Enhancing Effector T Cell Migration to Mucosal Regions

Using Rexinoids and Plasmid Adjuvants

by

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ABSTRACT

Mucosal membranes represent a major site of pathogen transmission and cancer development. Enhancing T cell migration to mucosal surfaces could improve immune-based therapies for these diseases, yielding better clinical outcomes. All-trans-retinoic acid (ATRA) is a biologically active form of vitamin A that has been shown to increase T cell migration to mucosal sites, however its therapeutic use is limited by its toxicity potential and unstable nature. ATRA-related compounds with lower toxicity and higher stability were assessed for their ability to induce similar immune migration effects as ATRA, using *in vitro* and *in vivo* model systems.

Chapter 2 summarizes the first project, in which synthetic, ATRA-like compounds called rexinoids were used to modulate T cell expression of mucosal homing proteins chemokine receptor 9 (CCR9) and integrin alpha 4 beta 7 ($\alpha 4 \beta 7$), and alter their physical migration *in vitro*. Several rexinoids independently mimicked the activity of ATRA to enhance protein expression and migration, while others worked synergistically with subtoxic doses of ATRA to produce similar results. Furthermore, rexinoid administration *in vivo* was well-tolerated by animal models, a finding not seen with ATRA.

Chapter 3 focuses on the second project, where plasmids containing ATRA-synthesizing proteins were assessed for their *in vivo* ability to act as mucosal vaccine adjuvants and enhance T cell migration to mucosal sites during DNA vaccination. Though increased mucosal migration was seen with use of the adjuvant plasmids, these findings were not determined to be significant. Immune-mediated protection following

viral challenge was also not determined to be significant in animal models receiving both vaccine and adjuvant plasmids.

The data shows that several novel rexinoids may possess enhanced clinical utility compared to ATRA, lending support for their use in immunotherapeutic approaches towards mucosal maladies. While the potential mucosal vaccine adjuvants did not show great significance in enhancing T cell migration or viral protection, further optimization of the model system may produce better results. This work helps advance knowledge of immune cell trafficking to afflicted mucosal regions. It can be used as a basis for understanding migration to other body areas, as well as for the development of better immune-based treatments.

To my MCB cohort, Peaches, Carrie, Cindy, Alex, Kevin, Mark, I am so glad we were able to share this journey.

And finally, to my family. Mom, Dad, Pa, Nim, Gurjeet, Jasmine, Meesha, you are the best family in this universe and all others. Thank you for loving and believing in me.

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9cRA	9-cis-retinoic acid
α4β7	integrin alpha 4 beta 7
ACK	Ammonium-Chloride-Potassium
ACT	adoptive cell transfer
ADH	alcