	0.3
AGACCCagacaTGCTCT	DR5	-1058	1.1
TGCTCTgccccTGACTG	DR5	-689	1.1
TGAACAaaTTTCCT	DR2	-603	1.4
TGGCCAgcaagAGGTCA	DR5	-463	0.4
TGAGCTcacacGGGTCT	DR5	-327	3.6
TGCCTTctttcTGCCCC[3]	DR5	-224	4.9
TCACATaaggtTGCCCT	DR5	-116	1.1
AGGTGAtgaatAGTTAA	DR5	-11	2.9
TTACCTgcTGAACG	DR2	15	0.2

[1]RARE type: The most frequent DRs with 1, 2, or 5 nucleotides spacing are termed DR1, DR2, and DR5 elements, respectively.

[2]RARE location was determined relative to the *Saa3* start site.

[3]RARE -224 was further validated by ChIP and luciferase reporter assay for *Saa3* promoter activity as shown in Fig. 3*B* and *C*.

2.3.4 SAA Promoter Activity is Dependent on a Functional RARE

To validate RARE -224 as a functional RARE, I showed promoter activity for the 4.1 kb region by a luciferase reporter assay (**FIGURE 2.7**). Introduction of point mutations into RARE -224 abolished reporter expression (**FIGURE 2.7**), establishing that this RARE is essential for *Saa3* promoter activity. Thus, RARβ activates *Saa3* transcription by binding directly to its promoter. *In silico* analysis of mouse *Saa1* and *Saa2* also identified multiple putative RAREs, suggesting that RARβ also binds directly to these promoters (**FIGURE 2.5**; **APPENDICES D** and **E**).

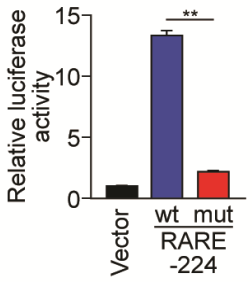

FIGURE 2.7. RARβ activates *Saa3* transcription by binding directly to RARE -224. A 4103 bp fragment of the *Saa3* promoter was fused to a firefly luciferase reporter and RARE -224 was mutated as shown in Figure 6. MODE-K cells were transfected with the wild-type (WT) or mutant (mut) reporter plasmids or empty vector and were treated with retinol and LPS for 24 hours. *N*=3 replicates/group; data represent two independent experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01 as determined by Student's *t*-test.

2.3.5 Disruption of IEC-intrinsic RAR Activity Has Systemic Effects

To determine if RARs govern SAA expression *in vivo* I studied mice with selective disruption of RAR activity in intestinal epithelial cells. The mice were derived from knock-in

mice carrying three *loxP*-flanked polyadenylation sequences upstream of an open reading frame encoding a dominant negative form of human RARα (dnRAR) that disrupts RAR activity (62, 70). I crossed mice carrying the epithelial cell-restricted *Villin-Cre* transgene (71) with the dnRAR knock-in mice to selectively disrupt RAR activity in epithelial cells (**FIGURE 2.8**). Expression of *Saa1-3* and SAA protein levels were lower in the dnRAR$^{Villin\text{-}Cre}$ mice as compared to *Villin-Cre* controls (**FIGURE 2.9**), indicating that RAR activity regulates intestinal SAA expression *in vivo*. I additionally found increased bacterial burdens of *Salmonella typhimurium* in spleen, liver, and ileum, 48 hours post-infection (**FIGURE 2.10**).

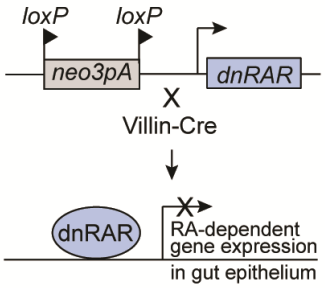

FIGURE 2.8. Selective disruption of RAR activity in intestinal epithelial cells. Knock-in mice carry a neomycin resistance gene and 3 loxP-flanked polyadenylation sequences that are located upstream of an open reading frame encoding a dominant negative (dn)RAR. Breeding to Villin-Cre mice results in selective expression of the dnRAR in intestinal epithelial cells.

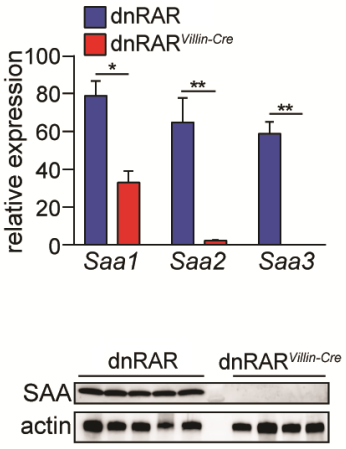

FIGURE 2.9. Intestinal SAA expression is dependent on intestinal epithelial cell-intrinsic RAR activity. qPCR analysis of *Saa* transcripts and Western blot of SAA in the distal small intestines of *dnRAR* mice and dnRAR$^{Villin\text{-}Cre}$ mice. *N*=4-5 mice/group; data represent two independent experiments.

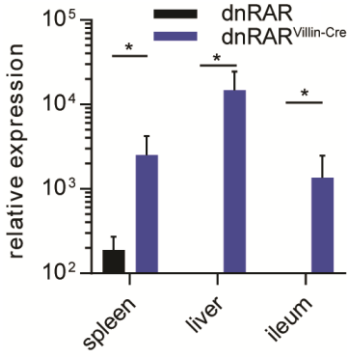

FIGURE 2.10. Intestinal epithelial cell-intrinsic RAR activity is critical for host defense. Bacterial burdens (cfu) in the distal small intestine (ileum), spleen, and liver of *dnRAR* and *dnRAR*$^{Villin\text{-}cre}$ littermates 48 hours after oral infection with 10^{10} cfu of *S.* Typhimurium. *N*=5 mice/group; data represent three independent experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01, ****P*<0.001 as determined by Student's *t*-test.

2.4 DISCUSSION

The intestinal epithelium is a vital interface between the environment and the underlying immune system. Epithelial cells sense key environmental factors, including the microbiota and the host diet, and use these cues to orchestrate adaptive immunity in subepithelial tissues. The *Saa* genes highlight the environmental sensing function of the gut epithelium by requiring both microbiota and vitamin A for expression in the intestinal epithelium. This study unravels the molecular basis for how vitamin A directs SAA expression by showing that RARβ activates the expression of *Saa* genes through direct promoter binding.

The next question I explore is whether epithelial RARβ has additional important roles in regulating immunity. By generating a mouse model with an intestinal epithelial cell-specific knockout of RARβ, both innate and adaptive immune responses could be studied in detail. As the three RAR isoforms (α, β, and γ) are conserved across species (72), suggesting a unique conserved function for each isoform, comparative models were considered. As the RARα isoform is the predominant isoform expressed in the gut, and the role of T-cell intrinsic RARα has been described in great detail, the role of epithelial cell-intrinsic RARα is another point of interest. In the next section, I explore the specific contributions of RARβ and whether RARα and RARβ have non-redundant functions in the regulation of intestinal immune function.

2.5 ACKNOWLEDGEMENTS

I thank Dr. Pierre Chambon for the kind gift of the *Rara*$^{fl/fl}$ and *Rarb*$^{fl/fl}$ mice and Dr. Shanthini Sockanathan for the *dnRAR* mice.

CHAPTER THREE
RARβ Regulates SAA and Intestinal Immunity

3.1 INTRODUCTION

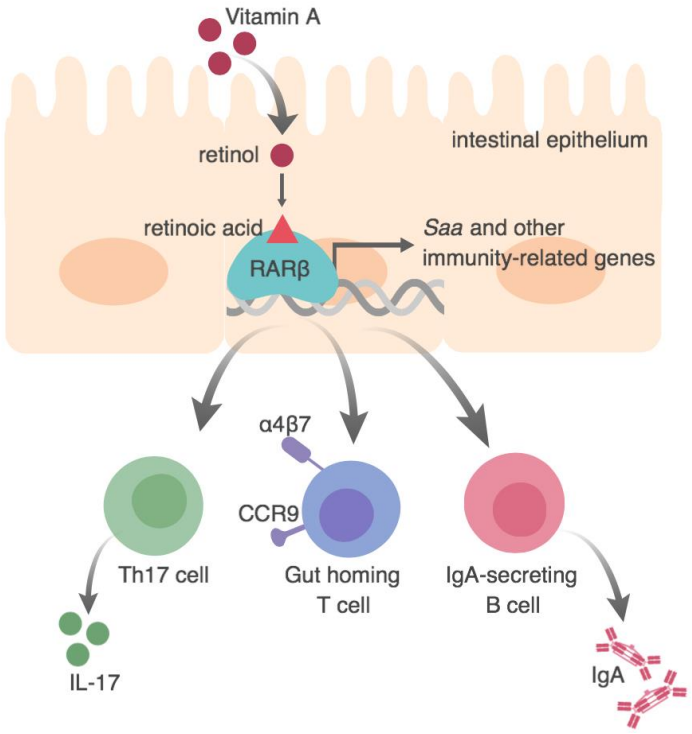

FIGURE 3.1. Epithelial RARβ regulates intestinal immunity. The transcription factor RARβ is activated by retinoic acid, which is derived from dietary vitamin A. Epithelial RARβ controls an immune transcriptional program that includes members of the *Saa* family. Epithelial RARβ promotes IL-17 production by Th17 cells, which is known to require SAA (Iwata et al.). Epithelial RARβ also promotes vitamin A-dependent immune responses that include the generation of gut-homing CD4+ T cells (marked by the gut homing receptors α4β7 and CCR9) (Iwata et al. Immunity) and IgA-secreting cells (Mora et al., Science). Our findings reveal a mechanism by which the intestinal epithelium regulates adaptive immunity in response to diet.

Having shown that RARβ activates *Saa* expression through direct binding to retinoic acid response elements (RAREs) in *Saa* promoters *in vitro*, I next generated a mouse model to verify the physiological significance of this finding. Consistent with the known role of

SAAs in regulating Th17 cell effector function (37), I find that epithelial RARβ additionally regulates IL-17 production by Th17 cells. More generally, I show that epithelial RARβ regulates other known vitamin A-dependent adaptive immune responses, including the development of gut-homing $CD4^+$ T cells and IgA-producing B cells. My findings thus provide insight into how the intestinal epithelium senses dietary vitamin A status to control vitamin A-dependent adaptive immunity (**FIGURE 3.1**)

3.2 METHODS

3.2.1 Animals and Animal Husbandry

Rarb$^{\Delta IEC}$ mice were generated by crossing *Rarb*$^{fl/fl}$ mice (73) with *Villin*-Cre mice (Jackson Laboratories), which express *Cre* recombinase under the control of the intestinal epithelial cell-specific *Villin* promoter (71). dnRAR$^{\text{Villin-Cre}}$ mice were generated by crossing dnRAR mice (62) with *Villin-Cre* mice. *Saa1/2*$^{-/-}$ mice were obtained from F. de Beer (23); RARa$^{fl/fl}$ mice were from P. Chambon (63) through Y. Belkaid (National Institutes of Health). 6-14 week-old mice were used for all experiments. Because microbiota composition is known to impact intestinal immune cells frequencies, I used age- and sex-matched littermates that were cocaged to minimize microbiota differences in all experiments. Experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of the University of Texas Southwestern Medical Center.

3.2.2 Histology

Paraffin embedded sections of Bouin's fixed mouse distal ileum were cut, and heat-induced epitope retrieval was performed in a 0.05% sodium citrate buffer (pH 6.0). The slides were incubated with rabbit anti-mouse SAA (32) (1:50 dilution) at 4^{o}C overnight. H&E and PAS staining were performed by the UTSW Histology Core.

3.2.3 *Salmonella* Infection Mouse Model

Salmonella enterica serovar *typhimurium* (SL1344) was grown in Luria-Bertani broth with ampicillin (100 μg/ml) at 37°C. Mice were infected intragastrically by gavage with *S. typhimurium* at 10^{10} colony-forming units (cfu) per mouse. cfu in the small intestine, liver, and spleen were determined by dilution plating on Luria broth plates containing ampicillin (100 μg/ml).

3.2.4 RNA-seq analysis

RNA was extracted and purified from ileums of 3 mice per experimental group. RNA quality was assessed on an Agilent 2100 Bioanalyzer. Sequencing libraries were prepared using the TruSeq RNA sample preparation kit v2 (Illumina) and sequenced on an Illumina HiSeq 2500 for signal end 50 bp length reads. Sequence data were mapped against the mm10 genome using TopHat (2) and FPKMs were generated using Cuffdiff (3) with default

parameters. Altered expression was defined as a >2 fold increase or decrease in average FPKM reads compared between the two groups.

832 genes were differentially expressed, greater than 2-fold, and with *p* values <0.05. These 832 genes were then analyzed with PANTHER Gene Ontology Classification System to generate GO Biological Processes enriched for in the gene list. The top 20 differentially regulated GO Biological Processes (gene categories) are shown here, organized as immunity-related and metabolism-related genes. Bonferroni correction for multiple testing and a *p* value filter of <0.05 were applied to the PANTHER analysis.

3.2.5 Laser Capture Microdissection

Laser capture microdissection was performed as described (74). In brief, 5 cm of distal small intestine was washed and snap-frozen in optimum cutting temperature (OCT) compound (Fisher 23-730.571). Frozen sections were cut to a thickness of 7 µm and fixed in 70% ethanol, then stained with Methyl Green and eosin. Freshly stained sections were immediately used for laser capture microdissection of intestinal epithelial cells using an Arcturus PixCell IIe system, and 5,000-10,000 pulses were obtained from each section. RNA was extracted from isolated IECs by incubating with 14 µl RNA Extraction Buffer from the PicoPure RNA Isolation Kit (Life Technology 12204-01) for 30 min, then stored at -80^{o}C until use. Extracted RNA was purified using PicoPure RNA Isolation Kit following the

manufacturer's protocol. RNA quality and concentration were determined by Agilent 2100 Bioanalyzer or RiboGreen S2 RNA Assay Kit (Thermo Fisher R11490).

3.2.6 SI Lamina Propria Lymphocyte Isolation

Small intestinal lamina propria lymphocytes were isolated as previously described (75). ~2 x 10^6 cells were treated with 50 ng/ml phorbol myristate acetate (PMA), 1 mM ionomycin, and 1 mg/ml brefeldin A for 4 hours. For the rescue of IL-17 production, 5 μg/mL recombinant mSAA1 (R&D Systems) was added to whole lamina propria samples during the PMA/ionomycin/brefeldin A stimulation. Cells were fixed and permeabilized for 30 minutes and stained with commercial antibodies from Biolegend, BD, and eBiosciences. Flow cytometry was performed using the LSRII and data were analyzed with FlowJo software (TreeStar).

3.2.7 Flow Cytometry

Foxp3/transcription factor staining reagents for fixation and permeabilization were utilized (eBiosciences). Zombie Yellow (Biolegend) fixable viability dye was used to gate live cells prior to further analysis. Antibodies used to characterize Th17 populations include: CD45-FITC (Biolegend), CD4-PECy7 (Biolegend), RORgt-PE (eBiosciences), IL-17a-BV421 (BD Bioscience), IL-22-APC (eBiosciences). Antibodies used to characterize gut homing T cells include: CD45-FITC (Biolegend), CD4-PECy7 (Biolegend), FoxP3-APC

(Biolegend), CCR9-PE (Biolegend), a4b7-Pacific Blue (BD Bioscience). Antibodies used to characterize IgA-producing B cells include CD45-BV650 (Biolegend), CD19-FITC (eBiosciences), B220-APC (eBiosciences), and IgA-PE (eBiosciences). Antibodies used to characterize dendritic cell populations include CD45-Pacific Blue (Biolegend), CD11c-APC-Cy7 (eBiosciences), CD103-FITC (Biolegend), MHCII-BV711 (eBiosciences), CD11b-PE (Biolegend), and F480-APC (eBiosciences).

3.2.8 IgA Quantification by ELISA

Total protein was isolated by homogenizing mouse feces in PBS with protease inhibitor cocktail (Roche). Samples were rotated at 4^{o}C for 4 hours and centrifuged at 16,000*g* for 15 minutes. Supernatants were serially diluted to quantify IgA levels per manufacturer instructions (Invitrogen).

3.2.9 Western Blot

Total protein was isolated from cells or homogenized tissues as previously described (60). Membranes were detected with antibodies against SAA (32), RARα (Affinity Bioreagents), RARβ (Invitrogen), RARγ (ThermoFisher), and actin (ThermoFisher). The anti-SAA antibody was generated by Cocalico and recognizes all isoforms (32).

3.2.10 Statistical Analysis

All statistical analyses were performed using two-tailed Student's *t* test. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; and ns, $P > 0.05$.

3.3 RESULTS

3.3.1 IEC-intrinsic SAA Expression is Dependent on RARβ Expression

To determine if RARβ controls intestinal SAA expression *in vivo*, I created mice with an intestinal epithelial cell-specific deletion of *Rarb*. I crossed mice with a *loxP*-flanked *Rarb* allele ($Rarb^{fl/fl}$) (73) with *Villin-Cre* transgenic mice (71) to produce $Rarb^{\Delta IEC}$ mice (**FIGURE 3.2**).

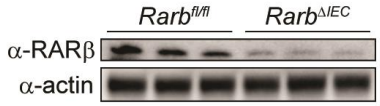

FIGURE 3.2. Validation of RARβ knockdown in intestinal epithelium of $Rarb^{\Delta IEC}$ mice. Western blot of small intestinal epithelial RARβ from $Rarb^{fl/fl}$ and $Rarb^{\Delta IEC}$mice, with actin as a control. Epithelial cells were crudely isolated with EDTA (1mM) in RPMI media. N=3 mice/group; data represent two independent experiments.

SAAs were expressed throughout the small intestinal epithelium of $Rarb^{fl/fl}$ mice but showed markedly reduced expression in $Rarb^{\Delta IEC}$ mice (**FIGURE 3.3**). qPCR analysis of laser capture microdissected epithelial cells showed reduced abundance of transcripts encoding all three mouse *Saa* isoforms (*Saa1*, *Saa2*, and *Saa3*) (**FIGURE 3.3**), and SAA protein levels were reduced in the small intestines of $Rarb^{\Delta IEC}$ mice (**FIGURE 3.3**).

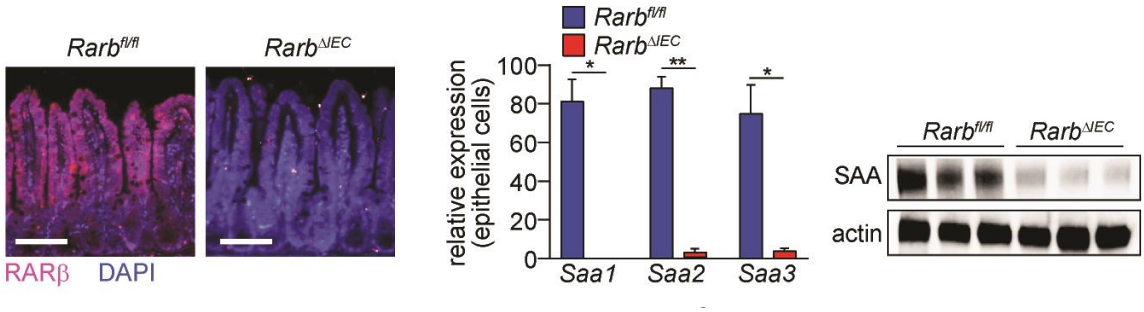

FIGURE 3.3. Epithelial RARβ controls SAA expression. Immunofluorescence detection of SAA in the small intestines of *Rarb*$^{fl/fl}$ and *Rarb*$^{\Delta IEC}$ mice. Scale bars, 50 μm. qPCR analysis of *Saa* expression in small intestinal epithelial cells acquired by laser capture microdissection. Western blot of small intestinal SAA from *Rarb*$^{fl/fl}$ and *Rarb*$^{\Delta IEC}$ mice, with actin as a control. *N*=3 mice/group; data represent three independent experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01, ****P*<0.001 as determined by Student's *t*-test.

3.3.2 IEC-intrinsic Ablation of RARβ Increases Susceptibility to Infection and Injury

Our finding that RARβ regulates the expression of immune genes in the intestinal epithelium suggested that RARβ might promote resistance to bacterial infection of the intestine. I orally challenged *Rarb*$^{\Delta IEC}$ and *Rarb*$^{fl/fl}$ mice with the gastrointestinal pathogen *Salmonella enterica* Serovar Typhimurium (*Salmonella* Typhimurium). 48 hours later, *Rarb*$^{\Delta IEC}$ mice had increased bacterial burdens in the ileum, liver, and spleen (**FIGURE 3.4**). Measurements of serum fluorescence following FITC-dextran administration indicated that the increased bacterial dissemination in the *Rarb*$^{\Delta IEC}$ mice did not arise from increased nonspecific barrier permeability (**FIGURE 3.5**). Thus, epithelial RARβ contributes to host resistance to intestinal bacterial infection and dissemination.

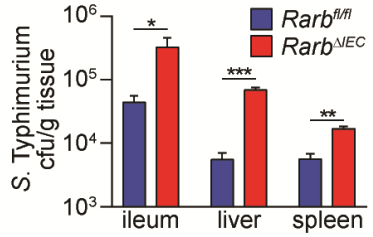

FIGURE 3.4. RARβ is critical for host resistance to intestinal bacterial infection. Bacterial burdens (cfu) in the distal small intestine (ileum), spleen, and liver of *Rarb*$^{fl/fl}$ and *Rarb*$^{\Delta IEC}$ littermates 48 hours after oral infection with 10^{10} cfu of *S*. Typhimurium. *N*=5 mice/group; data represent three independent experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01, ****P*<0.001 as determined by Student's *t*-test.

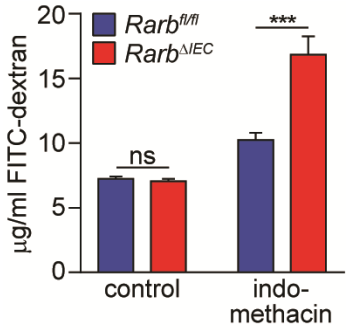

FIGURE 3.5. Intestinal permeability measurements in *Rarb*$^{fl/fl}$ and *Rarb*$^{\Delta IEC}$ mice. Serum concentrations of FITC-dextran 4 hours after oral gavage are shown. As a positive control, intestinal epithelial damage was induced by pretreatment with indomethacin (15 mg/kg in DMSO) for 1 hour prior to FITC-dextran administration (600 mg/kg body weight; 4 kDa; Sigma). N=3 mice/group, and each sample was analyzed in triplicate. Means±SEM are plotted. ***P<0.001 as determined by Student's t-test. ns, not significant.

3.3.3 IEC-intrinsic Ablation of RARβ Broadly Impacts Intestinal Immunity

To broaden our insight into epithelial RARβ function, I identified other intestinal genes that were regulated by RARβ. I used RNAseq to compare the transcriptomes of *Rarb*$^{fl/fl}$ and *Rarb*$^{\Delta IEC}$ mouse small intestines, finding 832 differentially-abundant transcripts. Gene

Ontology (GO) term analysis identified gene categories that were highly represented among the differentially expressed genes, including multiple categories related to immunity and metabolism (**FIGURES 3.6** and **3.7**). In addition to *Saa1*, *Saa2*, and *Saa3* transcripts, the immunological gene category included transcripts encoding epithelial proteins involved in antimicrobial defense, including several members of the *Defa* (defensin) gene family, *Reg3b*, *Reg3g*, and *Ang4* (**FIGURES 3.7**). Also represented were transcripts encoding proteins involved in inflammasome function, including *Card9*, *Aim2*, *Naip6*, *Casp4*, and *Gsdmc2*, *3*, *and 4* (**FIGURES 3.7**). Thus, RARβ controls an immune gene transcriptional program in intestinal epithelial cells. This suggests that epithelial sensing of vitamin A status may regulate antimicrobial defense as well as inflammasome function.

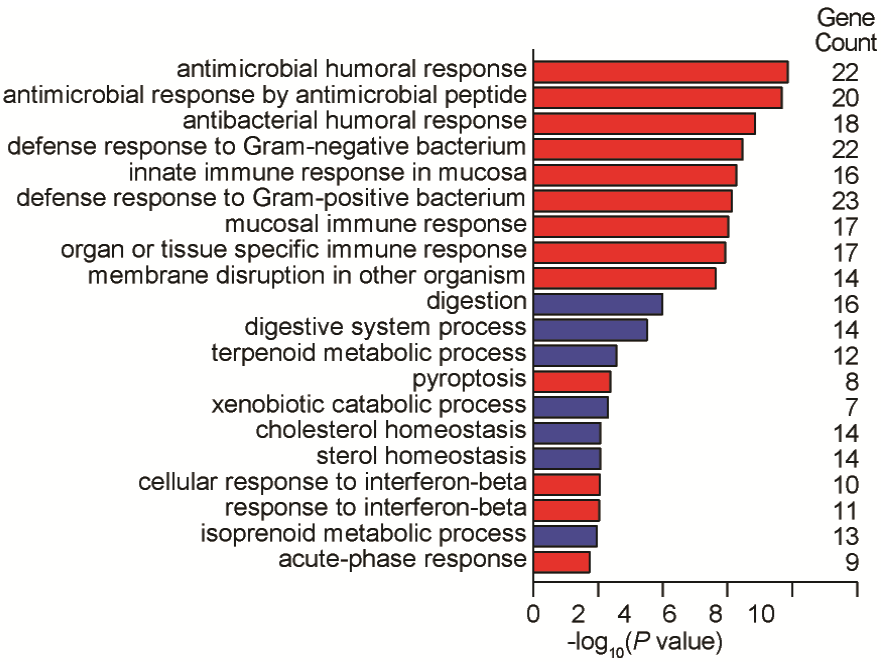

FIGURE 3.6. RARβ controls an epithelial transcriptional program. Gene ontology (GO) biological process enrichment analysis of genes identified by RNA sequencing analysis as being differentially regulated in whole tissue ileum from *Rarb*$^{fl/fl}$ and *Rarb*$^{\Delta IEC}$ mice. Immunological gene categories are highlighted in red, metabolic gene categories are highlighted in blue.

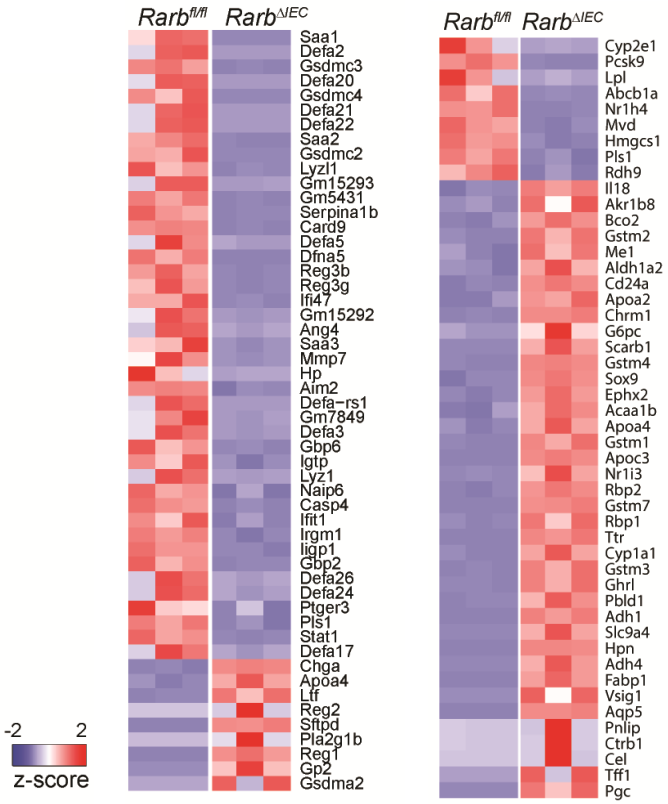

FIGURE 3.7. RARβ controls epithelial immune and metabolic gene expression programs. Heat maps displaying expression levels of the genes that were identified as having immune functions (left) or metabolic functions (right) by the GO analysis in Figure 13, and which had a - $\log_{10}$(*P* value) >5. *N*=3 mice/group. Means±SEM are plotted. **P*<0.05, ***P*<0.01, ****P*<0.001 as determined by Student's *t*-test.

3.3.4 IEC-intrinsic Ablation of RARβ Results in Defective Th17 Function

Th17 cells are a specialized subset of CD4$^+$ T cells that require the transcription factor RORγt for lineage commitment (76). Th17 cells secrete a distinct set of cytokines,

including IL-17A, IL-17F, and IL-22, and promote host defense against extracellular bacteria (77). SAA secretion by intestinal epithelial cells promotes Th17 cell effector functions (37). Although SAAs are not required for Th17 cell lineage commitment, they boost Th17 cell effector function by stimulating IL-17A production in differentiated Th17 cells (37).

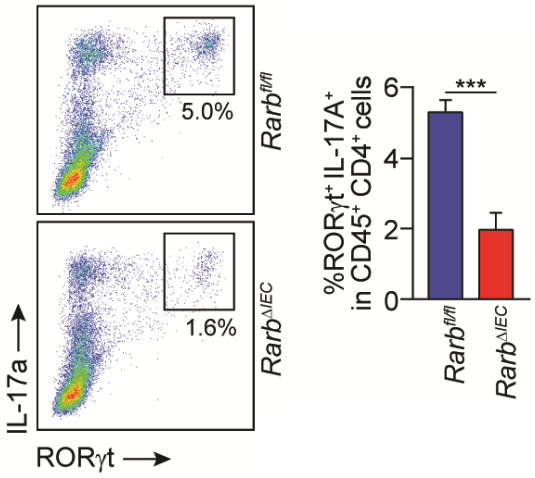

FIGURE 3.8. Epithelial RARβ regulates intestinal Th17 cell effector function. IL-17a+ RORγt+ Th17 cells as a percentage of CD45+CD4+ cells in the small intestinal lamina propria are shown in representative flow cytometry plots, with quantification. *N*=3 mice/group; data represent four independent experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01, as determined by Student's *t*-test. ns, not significant.

Our finding that epithelial RARβ governs SAA expression suggested that epithelial RARβ might also regulate IL-17 production by intestinal Th17 cells. In support of this idea, *Il17a* expression was lowered in the intestines of *Rarb*$^{\Delta IEC}$ mice, paralleling the lowered *Il17a* expression in *Saa1/2*$^{-/-}$ mouse intestines ((37); **FIGURE 4.1**). Overall frequencies of CD4^{+} RORγt^{+} Th17 cells were similar between *Rarb*$^{fl/fl}$ and *Rarb*$^{\Delta IEC}$ mice, consistent with

the fact that SAA is dispensable for Th17 cell lineage commitment (37). However, IL-17A production was lower in RORγt^{+} Th17 cells from *Rarb*$^{\Delta IEC}$ mice (**FIGURE 3.8**), indicating that epithelial RARβ regulates Th17 cell effector function. IL-17A production was restored by the addition of recombinant SAA1 to cultured intestinal lamina propria cells from *Rarb*$^{\Delta IEC}$ mice (**FIGURE 3.9**). This indicates that SAA1 is sufficient to rescue the defective Th17 effector function conferred by epithelial RARβ deficiency and suggests that the defect in Th17 IL-17 production in *Rarb*$^{\Delta IEC}$ mice is due to the lowered SAA expression. Thus, epithelial RARβ promotes intestinal Th17 cell effector function, likely by activating *Saa* expression.

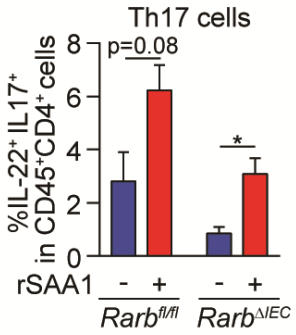

FIGURE 3.9. Recombinant SAA1 (rSAA1) rescues lowered IL-17+ IL-22+ T cell numbers from *Rarb*$^{\Delta IEC}$ mice. Small intestinal lamina propria cells were isolated and stimulated *ex vivo* with ionomycin, PMA, brefeldin A, and rSAA1 for 4 hours prior to flow cytometry analysis. *N*=3 mice/group; data represent four independent experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01, as determined by Student's *t*-test. ns, not significant.

3.3.5 IEC-intrinsic Ablation of RARβ Results in Disrupted T Cell Homing and IgA Production

Vitamin A is essential for the development of key intestinal adaptive immune cells, including gut homing $CD4^+$ T cells and IgA-producing B cells (2, 3). RA produced by intestinal DCs imprints gut homing receptors on activated T and B lymphocytes (2, 3) and induces IgA expression in gut homing B lymphocytes (2). Given the requirement for RA, I sought to determine if epithelial RARβ regulates the development of gut homing T cells and IgA-producing B cells. Supporting this idea, $Rarb^{\Delta IEC}$ mice had reduced frequencies of $CD4^+$ T cells imprinted with the gut homing receptors α4β7 and CCR9 in both the ileum and the mesenteric lymph nodes (**FIGURE 3.11**). The decreased frequencies of gut homing T cells was associated with decreased overall numbers of intestinal $CD4^+$ T cells (**FIGURE 3.10**). $Rarb^{\Delta IEC}$ mice also had reduced frequencies of small intestinal IgA^+ B cells and decreased fecal IgA concentrations (**FIGURE 3.12**). Thus, epithelial RARβ promotes the development of gut homing T cells and IgA-producing B cells.

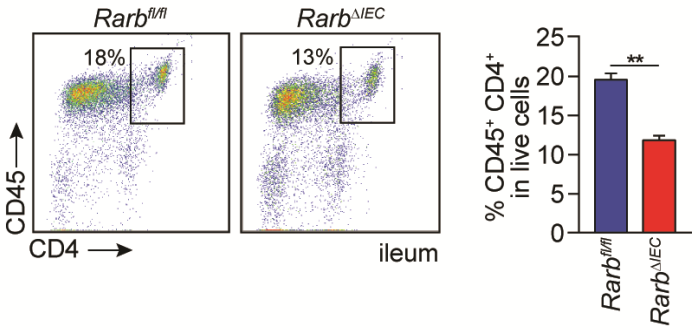

FIGURE 3.10. Decreased frequency of small intestinal CD4 T cells among total live cells in *Rarb*$^{\Delta IEC}$ mice. Representative flow cytometry plots and quantification data from multiple mice. *N*=3 mice/ group; data represent four independent experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01 as determined by Student's *t*-test.

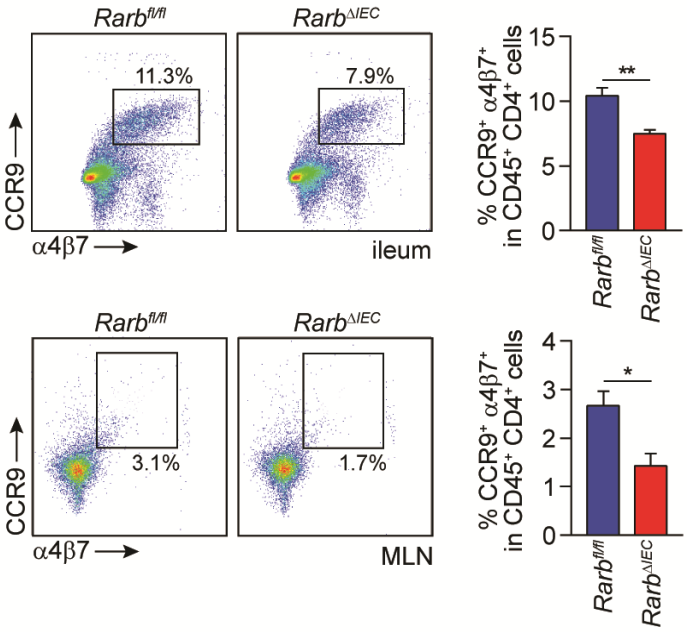

FIGURE 3.11. Decreased expression of the gut homing markers α4β7 and CCR9 on small intestinal T cells (CD45+ CD4+) in *Rarb*$^{\Delta IEC}$ mice. Representative flow cytometry plots and quantification data from multiple mice. *N*=3 mice/group; data represent four independent experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01 as determined by Student's *t*-test.

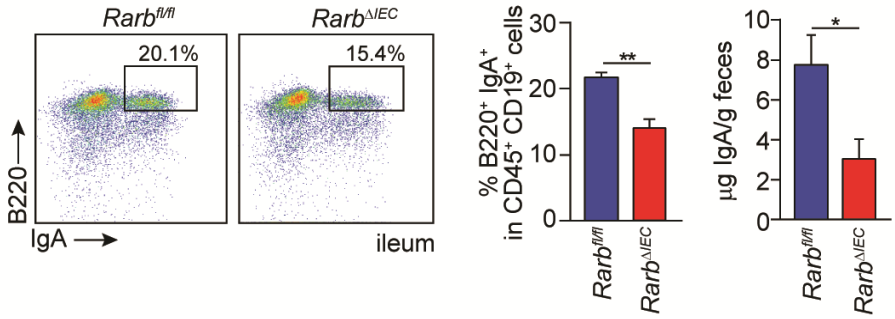

FIGURE 3.12. Decreased frequency of small intestinal IgA- producing B cells (B220+ IgA+) in *Rarb*$^{\Delta IEC}$ mice. Representative flow cytometry plots with quantification data from multiple mice. Quantification of fecal IgA by ELISA. *N*=3-4 mice/group; data represent two independent experiments.

3.3.6 IEC-intrinsic Ablation of RARβ Does Not Impact All Vitamin A-dependent Immune Populations

RARα is a closely related RAR isoform that also impacts several aspects of intestinal immunity, including Paneth and goblet cell development, numbers of RA-producing DCs, and overall B cell numbers (8). I considered whether RARα and RARβ might have overlapping functions in the control of intestinal adaptive immunity. I found that *RARb*$^{fl/fl}$ and *Rarb*$^{\Delta IEC}$ mice had similar numbers of small intestinal Paneth cells or goblet cells (**FIGURE 3.13**), CD11c^{+} CD103^{+} cells (which include RA-producing DCs) (**FIGURE 3.14**), and B220^{+} B cells (**FIGURE 3.15**).

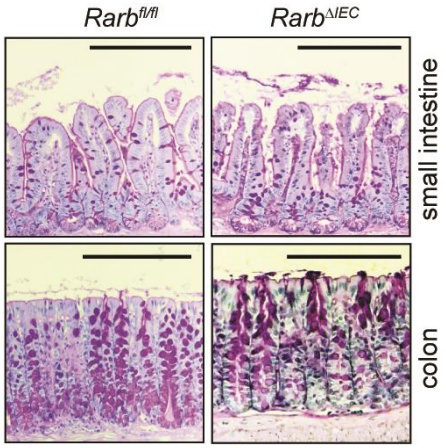

FIGURE 3.13. Numbers of Paneth cells and goblet cells are similar between Rarb$^{fl/fl}$ and Rarb$^{\Delta IEC}$ mice. Periodic Acid Schiff (PAS) staining was performed on small intestines and colons from Rarb$^{\Delta IEC}$ mice and their Rarbfl/fl littermates in order to detect goblet cells. Scale bars, 220 μm. Data represent three independent experiments.

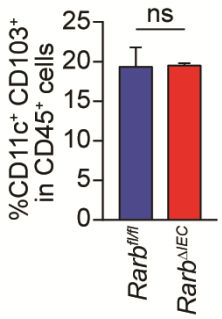

FIGURE 3.14. Numbers CD11c+ CD103+ dendritic cells are similar between Rarb$^{fl/fl}$ and Rarb$^{\Delta IEC}$ mice. Cell suspensions from the small intestinal lamina propria of Rarb$^{\Delta IEC}$ mice and their Rarb$^{fl/fl}$ littermates were analyzed by flow cytometry. DCs were identified using antibodies against CD45, CD11c, and CD103. Frequencies of CD11c+ CD103+ cells (which include RA-producing DCs) within the CD45+ cell subset are shown. N=3 mice per group; data represent three independent experiments. Means±SEM are plotted. ns, not significant as determined by Student's t-test.

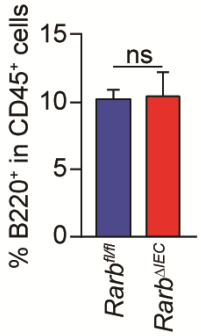

FIGURE 3.15. Numbers of total B cells are similar between Rarb$^{fl/fl}$ and Rarb$^{\Delta IEC}$ mice. Cell suspensions from the small intestinal lamina propria of Rarb$^{\Delta IEC}$ mice and their Rarb$^{fl/fl}$ littermates were analyzed by flow cytometry. B cells were identified using antibodies against CD45 and B220. Frequencies of B220+ cells within the CD45+ cell subset are shown. N=3 mice per group; data represent three independent experiments. Means±SEM are plotted. ns, not significant as determined by Student's t-test.

3.3.7 Vitamin A Directs Unique Pathways in Immunity through Specific RAR Isoforms

The integrity of RARs are important for the balancing act between differentiation of tissue stem cells and self-renewal. As these nuclear receptors are required for tissue homeostasis in adulthood, the best tools available in the study of these receptors involve more specific, cell-intrinsic disruption of specific RAR subtypes. Broadly speaking, our observations suggest that IEC-intrinsic ablation of RARα results in perturbations to intestinal homeostasis that are developmental in nature, owing to RARα being the predominantly expressed subtype in the intestine over the course of development. The IEC-intrinsic ablation of RARβ does not result in broad defects for barrier integrity or gross pathological differences as reported for RARα (8).

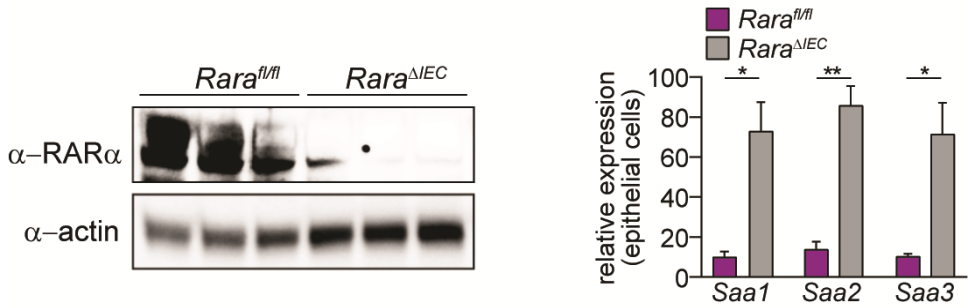

FIGURE 3.16. Epithelial cell RARα is dispensable for Saa expression in the small intestine. Rarα$^{\Delta IEC}$ mice were created by crossing Rara$^{fl/fl}$ with mice carrying a Villin-Cre transgene. RARa expression was assessed by Western blotting of small intestinal epithelial cells isolated by EDTA treatment as described (left). Actin was detected as a control. *Saa* expression was quantified by qPCR analysis of small intestinal epithelial cells isolated by EDTA treatment (right). N=3 mice per group. *P<0.05, **P<0.01 as determined by Student's t-test.

In contrast to *Rarb*$^{\Delta IEC}$ mice, *Rara*$^{\Delta IEC}$ mice had elevated intestinal *Saa* expression (**FIGURE 3.16**). Frequencies of gut homing T cells (**FIGURE 3.17**), and total intestinal CD4$^+$ T cell numbers (**FIGURE 3.18**) were also elevated relative to *Rara*$^{fl/fl}$ mice. Frequencies of intestinal IgA-producing B cells and fecal IgA quantities (**FIGURE 3.19**) were similar as compared to *Rara*$^{fl/fl}$ mice. These data indicate that RARα is dispensable for CD4$^+$ T cell homing and B cell expression of IgA, and show that epithelial RARα and RARβ regulate distinct aspects of intestinal immunity.

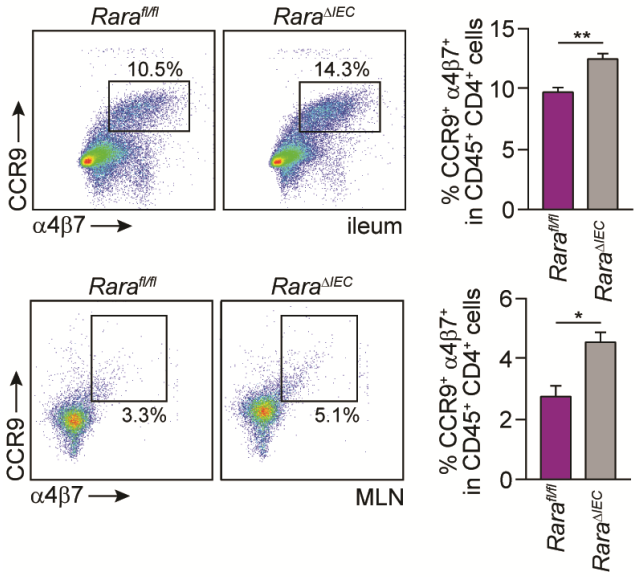

FIGURE 3.17. Epithelial cell RARα is dispensable for CD4+ T cell homing. Flow cytometry analysis and quantification of mouse small intestinal T cells (CD45+ CD4+) expressing a4b7 and CCR9, which define a gut homing phenotype. Representative flow cytometry plots for T cells isolated from the ileum (top) and MLN (bottom) of Rarα$^{fl/fl}$ and Rarα$^{\Delta IEC}$ littermates. N=3 mice per group; data represent three independent experiments. Means±SEM are plotted. *P<0.05, **P<0.01, ***P<0.001as determined by Student's t-test. ns, not significant.

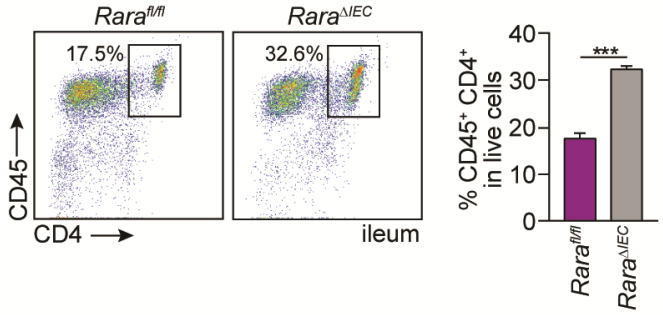

FIGURE 3.18. Epithelial cell RARα is dispensable for the generation of CD4+ T cells. Flow cytometry analysis and quantification of CD4+ T cells from the small intestines of Rarα$^{fl/fl}$ and Rarα$^{\Delta IEC}$ littermates. N=5 mice per group; data are representative of two independent experiments. Means±SEM are plotted. *P<0.05, **P<0.01, ***P<0.001as determined by Student's t-test. ns, not significant.

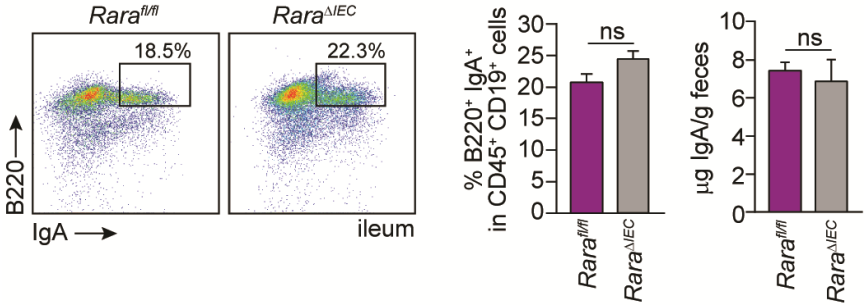

FIGURE 3.19. Epithelial cell RARα is dispensable for B cell expression and production of IgA. Flow cytometry analysis and quantification of IgA-secreting B220+ cells from Rarα$^{fl/fl}$ and Rarα$^{\Delta IEC}$ littermates (left and middle). N=5 mice per group; data represent two independent experiments. Quantification of fecal IgA by ELISA (right). N=5 mice per group; data represent two independent experiments. Means±SEM are plotted. *P<0.05, **P<0.01, ***P<0.001as determined by Student's t-test. ns, not significant.

Additional observations thought to owe to the pathology of *Rara$^{\Delta IEC}$* mice include increases in Klf4 and Reg3g gene expression (8), which are not found in *Rarb$^{\Delta IEC}$* mice (**FIGURE 3.20**), again supporting the ability of vitamin A to regulate immunity through distinct and unique mechanisms.

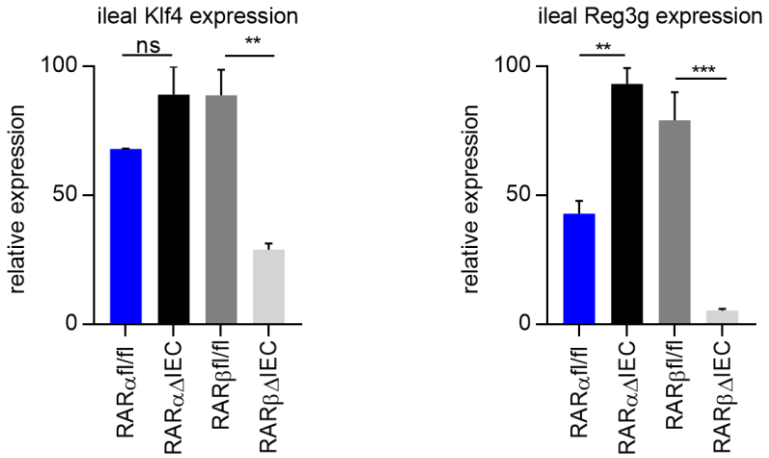

FIGURE 3.20. Klf4 and Reg3g overexpression in Rarα$^{\Delta IEC}$ mice not found in Rarβ$^{\Delta IEC}$ mice. The upregulation of genes contributing to pathology in Rarα$^{\Delta IEC}$ mice, are downregulated in Rarβ$^{\Delta IEC}$ mice. N=3 mice per group; data represent two independent experiments. Means±SEM are plotted. *P<0.05, **P<0.01, ***P<0.001as determined by Student's t-test. ns, not significant.

3.3.7 Microbiota-driven effect on RARβ activity

An initial observation that prompted my interest in IEC-intrinsic RARβ is that RARβ expression is induced in response to colonization of germ-free (microbiologically sterile) mice or bacterial infection of conventionally colonized mice (**FIGURE 3.21**)

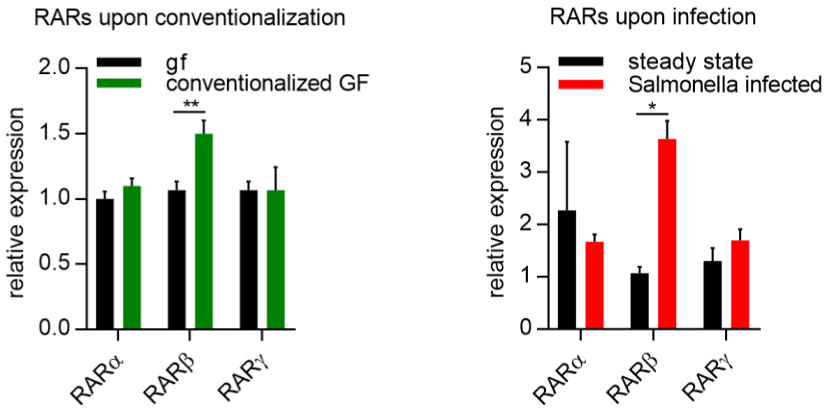

FIGURE 3.21. Bacterial stimulation drives intestinal epithelial RARβ expression. qPCR analysis of intestinal epithelial cells from germ-free (microbiologically sterile) mice sacrificed 48 hours post-stimulation (left) or conventionally colonized mice sacrificed 48 hours post-infection with *Salmonella typhimurium* (10^{10} cfu/mouse) (right). Intestinal epithelial cells were crudely isolated with EDTA. N=5 mice per group; data represent two independent experiments. Means±SEM are plotted. *P<0.05, **P<0.01, ***P<0.001as determined by Student's t-test. ns, not significant.

The induction of RARβ in response to microbial stimulation indicated the possibility of an inducible vitamin A-directed transcriptional program that might be important for an

early immediate immune response. The fact that SAA is a key immunomodulatory protein regulated by RARβ, also suggested that RARβ could be a useful tool in tightly regulating processes like the acute phase response, which require rapid induction and clearance to avoid serious pathology. The complexity of RAR regulation mixed with the ability of RXRs to heterodimerize with a variety of other nuclear receptors, puts vitamin A metabolism in a prime position to intersect with other pathways such as lipid metabolism and bile acid synthesis and transport.

3.4 DISCUSSION

The intestinal epithelium facilitates communication between the external environment and the underlying immune system. Epithelial cells sense key environmental factors, including the microbiota and the host diet, and use these cues to orchestrate adaptive immunity in subepithelial tissues. The *Saa* genes highlight the environmental sensing function of the gut epithelium by requiring both microbiota and vitamin A for expression in the intestinal epithelium. I have now unraveled the molecular basis for how vitamin A directs SAA expression by showing that RARβ activates the expression of *Saa* genes through direct promoter binding. More generally, I show that epithelial RARβ regulates the function of Th17 cells, the development of gut homing T cells, and the development of IgA-producing B cells. Our findings thus provide important mechanistic insight into how the intestinal epithelium senses vitamin A to regulate intestinal adaptive immunity.

The three RAR isoforms (α, β, and γ) are conserved across species (72), suggesting a unique conserved function for each isoform. Supporting this idea, I found that RARβ was uniquely required for expression of *Saa* genes, and that RARα and RARβ have non-redundant functions in the regulation of intestinal immune function. While RARα regulates the development of intestinal epithelial cell secretory lineages, RA-producing DCs, and overall B cell populations (8), our findings show that RARβ regulates Th17 cell effector function, the development of gut homing T cells, and IgA-producing B cells. Thus, epithelial RARα and RARβ are both essential for intestinal immune homeostasis but regulate distinct aspects of immunity.

Our finding that epithelial RARβ promotes IL-17 production by Th17 cells suggests that dietary vitamin A promotes Th17 cell effector function. This idea is supported by prior work showing that mice fed a vitamin A-deficient diet exhibit lowered intestinal IL-17 production (4). Furthermore, mice carrying a T cell-specific *Rara* deletion show lowered IL-17 production by Th17 cells (4), indicating that T cell-intrinsic RA signaling is required for Th17 cell effector function. Taken together, these findings indicate that Th17 cell effector function is a component of vitamin A-dependent intestinal immunity.

I propose that the function of SAAs in retinol transport could explain the requirement for both epithelial cell-intrinsic RARβ and T cell-intrinsic RARα in Th17 cell effector function. Given that production of IL-17 by Th17 cells requires vitamin A (4) and that SAAs

transport the vitamin A derivative retinol, it is possible that SAAs deliver retinol directly to Th17 cells for conversion to RA and activation of RARα. Alternatively, SAAs could deliver retinol to antigen presenting cells (such as dendritic cells or macrophages) for conversion to RA and delivery to Th17 cells. Defective Th17 cell function could also help explain the increased susceptibility of *Rarb*$^{\Delta IEC}$ mice to *Salmonella typhimurium* infection, which require Th17 responses for effective clearance.

SAA could also in part account for the essential role of epithelial RARβ for the development of gut-homing T cells and IgA-producing B cells. Development of both groups of cells requires RA-producing DCs. These DCs convert retinol to RA, which imprints gut homing receptors on T cells and induces IgA expression in B cells (2, 3). Given the retinol transport function of SAAs, I propose that SAAs could deliver retinol from the epithelium to RA-producing DCs to serve as substrate for RA production. Future work will be directed at testing this idea.

Altogether, our findings provide new insight into how the intestinal epithelium uses dietary cues to orchestrate adaptive immunity in the intestine (Fig. S10). By showing that epithelial RARβ is a key regulator of vitamin A-dependent immunity, I highlight epithelial RARs as potential therapeutic targets for the modulation of intestinal immunity during infection or inflammation.

3.5 ACKNOWLEDGEMENTS

I thank Dr. Pierre Chambon for the kind gift of the *Rara*$^{fl/fl}$ and *Rarb*$^{fl/fl}$ mice.

CHAPTER FOUR
Characterization of SAA Function

4.1 INTRODUCTION

As previously described, our study of SAA illustrates that mouse and human SAAs are retinol binding proteins. We demonstrated that SAA expression in mice requires dietary vitamin A, that mouse and human SAAs bind tightly to retinol, and that SAA recovered from serum following bacterial infection is associated with retinol. We found that *Saa1/2−/−* mice, which harbor deletions of both the *Saa1* and *Saa2* genes, have higher bacterial burdens in spleen and liver following an acute bacterial infection, supporting an essential role for SAAs in the response to microbial challenge. Finally, we provided structural insight into the binding interaction by solving the mouse SAA3 crystal structure, which revealed a tetrameric assembly with a hydrophobic binding pocket that can accommodate retinol. Our published findings suggest that SAAs mediate retinol transport during microbial challenge and thus constitute a key component of the physiological response to infection. We have additional ongoing studies to further understanding of the biochemical and biophysical properties of SAAs. We are also working to identify the receptor for SAA on immune cells and delineate the transport mechanisms of retinol that are at play during infection.

The concept of nutrient mobilization during infection is a broader theme of interest that has emerged during our investigation of SAA. The requirement for vitamin A or other nutrients at specific tissue sites during infection or inflammation is not always met by the

transport proteins defined at steady state, as we see is the case for vitamin A transport by RBP versus SAA. The technical challenges met in my attempts to identify from where and to where SAA is transporting retinoids, directly, were not surmountable. The biochemical quantification and/or visualization of retinoids at a cellular level will prove indispensable in defining this type of question.

SAA is also of thematic interest at the intersection of lipid metabolism, vitamin A metabolism, and immunity. The nature of its relationship with lipophilic molecules—as a transporter, as a sequestering agent—and how this impacts various disease states, is unclear. As an amyloid protein, its tight regulation and appropriate clearance, are paramount to avoiding severe pathologies—what contributes to SAA amyloidosis is certainly a big question beyond the scope of our current investigation. I highlight here some of my initial findings regarding the function of SAA *in vivo*, and discuss potential technical solutions or alternatives for furthering this line of study beyond my work.

4.2 METHODS

4.2.1 Animals and Animal Husbandry

C57BL/6 wild-type mice were maintained in the barrier at the University of Texas Southwestern Medical Center. *Saa1/2*$^{-/-}$ mice were obtained from Dr Frederick C de Beer (*Eckhardt et al., 2010*) at the University of Kentucky and were maintained in the barrier at the University of Texas Southwestern Medical Center. 6–12 weeks 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.

4.2.2 Animal Treatment and Diet

Vitamin A-deficient (TD.09838) and control (~20,000 IU vitamin A/kg; TD.09839) diets were purchased from Harlan Laboratories (South Easton, MA). At day 10 of gestation, pregnant females were placed on the standard diet or the vitamin A-deficient diet (*Hall et al., 2011*). Mothers and pups were maintained on the diets until weaning, and pups stayed on the diet for two additional months prior to sacrifice.

4.2.3 Immunofluorescence and Microscopy

Anti-SAA antiserum was raised against purified recombinant mSAA1. Retinol, retinoic acid, β-carotene, retinyl acetate, retinyl palmitate, and cholesterol were from Sigma-Aldrich (St. Louis, MO) and were reconstituted into ethanol, DMSO, or dioxane, depending on the experiment. IL-1β and IL-6 were from Invitrogen.

4.2.4 Quantitative RT-PCR

Total RNA was isolated from homogenized tissues or cells using the Qiagen RNeasy RNA isolation kit. Random primed cDNAs were assayed by SYBR Green-based real-time PCR

using *SAA*-specific primers as given in **TABLE 2.1**. Signals were normalized to 18S rRNA or *Gapdh*.

4.2.5 Microarray experiments

Total RNAs were isolated from mouse ileum using the Qiagen Midi-Prep RNA isolation kit. For each condition, RNA was isolated from two independent groups of five to eight 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 GeneChips in the University of Texas Southwestern Microarray Core.

To identify genes that are differentially expressed between germ-free and conventional mice, we performed two-way comparisons between germ-free and conventional groups, with germ-free samples designated as baseline. Raw data were imported into Affymetrix (Santa Clara, CA) GeneChip software for analysis, and previously established criteria were used to identify differentially expressed genes (*Cash et al., 2006*). Briefly, a twofold difference was considered significant if three criteria were met: (1) the GeneChip software returned a difference call of increased or decreased; (2) the mRNA was called present by GeneChip software in either germ-free or conventional samples; and (3) the difference was observed in duplicate microarray experiments. We performed a similar analysis to identify genes that are differentially regulated between mice fed a normal diet vs those fed a vitamin A-deficient diet. Finally, we 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 heatmaps using Java TreeView software.

4.2.6 Intestinal explant culture

Terminal ileum (5 cm) was collected from mice post-sacrifice and flushed with a solution of phosphate-buffered saline with penicillin (100 units/ml) and streptomycin (100 μg/ml). Ileal segments were cultured on equilibrated cell culture plate inserts (PIHA03050; Millipore) at 37°C and 95% oxygen for 6 hr in Dulbecco's modified Eagle's medium (4 g/l glucose and L-glutamine; Invitrogen, Carlsbad, CA) supplemented with 10% charcoal-stripped heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA), 10% NCTC135 media (Sigma), 25 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, and either 0.1% DMSO or 1 μM retinol in 0.1% DMSO. After culture for 6 hr, segments were flash-frozen and processed for total RNA extraction.

4.2.7 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. Cells were maintained at 5% CO2. Prior to addition of retinoids, the cells were grown overnight in

DMEM containing 10% charcoal-stripped FBS (to removes retinoids) and were treated with 1 μM retinol or 100 nM retinoic acid, 10 ng/ml of IL-1β, and 10 ng/ml IL-6.

4.2.8 Liquid Chromatography Mass Spectrometry

Retinoid extraction was modified and scaled from a previously described procedure (78). SAA-containing fractions purified by size exclusion chromatography were pooled, added to an equal volume of 1:1 1-butanol:acetonitrile, and vortexed for 60 s. 20 μl of 20.6 M K2HPO4 was added for each 1 ml of pooled fractions. Samples were then vortexed 30 s and 5 ml of hexane per 1 ml sample was added. Samples were vortexed for another 30 s and centrifuged at 1,000×*g* for 5 min and the top organic phase was dried in a nitrogen evaporator (Organomation Associates, Berlin, MA). Samples were prepared the day before the assay and stored at 80°C. Standard solutions were resuspended 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 NaCl and processed as for serum samples. Samples were resuspended 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-H2O]+) and 301.2 → 123.1 for retinoic acid (pos.

mode; M+H+). An Agilent (Santa Clara, CA) Eclipse XDB C18 column (150 × 4.6 mm, 5 micron packing) was used for chromatography with the following conditions: mobile phase A: acetonitrile:methanol:H2O: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. Stoichiometries 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 anti-SAA antiserum and densitometry.

4.2.9 Statistical Analysis

Statistical differences were calculated by the unpaired two-tailed Student's *t* test or Mann–Whitney test using GraphPad Prism software. Results are expressed as the mean ± standard error of the mean (SEM).

4.3 RESULTS

4.3.1 SAA1/2-/- Mice Produce Decreased IL-17

In characterizing SAA1/2-/- mice, I did not observe any gross histopathology at steady state or in response to *Salmonella typhimurium* infection. In searching for immune

defects, I observed a decrease in *Il17a* expression at the level of whole tissue ileum (**FIGURE 4.1**). While trying to identify the cellular source of this IL-17 defect by flow cytometry, larger strides were made by the Littman group in characterizing the immune contribution of SAA towards Th17 effector function in the small intestine (37).

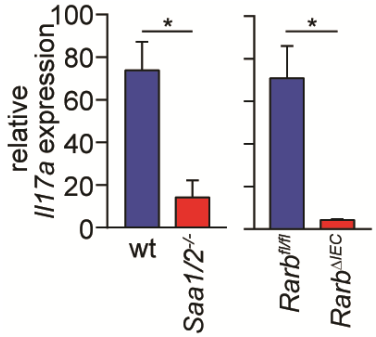

FIGURE 4.1. Decreased expression of IL-17A in SAA1/2-/- mice. qPCR analysis of *Il17a* transcripts in small intestines from wild-type (WT) and *Saa1/2*$^{-/-}$ mice (Eckhardt 2010) and *Rarb*$^{fl/fl}$ and *RarbΔIEC* mice.

Sano and colleagues neatly connect the microbiota to adaptive immunity, with SAA at the forefront of this story. They show the ability of segmented filamentous bacteria to promote epithelial SAA-mediated production of IL-17 by Th17 cells. Also important was the finding that this process is regulated by STAT3, thus adding to our understanding of how SAAs—and perhaps their multitude of functions—are tightly regulated by a variety of factors, likely in a tissue-specific context.

4.3.2 SAA1/2-/- Mice Have Defective Retinoid Storage

With the knowledge that SAAs bind retinol, I sought to further test the hypothesis that SAAs transport retinol. To answer whether global retinoid trafficking was disrupted in the SAA1/2-/- mice, I looked to biochemically quantify retinol and retinal stores in different organs and compare to wildtype counterparts. The SAA1/2-/- mice exhibited increased retinol stores in the small intestine and serum, but decreased stores in the liver (**FIGURE 4.2**).

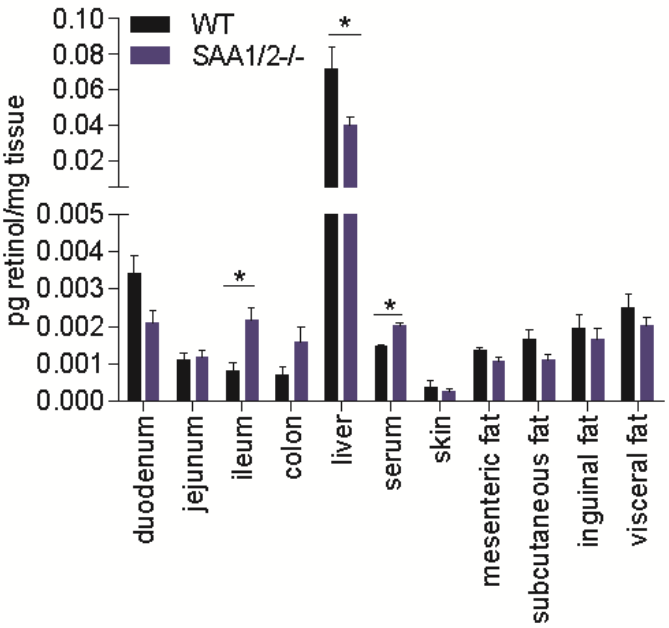

FIGURE 4.2. SAA1/2-/- mice have a defect in retinol storage. Retinoids were extracted and quantified by mass spectrometry as described previously (78).

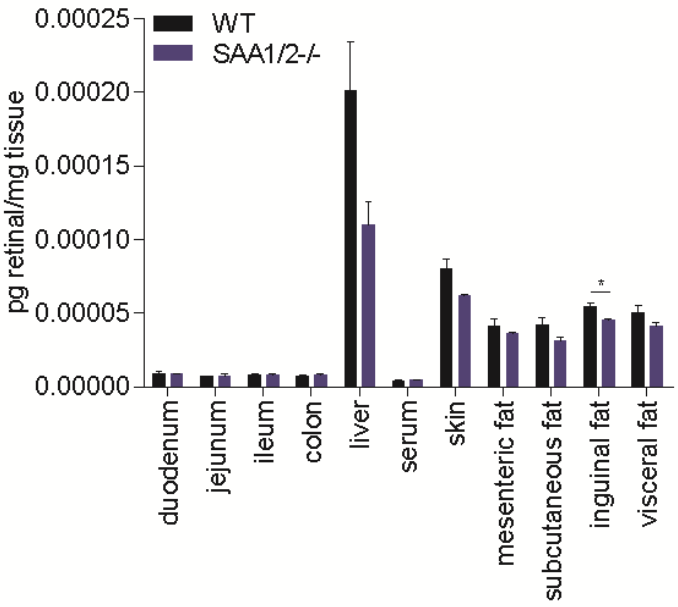

FIGURE 4.3. SAA1/2-/- mice have a defect in retinal storage. Retinoids were extracted and quantified by mass spectrometry as described previously (78).

We also tested retinal stores, which suggested that SAA1/2-/- mice have decreased retinal in inguinal fat (**FIGURE 4.3**), and potentially also in liver though this was not statistically significant in this preliminary experiment. Other vitamin A metabolites could be tested in the future.

In addition to biochemical quantification of global retinoid stores, I sought to establish *in vivo* models wherein RAR activity could be tracked with a reporter. By crossing a RARE-lacZ reporter mouse to SAA1/2-/- mice, I was able to generate mice that yielded increased lacZ expression in response to RAR activity. For preliminary studies, this mouse model was used to track changes in RAR activity at the level of whole organ tissues. The most notable difference detected was a decrease in RAR activity in the mesenteric lymph

nodes (MLNs) of SAA1/2-/- mice, quantified by expression of the lacZ reporter by qPCR (**FIGURE 4.4**).

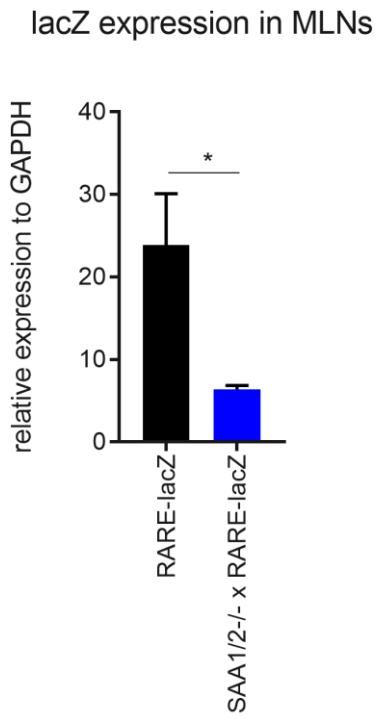

FIGURE 4.4. SAA1/2-/- x RARE-lacZ mice exhibit lower levels of RAR activity in mesenteric lymph nodes.

This finding is congruent with our general hypothesis that SAA is involved in the trafficking of retinoids to immune sites, though the cellular sources of RA or high RAR activity remain elusive. Current exploration for the receptor for SAA points to a cell in the myeloid compartment, but understanding of these intricate processes at the immune synapses I am curious about requires finer model systems and techniques.

4.4 DISCUSSION

The SAA1/2-/- model provided an important starting point to exploring the physiological role of SAAs and has more avenues for exploration in systems beyond the small intestine, including the skin. I noted skin pathology in aging SAA1/2-/- mice characterized by patches of missing fur and occasional lesions, which I have not explored. As retinoic acid is the functional metabolite that can act upon nuclear receptors to regulate transcriptional programming, the goal was to next quantify retinoic acid levels in tissue. Unfortunately, my early attempts using HPLC technology suggested that my extraction protocols were not yielding sufficient retinoic acid and remained under the range of detection. Having now understood the sensitivity of retinoids to light and air, and also gained access to improved methodology and resources at the UT Southwestern Metabolic Core, the quantification of retinoic acid and other retinoids at different tissue sites is worth revisiting. Importantly, a comparison of this data between steady state and post-infection would be useful in understanding global retinoid trafficking in response to infection.

As SAA3 is the predominant isoform expressed in the intestinal epithelial cell, we set out to create a full SAA1-4 knockout mouse that would enable us to better characterize the role of SAA family of proteins in intestinal immunity. The full knockout mouse crossed with the RARE-lacZ mouse is another tool we developed, which we have not yet utilized fully due to difficulties in quantifying lacZ expression for specific immune cell populations. However, due to improved protocols and technology available to us through the UT Southwestern

Metabolic Core, the quantification of retinoids at different tissue sites, and over the course of infection, have become feasible. I hypothesize that these data sets will prove invaluable to the understanding of how SAA contributes to defective retinoid trafficking. Importantly, current efforts to identify the SAA receptor on myeloid cells are promising; unlocking this information will enable us to further investigate the role of SAA at specific immune cell synapses, and disrupt retinoid trafficking with far greater precision than the dietary, whole body, developmental models of yesterday.

4.5 ACKNOWLEDGEMENTS

I thank Tess Leal, Clare Zlatkov, and Andrew Chara for assistance with mouse experiments. The contributions of Clare Zlatkov and Mehabaw Derebe were foundational to my dissertation research, and that legacy is continued by Zehan Hu and Ye-ji Bang. I owe particular thanks to Ye-ji Bang for being an excellent partner at the bench, both intellectually and experimentally. Our conversations regarding the function of individual SAA isoforms, the physiological relevance of the LRP-SAA interaction, and much more, have been a source of motivation to plow forward with these studies on SAA and the role of vitamin A transport in immunity. Moreover, Ye-Ji Bang is an exceptional bench scientist with a level of attention to detail that I respect and found essential to our ability to collaborate and learn new techniques together. I found a mini-mentor in her that I hope many trainees in her future will

find and cherish—she is a rare form of excellence and working with her will remain a bright memory of my graduate experience.

CHAPTER FIVE
Role of Vitamin A in Skin Immunity

5.1 INTRODUCTION

Like the mucosal surface of the intestines, the skin is an essential barrier between the body's internal tissues and the external environment. It is colonized by a diverse and complex community of microorganisms that includes bacteria, viruses, and fungi. Antimicrobial proteins are evolutionarily ancient immune effectors that maintain mutually-beneficial host-microbial relationships at multiple epithelial surfaces, such as those of the intestine. However, we still have a limited understanding of how antimicrobial proteins promote homeostasis with the diverse microbial communities of the skin. I explore here the role of one particular antimicrobial protein—resistin.

Human resistin (*RETN*), and the mouse homolog resistin-like molecule alpha (*RELMa*), were characterized initially as hormones that modulate insulin production (79, 80). However, it was recently discovered in our lab that RELMβ is directly bactericidal and kills Gram-negative bacteria at the surface of the colon and thus promotes host-bacterial mutualism in the intestine. This prompted parallel studies that show how RELMα is a bactericidal protein of the skin (manuscript submitted).

Skin immunity is highly sensitive to the presence of dietary vitamin A (81, 82). Furthermore, synthetic retinoids (compounds related to vitamin A) are widely used as treatments for skin inflammation (83). Despite the effectiveness of these treatments, little is known about the mechanisms by which vitamin A and synthetic retinoids regulate

cutaneous immunity. Both mouse RELMα and human RETN require vitamin A for expression in skin epithelial cells, and RELMα is required for retinoid-dependent protection against skin infection. While vitamin D regulates the expression of the skin antimicrobial protein cathelicidin (49, 84), expression of RELMs is uniquely sensitive to vitamin A. Though synthetic vitamin A derivatives are highly effective in treating skin conditions, such as psoriasis and acne vulgaris, there has been little molecular insight about their mechanism of action. Findings in the lab suggest RELM proteins as possible molecular targets of therapeutic retinoids that could in part account for protection against disease, which made the use of what I have learned about vitamin A-directed gut immunity potentially useful for the study of skin immunity. I establish here a model that will enable exploration of vitamin A-directed immunity in sebocytes, similar to the cell culture models I developed in Chapters 2 and 3.

5.2 METHODS

5.2.1 Cell Culture

SZ95 cells were obtained from Dr. Bruce Beutler with permission from the creator of the cell line, Dr. Christos Zouboulis [25]. SZ95 cells were cultured in F12:DMEM (Invitrogen) supplemented with 0.1 ng/ml human epidermal growth factor (ThermoFisher) and 10% fetal calf serum (Gibco). Cells were maintained at 5% CO2 at 37^{o} C. Prior to stimulation, cells were adapted to serum-free medium for 48 hours. Cells

were stimulated with retinol (100 nM) (Sigma) and IL-1b (50 pg/ml) (ThermoFisher 10139HNAE). Retinoic acid receptor activity was inhibited with BMS493 (TOCRIS) for 3 hours prior to stimulation. 24 hours post-stimulation cells were harvested and human *RETN* and *GAPDH* transcripts were analyzed as described above. Amber lighting was utilized to minimize retinoid degradation.

5.2.2 Quantitative RT-PCR

RNA was isolated from whole mouse skin using the RNAeasy Plus universal kit (Qiagen 73404). 2 μg of RNA were converted to cDNA and qPCR was performed using TaqMan Gene Expression Assays and the QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems). Relative expression values were determined using the comparative Ct (ΔΔCt) method, and transcript abundances were normalized to *Gapdh* transcript abundance.

5.2.3 Chromatin Immunoprecipitation of RAR Targets

SZ95 sebocyte cells were crosslinked in PBS with 1% formaldehyde for 8 minutes at room temperature and quenched in 125 mM glycine at 4ºC for 10 min. Nuclei from fixed cells were pelleted and used for chromatin immunoprecipitation per manufacturer's instructions (Diagenode). Each immunoprecipitation reaction included chromatin from 5 x 10^6 cells, 5 μg of goat anti-RAR (Santa Cruz) or total goat IgG

(Millipore), and 20 μl of Magna protein A beads (Millipore). Bound *RETN* promoter sequences were quantified using SYBR Green-based real-time PCR. Relative enrichment of the *RETN* promoter was calculated as the ratio of specific antibody pull-down to input DNA.

5.2.4 Statistical Analysis

All statistical analyses were performed using two-tailed Student's *t* test. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; and ns, $P > 0.05$.

5.3 RESULTS

5.3.1 Resistin Expression is IL-1β- and Retinoic Acid-Dependent

To determine if expression of human *RETN* was dependent on retinoids, I added retinol to cultured SZ95 cells (a human sebocyte cell line) (85). Retinol enhanced expression of *RETN* transcripts mediated by the proinflammatory cytokine IL-1β (**FIGURE 5.1**), indicating that retinol acts synergistically with a proinflammatory stimulus to stimulate *RETN* expression in sebocytes.

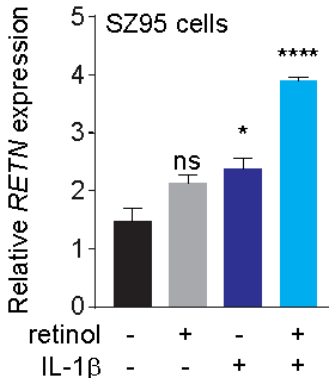

FIGURE 5.1. Resistin expression is IL-1β- and retinoic acid-dependent. qPCR analysis of human *RETN* expression in the human sebocyte cell line SZ95. Cells were treated with retinol, IL-1β, or a combination. N=3; data represent two experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01, ****P*<0.001 as determined by one-way ANOVA.

5.3.2 Resistin Expression is Dependent on RAR Activity

As described before, retinol typically regulates gene transcription through its derivative, retinoic acid, which binds to retinoic acid receptors (RARs) to activate transcription of specific target genes. Addition of BMS493, a pharmacological inhibitor of RARs, abrogated the increase in *RETN* expression (**FIGURE 5.2**), indicating that retinol-stimulated *RETN* expression requires RAR activity.

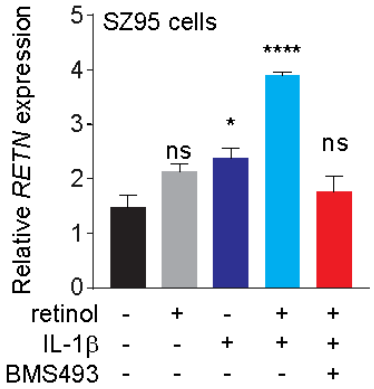

FIGURE 5.2. Resistin expression is dependent on RAR activity. qPCR analysis of human *RETN* expression in the human sebocyte cell line SZ95. Cells were treated with retinol, IL-1β, the pan-RAR inhibitor BMS493, or a combination. N=3; data represent two experiments. Means±SEM are plotted. **P*<0.05, ***P*<0.01, ****P*<0.001 as determined by one-way ANOVA.

5.3.3 RARs Bind Directly to the Resistin Promoter

To test whether RARs directly bind to the resistin promoter, I utilized NubiScan predictive software (69) as before to determine likely RAR binding sites. Predicted RARE sequences in the human *RETN* promoter in **TABLE 5.1** include RARE locations determined based on the *RETN* promoter construct purchased from Genecopoeia; 1 represents the farthest downstream location from the transcriptional start site of the *RETN* gene. I generated primers targeting 50-500 bp segments of the resistin promoter, to test for enrichment of DNA sequences bound during chromatin immunoprecipitation of RAR targets. Multiple regions of the resistin promoter were enriched for, supporting future endeavors to narrow down specific binding sites within the promoter.

TABLE 5.1. Predicted RARE sequences in human RETN promoter

Region Amplified in RETN promoter	Fold Enrichment of RAR targets over Input	Primer Set	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
35-518	0.05	9	AGGGATAGGGAGTGAGTGTT	GCTACAGGTATGCACCATCTT
60-193	**133.77**	**21**	GGGACAGAGTTTCAGTTTGGA	CACCATGCCCAGCCTATTT
83-570	1.12	11	GAGGAGAAAGTTCTGGAGATGG	CAGACTCCTGGCTTCAAGTAAT
108-434	**26.79**	**4**	GCAGTGGTGGCTACATAACA	CTGGAGTACAGTGGCTCAATC
410-777	0.66	15	CCAAGATTGAGCCACTGTACT	ACCACGCCCAGCTAATTT
533-666	**5.14**	**5**	AGTCCAAGGTGGGAGGATTA	TCCCAAAGTGCTGGGATTAC
545-735	1.24	7	GAGGATTACTTGAAGCCAGGAG	AGATGGAGTTTCACCGTGTTAG
633-1061	**584.82**	**10**	GGTGGTTCACGCCTGTAAT	GGTCCAGCTCATGTTTCTTCT
718-1167	0.21	1	CACGGTGAAACTCCATCTCTAC	CACCATAGCAAGACTCCATCTC
808-1100	ND	22	AGGCTGAAGCAGGAGAATG	CCTGCAACAGAAGTTGGAAAG
975-1033	**34.52**	**18**	CCCGTTGTACTGGAAACAAAGA	CCCATGAGCTTGGAATGGTTAT
1028-1341	0.01	8	CATGGGTGGTCCAAGAAGAA	TGATGAAACCCTGTCTCCATTAG
1071-1293	**35.02**	**14**	GAGCAATGGCTTTCCAACTTC	TGGCGTGTGCCTGTAATC
1144-1640	ND	13	TTGAGATGGAGTCTTGCTATGG	AGGCTGACGTGAGAGAATTG
1236-1420	**8.50**	**17**	GTTCAAGTGATTCTCCCACCTC	GCCTGTAATCCCAGCACTTT
1261-1352	0.35	16	CTCCCAAGTAGCTGGGATTAC	CCTGGCCAACATGATGAAAC
1291-1555	**4.61**	**23**	CCACCACGCCCAGTTAAT	CAGAGCAAGACCACTTCTCTAAA
1317-1513	0.31	24	GTCTAATGGAGACAGGGTTTCA	CTCTGGGTGAGAATGACATACC
1397-1721	0.00	3	TCCCAAAGTGCTGGGATTAC	GTGGCTGATGCAAACATGAC
1469-1955	**5.10**	**2**	CATGGGCATTTGGGTATGAATG	GCTTCCTGGCTTGGCTAATA
1492-1762	1.97	12	GGTATGTCATTCTCACCCAGAG	GTCTGAGGAACAGGTAGACAAG
1537-1609	ND	20	GAGAAGTGGTCTTGCTCTGTT	GAGTTCGAAGCTACAGTGACTTAT
1629-2028	ND	6	TCACGTCAGCCTCCTTAGTA	TGGGCTCAGCTAACCAAATC
1676-1825	ND	19	CATGCCTAGCAAGAGGCATTA	TCCGTCTTCATGTCCAGAGA
1736-1990	ND	25	CTCTGCTTGTCTACCTGTTCC	ACCGCAGCTCTTTCTTTGA

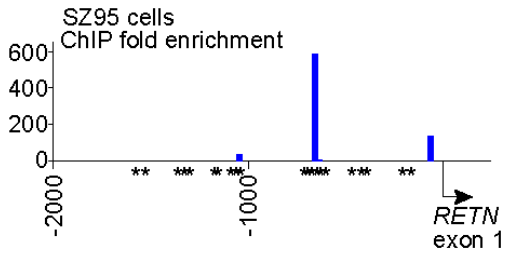

FIGURE 5.3. RARs bind directly to the human *RETN* promoter. RAR binding to the *RETN* promoter was measured by chromatin immunoprecipitation (ChIP) with an anti-RAR antibody. Predicted retinoic acid response elements (RAREs) were determined by *in silico* analysis using NUBIScan,and are indicated by an *. Data represent two experiments.

5.4 DISCUSSION

By extending what I learned about RAR-regulated immunity in the intestinal tract, to the skin, I hoped to establish a useful model to probe truly basic questions that remain unanswered in the skin. The sebocyte has not previously been appreciated as a source of resistin, and it is only recently that we have come to appreciate that resistin—like SAA—is regulated by both the microbiota and dietary vitamin A. The sebocyte shares similar qualities with the Goblet cell of the intestines, and its contribution to skin immunity remains unclear and underappreciated. In establishing that the sebocyte can produce resistin *in vitro*, and that this process can be manipulated by pro-inflammatory cytokines and retinoids, we have a new tool to explore sebocyte-intrinsic immune processes.

5.5 ACKNOWLEDGEMENT

This work was the result of a collaboration with my colleague, Dr. Tamia Harris-Tryon, who was instrumental in shaping the flavor of science that appeals most to me. Her perspectives are unique, and evidence of the value of bridging the gap between scientific exploration and clinical observation. She additionally taught me important lessons about team dynamics and different lab environments, which became important points of introspection and personal growth.

CHAPTER SIX
Perspectives and Future Directions

6.1 INTRODUCTION

We are what we eat. To be able to delineate the details of how exactly a nutrient shapes development and maintenance of a robust immune system is the broader goal of these studies. The combination of dietary choices, efficient metabolism and trafficking of nutrients, training and maintenance of immunity, and the composition of the microbiota is a complex web of interactions. But it is in the complex interactions between diet, immunity, and metabolism that we even begin to define “healthy” or steady state.

In SAA, we find a protein with implications for cardiovascular health, immunity, and neurodegenerative diseases. On one hand, SAA has been historically villainized as a biomarker for disease states, as it is found at elevated levels in the serum in response to

serious infection and inflammation. On the other hand, this feature highlights the importance and complexity of its regulation: the rapid induction and clearance of this acute phase response protein is critical. Thus it is of no surprise that numerous transcription factors contribute to SAA transcriptional regulation, and that these processes can vary significantly between tissue and cell types. Through our study of RAR regulation of SAAs, we have opened a new chapter of interest in the lab: vitamin directed immunity through nuclear hormone receptors.

In many instances, the field of immunology has represented the biological activity of retinoic acid as that of a cytokine. Moreover, where RAR activity has been studied, it is typically intrinsic to lymphocytes. The role of RARs and retinoids within the intestinal epithelial cell has been understudied, despite the fact that the epithelium is the site of both absorption and sampling of luminal antigens—biological events that fine tune the foundations of our immune system. The intestinal epithelial cell is a critical integrator of microbial and dietary cues, and represents the interface between the external world and our internal biology. The next sections outline the importance of intestinal epithelial cell-intrinsic nuclear receptors with dietary vitamins as cues—retinoic acid receptors and vitamin D receptor. In considering the literature around vitamin D-directed immune pathways, we can find valuable inspiration for experimental design that is useful in the further study of the role of IEC-intrinsic vitamin A-directed activity for the enterohepatic system, at large.

6.2 AT THE INTERSECTION OF IMMUNITY AND METABOLISM

Analysis of global transcriptional changes in the RARβ$^{\Delta IEC}$ ileum reveals that numerous metabolic and immune pathways are dysregulated. This data also suggests that RARβ may help bridge the gap in our understanding between vitamin A metabolism and lipid metabolism (though our current exploration has focused on the immune aspects). The RNA-seq data show dysregulation of inflammasome pathways, bile acid metabolism, lipid metabolism, interferon beta stimulated genes, cholesterol metabolism, M cell development, antimicrobial proteins and more.

The dysregulation of bile acid metabolism and inflammasome pathways, in particular, are of interest to me—especially in relation to the microbiota. Bile salts are absorbed in the ileum and are sent to the liver as bile acids for secretion as bile; this enterohepatic circulation unifies intestinal and liver function, and presents an opportunity for us to venture a little further out of the gut. It has been shown that when bile salts in circulation reach a critical concentration, as in sepsis for example, bile salts can serve as a danger signal to activate the NLRP-3 inflammasome in macrophages in a dose- and time-dependent manner (86, 87). Bile salts can effectively provide both of the necessary signals for strong activation of the NLRP3 inflammasome in liver macrophages: caspase 1 and IL-1β mRNA expression, plus cleavage of caspase 1 and maturation of pro-IL-1β. Bile salts at physiological levels are unlikely to activate the NLRP-3 inflammasome (87), but I am curious to understand the

impact of dysregulated bile acid metabolism and inflammasome pathways in the RARβ$^{\Delta IEC}$ mice.

The role of FXR in this model compared to its wildtype counterpart may be interesting to investigate as well, as it acts as a negative regulator of NLRP-3 by direct interaction with NLRP-3 and caspase 1, thereby preventing the assembly of the NLRP-3 inflammasome. While bile salt signaling in liver myeloid cells has been characterized to some extent, even less is known about the role of bile salts in lymphoid cells, like CD4+ or CD8+ T cells, or B cells. The bile salt-dependent shift in metabolomic activity of the microbiome during dysbiosis is another variable that clouds our understanding of these processes. To that end, it would be invaluable to breed the RARβ$^{\Delta IEC}$ mice under germ-free or microbiologically sterile conditions, and probe the individual contributions of specific microbial communities. Removing the variable of the microbiota in this model would allow for a cleaner system to assess the relationship between immune and metabolic pathways, and the relationship between gut and liver function as well. Work from the Stappenbeck lab has illustrated how a microbiota-derived metabolite impacts macrophage function, and in connecting a metabolic process with a robust immune response, inspires discussion on how to regulate immunity through metabolism (Steed 2017). As morbidity associated with infectious diseases can be immunopathological, methods of fine-tuning an immune response without altogether ablating it, should prove useful.

6.3 VITAMINS AS HORMONES

Vitamins A and D are the first group of substances reported to share properties with skin hormones. In the context of skin, vitamin A and its two important metabolites, retinaldehyde and retinoic acids, are fat-soluble unsaturated isoprenoids necessary for growth, differentiation and maintenance of epithelial tissues, and also for reproduction. On one hand, natural vitamin A metabolites are vitamins, because vitamin A cannot be synthesized in the body and must be derived from carotenoids or retinyl esters in the diet. On the other hand, retinoids are also hormones with intracrine activity: retinol is metabolized into retinoic acid, which binds to and activates specific nuclear receptors and when unbound are subsequently inactivated through degradation. The skin has been extensively studied as the human body's vitamin D endocrine system: site of absorption and metabolism, but also the site of regulation of whole body growth and differentiation, including cells derived from prostate, breast and bone. As a result, vitamin D analogues have been introduced for the treatment of skin diseases such as psoriasis. This vitamin-hormone that often begins its journey in the skin has been shown to regulate a variety of diseases, from cancer to inflammatory bowel disease to autoimmune disorders. Perhaps the seat of retinoids as a vitamin-hormone can be studied with a similar lens, but in the intestines—the site of absorption, metabolism, and self-renewal of the intestinal epithelium—with implications beyond intestinal homeostasis.

6.4 THE MICROBIOTA, LIPID METABOLISM, AND VITAMIN A

The intestines are essential to nutrient absorption, elimination of unwanted dietary components, and the balancing act of promoting both tolerance and active defense. The gastrointestinal tissues express nuclear hormone receptors that act as sensors for absorbed lipid molecules, including vitamin A. These receptors enable enterocytes to activate appropriate transcriptional programs in response to nutrients, microbial metabolites, and luminal antigens. With the burden of such mighty tasks, the activities of the nuclear hormone receptor family are tightly regulated and can illustrate high tissue- and cell-specificity over the course of development. Subtypes are well-conserved across species, as discussed previously, and are likely to be responsible for non-redundant physiological processes. The inducibility of RARβ with microbial stimulation suggests that there is much to be learned about the regulation of nuclear receptors themselves, in the context of infection.

As mounting evidence implicates the microbiota in regulating lipid metabolism and obesity, I am curious about further exploring the relationship between vitamin A metabolism and the microbiota. First, the possibility of microbes themselves as a source of vitamin A has not been illustrated definitively, though it is a topic currently being explored in the context of skin commensals. Second, if $RAR\beta^{\Delta IEC}$ mice and VAD mice can be bred under germ-free conditions, we can assess how global vitamin A deficiency versus a defect in vitamin A-directed transcription within the IEC contributes to pathology independent of exacerbation by microbiota. Removing the microbiota variable will allow for more careful consideration of

the relationship between metabolism and immunity in these mice, and to better understand how vitamin A, through RARβ, regulates enterohepatic physiological processes.

6.5 CLOSING REMARKS

My dissertation work began as a mission to delineate the transcriptional regulation of the acute phase protein serum amyloid A, which the lab had recently attributed retinol-binding function to. SAA made the short list of genes regulated by both the microbiota and dietary vitamin A. The fact that dietary vitamin A regulated SAA expression, led to the hypothesis that, if directly regulated, then vitamin A was acting through RARs. Beyond delineating how epithelial RARβ regulates SAAs, this study became a broader exploration of RAR activity within the intestinal epithelium, and its implications for intestinal immunity.

My findings provide insight into how the intestinal epithelium senses dietary vitamin A status to control vitamin A-dependent adaptive immunity. I explored the role of a critical immunoregulatory protein, SAA, which requires both dietary vitamin A and microbial colonization for its expression. I delineated how vitamin A regulates SAA expression through RARβ and explore how RARβ more broadly regulates vitamin A-dependent adaptive immune responses. I provide preliminary insight into the physiological role of SAA as a transporter of retinol, but look forward to future Hooper Lab contributions to the understanding of vitamin A trafficking in immunity. Lastly, I expand my study of RAR-driven immunity within the intestinal epithelium to an exploration of vitamin A-dependent

immunity in the skin. These final studies were invaluable in projecting my expertise to a different system, with many shared similarities, but much to learn from with regards to thinking of vitamins A and D as “vitamin-hormones”. The multiple and unique perspectives within the lab were essential to navigating the vast territory of vitamin A-directed immunity, and I hope that the RARβ$^{\Delta IEC}$ mice continue to provide an avenue to further explore the themes outlined here.

APPENDIX A
QUANTITATIVE PCR PRIMER SEQUENCES

Target gene for cDNA amplification	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
mGAPDH	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG
mSAA1	ATCACCAGATCTGCCCAGGA	CCTTGGAAAGCCTCGTGAAC
mSAA2	ACCAGATCTGCCCAGGAGAC	GCATGGAAGTATTTGTCTCCATCT
mSAA3	GACATGTGGCGAGCCTACTC	TTGGCAAACTGGTCAGCTCT
hGAPDH	CCTGGTCACCAGGGCTGCTTTTAAC	GTCGTTGAGGGCAATGCCAGCC
hSAA1	GGCATACAGCCATACCATTC	CCTTTTGGCAGCATCATAGT
hSAA2	GCTTCCTCTTCACTCTGCTCT	TGCCATATCTCAGCTTCTCTG

APPENDIX B
SIRNA PROBE SEQUENCES

Target gene	Probe Sequences
human RARA	GCAAAUACACUACGAACAA CCAAGGAGUCUGUGAGAAA GAGCAGCAGUUCUGAAGAG GAACAACGUGUCUCUCUGG
human RARB	CAGCUGAGUUGGACGAUCU CGAGAUAAGAACUGUGUUA GGCCUUACCCUAAAUCGAA UCACAGAUCUCCGUAGCAU
human RARG	GAAAGGGCCAUUACUCUGA CAAGGAAGCUGUGCGAAAU GGAGAACCCUGAAAUGUUU UAGAAGAGCUCAUCACCAA
mouse RARA	GCAAGUACACUACGAACAA AAGACAAAUCAUCCGGCUA CGGUGCGAAACGAUCGAAA CGAAUCUGCACGCGGUACA
mouse RARB	GAUAAGAACUGCGUCAUUA GAAAGGUGCCGAACGUGUA GAUCUACACUUGCCAUCGA AAGAGUCUGUUAGGAAUGA
mouse RARG	GUAAGGAACGAUCGAAACA GCGGAUCUGUACAAGGUAU GCAGGACACUAUGACAUUC GGAGCAGGCUUCCCAUUCG

APPENDIX C
CHIP PCR PRIMER SEQUENCES FOR SAA3 PROMOTER

Target Sequence in mSAA3 promoter	Forward Primers (5' to 3')	Reverse Primers (5' to 3')
RARE -3895 RARE -3556	GCAAAGAGGAAATGCTGGAAAG AAAGTTCTGGCAACTCACCTC	TCAAACAGGGATTGCTCCATTA TAGATATCCTCGGCCACATCTC
RARE -3895	GCTGCGAGCTCCTTCTG	GGGTAAGTGGGATTATGCAAGA
RARE -3556 RARE -3537	CGCCATCCTGGTTGGAAATAA TCATGGGATCCAATCCAAAGG ATGCTTTATATCCAGTTTCTTGGG	CCCATCCTCCTGTTCCCTAA TGCTCCCTGACCTTCCATA CAGCGTTGGGTGCTAAATG
RARE -3142 RARE -2949	CATTTGGCCTCCTGCTCTAA	CCTTCCAGCTTACGAGGTTTAT
RARE -3142	TTGCCGTGGGAGTTTATCTTAT CAGCTGCTAGAGCCAGAAA GCAAGAGGAGACACAGGAATG	GCAGCTATACCGTCCTTCTTT GTGATACCTTCACCACACCTAC CCTTCTTTCTCTAGGGAACTCAATAC
RARE -2949	GGTGTGGTGAAGGTATCACAT GAAGCAGAGGAAAGCAGAGG CCTAGAGAAAGAAGGACGGTATAG	CGAGATAGCCACAGTTCTCTTC CGGGTATTATTCACCACCTTCC CTTGGTAGCAAACAACTTGGG
RARE -2490 RARE -2479	GCTGCCTTTGGCTTAGGA TGGTCCTCAGCCAAGACA	AATCCCGGAACCAAATGTTTATG GCCTAGGAAGTCAGAACAAAGG
RARE -2490	GCTCTGTGACTGAAAGGGAAA	GGGAAGTTGTAGCTGCTTACTG
RARE -2479	AGTAAGCAGCTACAACTTCCC	GTGGCTGAGGAAGTCAATACA
RARE -2230 RARE -2116	CCTCCCTCTCGTCCCTTT CCAAATGGATGCATGTGACC	CCAGAGGACCTGGGTTTAATTC GTACTCATGGTAGCTCACAACT
RARE -2007	CCAAATGGATGCATGTGACC	AAAGGGTAAAGGGTGGATATGAG
RARE -1915 RARE -1832	TCTTTGATAGAAGGCAAGTGAGT	AAAGGGTAAAGGGTGGATATGAG
RARE -1740 RARE -1737	GCAACCTTGCCCGAAATAAA TCATATCCACCCTTTACCCTTTG	GTGAAGATGTGTGGACAGAAGA CACAGTGACACACTCTAGCTTC
RARE -1563	GTTCCTCGGAACAAGTCCATT TCTTCTGTCCACACATCTTCAC GTCACTGTGTCTCTCCAATGTAT CAGTACCAATACTGGGTCCTTT	GGCCCAGCTCACAATCTATATC TGGGATTCAAGGGACAGTTATG GCTGACCCAAAGCTGTAGAA CATCTGAGAATGGCTGGGATT
RARE -1357 RARE -1310 RARE -1255 RARE -1244	CTTTCCCAATTGCCAGAAGTG	GATTGCAACATTCTGGAGAGC
RARE -1357 RARE -1310	GTTCACTGAGCTGGTCTCATATTC	GCAGGAGTGTGGGAGAGT
RARE -1357	CCCAGCCATTCTCAGATGATATAG	GTGGTGCTGGCACAGAG
RARE -1310 RARE -1255 RARE -1244	GCTCATGACCCTGGGAATAG	GGTCCCATCTCTCACCAATAG

Target Sequence in mSAA3 promoter	Forward Primers (5' to 3')	Reverse Primers (5' to 3')
RARE -1160 RARE -1134 RARE -1058	GAATGTTGCAATCAGTGAGGAG	CAGCCTGAGATGATGGTGAA
RARE -1160	CTGCTGCTATTGGTGAGAGATG	TGTTATCACTAGAGGACATGGAGATA
RARE -1058	GGGATATCTCCATGTCCTCTAGT	GTGGAGCAGCTTGAGCATTA
RARE -689 RARE -603	CTTCAAGCTAGGATGAACAGAGG	GGGAAAGAGAGAAGAAAGCATCA
RARE -689	CTGTGCTGCCTGGATATGAT GGTCTGCAGGTGTCTATCTTC	CACCTTGATGTTGGCATTGTT CCCTGGCAATGGGCATA
RARE -603	GGAACAATGCCAACATCAAGG	CACACACTGGATTGGATGGA
RARE -463	GTGTGAGCCAACTGCTCTTA ACCCAGCTTGATGCTTTCT CCATCCAATCCAGTGTGTGT	ATCTTAGCATGGACGGTGTG GGTGAGAAGCTATTGGCATTTG GCAGAGGAAGGGTTGGTTT
RARE -327	CACACCGTCCATGCTAAGAT CAAATGCCAATAGCTTCTCACC AACCAACCCTTCCTCTGCTA	GGTAGAGGTGATGGTTGACTTC CAAGAGGGTGGACTCACAAG GGACTGTGACAGCATTGCATA
RARE -224	GAAGTCAACCATCACCTCTACC CTTGTGAGTCCACCCTCTTG	AAATATCCTCGGACACACCATC CAAGGTTCGAGGGTCCTTT
RARE -116	GATGGTGTGTCCGAGGATATTT AAAGGACCCTCGAACCTTG	GACAGTGAGACAGATGACACAG AAGAATCTGTGCGACAGTGA
RARE-11 RARE 15	CTGTGTCATCTGTCTCACTGTC ACTGTCGCACAGATTCTTCTC	AACTAGCATGCTGTCCTCAAA AGGCTCAGTACCATCCAAAC
RARE 15	CTGTGGGTTGGGATCTTGT	CTTTAGAGTGCTCCTCCAGTG
RARB RARE Positive Control	CGGGTAGGGTTCACCGAAAGTTCACTCGCA	TGCGAGTGAACTTTCGGTGAACCCTACCCG
H3K27Ac Binding Positive Control	TCTCCCTAAACTCTTCCACTCG	ATGTGCCTAAAACGCATCACTACTA
H3K27Ac Binding Negative Control	ACAACTGAGGGGAGGAGAGAAG	GCTGCATTTGTTTTCATTCAGT

APPENDIX D
Predicted RAREs in mouse SAA1 Promoter

Predicted RARE sequences in the mouse Saa1 promoter, where RARE location was determined relative to the *Saa1* start site.

Predicted RARE sequence	RARE type	RARE location
AGGACAcaccaTGATCT	DR5	-3758
GGATCAcaacaATCCCA	DR5	-3666
AGGTCTtaGGGCCA	DR2	-3443
ACTTCAgtataGGAACA	DR5	-3308
AGAGCTagctgGGGACA	DR5	-3127
GGATGAagAGCTCA	DR2	-2945
AGGTCActtctTGTAAA	DR5	-2888
AATTCAcaCGGACA	DR2	-2697
TGTCCActaggCGGACA	DR5	-2312
AGACCTgtagcAGGGCA	DR5	-1983
AGGGCAgtagtAGCACA	DR5	-1972
GGCTCAgcAGGTCA	DR2	-1767
AGGGCAtcAGATCT	DR2	-1671
AGACCAggccaGTCTCA	DR5	-1353
AGAGCAagACGACA	DR2	-1161
ATGGCAgaaggAGGAGA	DR5	-1138
AGCAGAcaAGCTCA	DR2	-1103
GGTTAAgaAGCACA	DR2	-950
AGGGGAaggctGGGCCA	DR5	-819
AGTTCCctgtgGGGCCA	DR5	-697
AGGTAAaatggGGGGCA	DR5	-348
GGGGCAgggggAGAACA	DR5	-337
GGGGCAggGGGAGA	DR2	-337
TGTGCAatgggAGCACA	DR5	-176
GGATGAagAGCTCA	DR2	-156
TCTTCAtccacAGGTCA	DR5	-148
AGGACAgcCTGGCA	DR2	-59
AGGTGAgggcaAGGACA	DR5	-48
AGATCAccAGATCT	DR2	3
AGGTGAgaggcAGATCC	DR5	31

APPENDIX E
Predicted RAREs in mouse SAA2 Promoter

Predicted RARE sequences in the mouse Saa2 promoter, where RARE location was determined relative to the *Saa1* start site.

Predicted RARE sequence	RARE type	RARE location
GGTACAcaGTGTCA	DR2	-3667
AGGACTggttgAGAGCA	DR5	-3568
AGGGCAgaGGTAGA	DR2	-3479
GGGCCAagAGAACG	DR2	-2871
GGGTCAagagcAGCCAA	DR5	-2835
AGCACAatagcAGGGCC	DR5	-2724
AGATCTcaTGTGCA	DR2	-2512
GGATCAtctcaAGTGCC	DR5	-2371
AAGTGAgttccAGGACA	DR5	-2299
AGATGAgagacTGGGCA	DR5	-2123
AGTGCAgctatGGGGCA	DR5	-1905
AAGCCActGGTCCA	DR2	-1853
TGGTCCacaagAGGACA	DR5	-1846
AGTACAgcAGGACT	DR2	-1730
AAGTCAtgcagTGGTCA	DR5	-1573
AATTCAagAGTTTA	DR2	-1568
AGGGGAacTGGGCA	DR2	-1530
GGGGCAgtttaATGTCA	DR5	-1437
AGGTCTgtGGGGCA	DR2	-1429
AGGACCctggtAGGTCT	DR5	-1418
AGTGTAtatttTGTTCA	DR5	-588
AGCTCAttAGTGTA	DR2	-580
TGTTCCtgcagAGAACA	DR5	-331
AGCACAggAGACAA	DR2	-188
TGTGCAatgggAGCACA	DR5	-177
GGATAAagAGCTCA	DR2	-157
AGGACAgcCTGGCA	DR2	-60
TGCTCAggtgaGGGGCA	DR5	-43
AGACCAccAGATCT	DR2	3
AGGTGAgaggcAGATCC	DR5	31

BIBLIOGRAPHY

1. Peterson LW & Artis D (2014) Intestinal epithelial cells: regulators of barrier function and immune homeostasis. *Nat Rev Immunol* 14(3):141-153.
2. Mora JR, *et al.* (2006) Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science* 314(5802):1157-1160.
3. Iwata M, *et al.* (2004) Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 21(4):527-538.
4. Hall JA, *et al.* (2011) Essential role for retinoic acid in the promotion of CD4(+) T cell effector responses via retinoic acid receptor alpha. *Immunity* 34(3):435-447.
5. Bhaskaram P (2002) Micronutrient malnutrition, infection, and immunity: an overview. *Nutr Rev* 60(5 Pt 2):S40-45.
6. Germain P, *et al.* (2006) International Union of Pharmacology. LX. Retinoic acid receptors. *Pharmacol Rev* 58(4):712-725.
7. Harrison EH (2005) Mechanisms of digestion and absorption of dietary vitamin A. *Annu Rev Nutr* 25:87-103.
8. Jijon HB, *et al.* (2018) Intestinal epithelial cell-specific RARalpha depletion results in aberrant epithelial cell homeostasis and underdeveloped immune system. *Mucosal Immunol* 11(3):703-715.
9. Lawson ND & Berliner N (1999) Neutrophil maturation and the role of retinoic acid. *Exp Hematol* 27(9):1355-1367.
10. Stephensen CB (2001) Vitamin A, infection, and immune function. *Annu Rev Nutr* 21:167-192.
11. Spencer SP, *et al.* (2014) Adaptation of innate lymphoid cells to a micronutrient deficiency promotes type 2 barrier immunity. *Science* 343(6169):432-437.
12. Osanai M, *et al.* (2007) Cellular retinoic acid bioavailability determines epithelial integrity: Role of retinoic acid receptor alpha agonists in colitis. *Mol Pharmacol* 71(1):250-258.
13. Sommer A (2008) Vitamin a deficiency and clinical disease: an historical overview. *J Nutr* 138(10):1835-1839.
14. Underwood BA (2004) Vitamin A deficiency disorders: international efforts to control a preventable "pox". *J Nutr* 134(1):231S-236S.
15. Penniston KL & Tanumihardjo SA (2006) The acute and chronic toxic effects of vitamin A. *Am J Clin Nutr* 83(2):191-201.
16. Iliev ID, Mileti E, Matteoli G, Chieppa M, & Rescigno M (2009) Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol* 2(4):340-350.
17. Vicente-Suarez I, *et al.* (2015) Unique lamina propria stromal cells imprint the functional phenotype of mucosal dendritic cells. *Mucosal Immunol* 8(1):141-151.
18. McDonald KG, *et al.* (2012) Epithelial expression of the cytosolic retinoid chaperone cellular retinol binding protein II is essential for in vivo imprinting of local gut dendritic cells by lumenal retinoids. *Am J Pathol* 180(3):984-997.

19. Villablanca EJ, *et al.* (2011) MyD88 and retinoic acid signaling pathways interact to modulate gastrointestinal activities of dendritic cells. *Gastroenterology* 141(1):176-185.
20. Persson EK, *et al.* (2013) IRF4 transcription-factor-dependent CD103(+)CD11b(+) dendritic cells drive mucosal T helper 17 cell differentiation. *Immunity* 38(5):958-969.
21. Pantazi E, *et al.* (2015) Cutting Edge: Retinoic Acid Signaling in B Cells Is Essential for Oral Immunization and Microflora Composition. *J Immunol* 195(4):1368-1371.
22. Reis BS, Rogoz A, Costa-Pinto FA, Taniuchi I, & Mucida D (2013) Mutual expression of the transcription factors Runx3 and ThPOK regulates intestinal CD4(+) T cell immunity. *Nat Immunol* 14(3):271-280.
23. Eckhardt ER, *et al.* (2010) Intestinal epithelial serum amyloid A modulates bacterial growth in vitro and pro-inflammatory responses in mouse experimental colitis. *BMC Gastroenterol* 10:133.
24. Uhlar CM & Whitehead AS (1999) Serum amyloid A, the major vertebrate acute-phase reactant. *Eur J Biochem* 265(2):501-523.
25. Steel DM & Whitehead AS (1994) The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunol Today* 15(2):81-88.
26. Ivanov, II, *et al.* (2009) Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* 139(3):485-498.
27. Reigstad CS & Backhed F (2010) Microbial regulation of SAA3 expression in mouse colon and adipose tissue. *Gut Microbes* 1(1):55-57.
28. Reigstad CS, Lunden GO, Felin J, & Backhed F (2009) Regulation of serum amyloid A3 (SAA3) in mouse colonic epithelium and adipose tissue by the intestinal microbiota. *PLoS One* 4(6):e5842.
29. Chiba T, *et al.* (2009) Serum amyloid A3 does not contribute to circulating SAA levels. *J Lipid Res* 50(7):1353-1362.
30. Meek RL & Benditt EP (1986) Amyloid A gene family expression in different mouse tissues. *J Exp Med* 164(6):2006-2017.
31. Whitehead AS, *et al.* (1992) Identification of novel members of the serum amyloid A protein superfamily as constitutive apolipoproteins of high density lipoprotein. *J Biol Chem* 267(6):3862-3867.
32. Derebe MG, *et al.* (2014) Serum amyloid A is a retinol binding protein that transports retinol during bacterial infection. *Elife* 3:e03206.
33. Blaner WS (1989) Retinol-binding protein: the serum transport protein for vitamin A. *Endocr Rev* 10(3):308-316.

34. Rosales FJ, Ritter SJ, Zolfaghari R, Smith JE, & Ross AC (1996) Effects of acute inflammation on plasma retinol, retinol-binding protein, and its mRNA in the liver and kidneys of vitamin A-sufficient rats. *J Lipid Res* 37(5):962-971.
35. Noy N (2000) Retinoid-binding proteins: mediators of retinoid action. *Biochem J* 348 Pt 3:481-495.
36. Tiwari P, *et al.* (2013) Prediction and experimental validation of a putative non-consensus binding site for transcription factor STAT3 in serum amyloid A gene promoter. *Biochim Biophys Acta* 1830(6):3650-3655.
37. Sano T, *et al.* (2015) An IL-23R/IL-22 Circuit Regulates Epithelial Serum Amyloid A to Promote Local Effector Th17 Responses. *Cell* 163(2):381-393.
38. Kumon Y, *et al.* (2002) Transcriptional regulation of serum amyloid A1 gene expression in human aortic smooth muscle cells involves CCAAT/enhancer binding proteins (C/EBP) and is distinct from HepG2 cells. *Scand J Immunol* 56(5):504-511.
39. Mangelsdorf DJ, *et al.* (1995) The nuclear receptor superfamily: the second decade. *Cell* 83(6):835-839.
40. Chawla A, Repa JJ, Evans RM, & Mangelsdorf DJ (2001) Nuclear receptors and lipid physiology: opening the X-files. *Science* 294(5548):1866-1870.
41. Makishima M, *et al.* (2002) Vitamin D receptor as an intestinal bile acid sensor. *Science* 296(5571):1313-1316.
42. Nehring JA, Zierold C, & DeLuca HF (2007) Lithocholic acid can carry out in vivo functions of vitamin D. *Proc Natl Acad Sci U S A* 104(24):10006-10009.
43. Shulman AI, Larson C, Mangelsdorf DJ, & Ranganathan R (2004) Structural determinants of allosteric ligand activation in RXR heterodimers. *Cell* 116(3):417-429.
44. Bouillon R, Van Cromphaut S, & Carmeliet G (2003) Intestinal calcium absorption: Molecular vitamin D mediated mechanisms. *J Cell Biochem* 88(2):332-339.
45. Christakos CN, Papadimitriou NA, & Erimaki S (2006) Parallel neuronal mechanisms underlying physiological force tremor in steady muscle contractions of humans. *J Neurophysiol* 95(1):53-66.
46. Deluca HF & Cantorna MT (2001) Vitamin D: its role and uses in immunology. *FASEB J* 15(14):2579-2585.
47. Gregori S, *et al.* (2001) Transplantation tolerance by 1,25-dihydroxyvitamin D(3)-induced costimulation blockade. *Transplant Proc* 33(1-2):219-220.
48. Piemonti L, *et al.* (2000) Vitamin D3 affects differentiation, maturation, and function of human monocyte-derived dendritic cells. *J Immunol* 164(9):4443-4451.

49. Liu PT, *et al.* (2006) Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. *Science* 311(5768):1770-1773.
50. Zasloff M (2002) Antimicrobial peptides of multicellular organisms. *Nature* 415(6870):389-395.
51. Dubuquoy L, *et al.* (2006) PPARgamma as a new therapeutic target in inflammatory bowel diseases. *Gut* 55(9):1341-1349.
52. Kelly D, *et al.* (2004) Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. *Nat Immunol* 5(1):104-112.
53. Inagaki T, *et al.* (2006) Regulation of antibacterial defense in the small intestine by the nuclear bile acid receptor. *Proc Natl Acad Sci U S A* 103(10):3920-3925.
54. Rochette-Egly C & Germain P (2009) Dynamic and combinatorial control of gene expression by nuclear retinoic acid receptors (RARs). *Nucl Recept Signal* 7:e005.
55. Dolle P (2009) Developmental expression of retinoic acid receptors (RARs). *Nucl Recept Signal* 7:e006.
56. Fisher GJ, *et al.* (1994) Immunological identification and functional quantitation of retinoic acid and retinoid X receptor proteins in human skin. *J Biol Chem* 269(32):20629-20635.
57. Cras A, *et al.* (2007) Epigenetic patterns of the retinoic acid receptor beta2 promoter in retinoic acid-resistant thyroid cancer cells. *Oncogene* 26(27):4018-4024.
58. Farias EF, *et al.* (2010) Interference with Sin3 function induces epigenetic reprogramming and differentiation in breast cancer cells. *Proc Natl Acad Sci U S A* 107(26):11811-11816.
59. Vidal K, Grosjean I, evillard JP, Gespach C, & Kaiserlian D (1993) Immortalization of mouse intestinal epithelial cells by the SV40-large T gene. Phenotypic and immune characterization of the MODE-K cell line. *J Immunol Methods* 166(1):63-73.
60. Wang Y, *et al.* (2017) The intestinal microbiota regulates body composition through NFIL3 and the circadian clock. *Science* 357(6354):912-916.
61. Gundersen TE & Blomhoff R (2001) Qualitative and quantitative liquid chromatographic determination of natural retinoids in biological samples. *J Chromatogr A* 935(1-2):13-43.
62. Rajaii F, Bitzer ZT, Xu Q, & Sockanathan S (2008) Expression of the dominant negative retinoid receptor, RAR403, alters telencephalic progenitor proliferation, survival, and cell fate specification. *Dev Biol* 316(2):371-382.
63. Chapellier B, *et al.* (2002) A conditional floxed (loxP-flanked) allele for the retinoic acid receptor alpha (RARalpha) gene. *Genesis* 32(2):87-90.

64. Slaats J, Ten Oever J, van de Veerdonk FL, & Netea MG (2016) IL-1beta/IL-6/CRP and IL-18/ferritin: Distinct Inflammatory Programs in Infections. *PLoS Pathog* 12(12):e1005973.
65. Tolleson WH, *et al.* (2005) Photodecomposition and phototoxicity of natural retinoids. *Int J Environ Res Public Health* 2(1):147-155.
66. Blomhoff R, Green MH, Berg T, & Norum KR (1990) Transport and storage of vitamin A. *Science* 250(4979):399-404.
67. Rossi E, *et al.* (2007) Forced expression of RDH10 gene retards growth of HepG2 cells. *Cancer Biol Ther* 6(2):238-244.
68. Moutier E, *et al.* (2012) Retinoic acid receptors recognize the mouse genome through binding elements with diverse spacing and topology. *J Biol Chem* 287(31):26328-26341.
69. Podvinec M, Kaufmann MR, Handschin C, & Meyer UA (2002) NUBIScan, an in silico approach for prediction of nuclear receptor response elements. *Mol Endocrinol* 16(6):1269-1279.
70. Damm K & Evans RM (1993) Identification of a domain required for oncogenic activity and transcriptional suppression by v-erbA and thyroid-hormone receptor alpha. *Proc Natl Acad Sci U S A* 90(22):10668-10672.
71. Madison BB, *et al.* (2002) Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine. *J Biol Chem* 277(36):33275-33283.
72. Blomhoff R & Blomhoff HK (2006) Overview of retinoid metabolism and function. *J Neurobiol* 66(7):606-630.
73. Chapellier B, *et al.* (2002) A conditional floxed (loxP-flanked) allele for the retinoic acid receptor beta (RARbeta) gene. *Genesis* 32(2):91-94.
74. Stappenbeck TS, Hooper LV, Manchester JK, Wong MH, & Gordon JI (2002) Laser capture microdissection of mouse intestine: characterizing mRNA and protein expression, and profiling intermediary metabolism in specified cell populations. *Methods Enzymol* 356:167-196.
75. Ivanov, II, *et al.* (2008) Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe* 4(4):337-349.
76. Ivanov, II, Zhou L, & Littman DR (2007) Transcriptional regulation of Th17 cell differentiation. *Semin Immunol* 19(6):409-417.
77. Korn T, Bettelli E, Oukka M, & Kuchroo VK (2009) IL-17 and Th17 Cells. *Annu Rev Immunol* 27:485-517.
78. McClean SW, Ruddel ME, Gross EG, DeGiovanna JJ, & Peck GL (1982) Liquid-chromatographic assay for retinol (vitamin A) and retinol analogs in therapeutic trials. *Clin Chem* 28(4 Pt 1):693-696.

79. Rajala MW, Obici S, Scherer PE, & Rossetti L (2003) Adipose-derived resistin and gut-derived resistin-like molecule-beta selectively impair insulin action on glucose production. *J Clin Invest* 111(2):225-230.
80. Steppan CM, *et al.* (2001) A family of tissue-specific resistin-like molecules. *Proc Natl Acad Sci U S A* 98(2):502-506.
81. Everts HB (2012) Endogenous retinoids in the hair follicle and sebaceous gland. *Biochim Biophys Acta* 1821(1):222-229.
82. West KP, Jr., *et al.* (1995) Mortality of infants < 6 mo of age supplemented with vitamin A: a randomized, double-masked trial in Nepal. *Am J Clin Nutr* 62(1):143-148.
83. Orfanos CE, Ehlert R, & Gollnick H (1987) The retinoids. A review of their clinical pharmacology and therapeutic use. *Drugs* 34(4):459-503.
84. Schauber J, Dorschner RA, Yamasaki K, Brouha B, & Gallo RL (2006) Control of the innate epithelial antimicrobial response is cell-type specific and dependent on relevant microenvironmental stimuli. *Immunology* 118(4):509-519.
85. Zouboulis CC, Korge BP, Mischke D, & Orfanos CE (1993) Altered proliferation, synthetic activity, and differentiation of cultured human sebocytes in the absence of vitamin A and their modulation by synthetic retinoids. *J Invest Dermatol* 101(4):628-633.
86. Gong Z, *et al.* (2016) Chenodeoxycholic acid activates NLRP3 inflammasome and contributes to cholestatic liver fibrosis. *Oncotarget* 7(51):83951-83963.
87. Hao H, *et al.* (2017) Farnesoid X Receptor Regulation of the NLRP3 Inflammasome Underlies Cholestasis-Associated Sepsis. *Cell Metab* 25(4):856-867 e855.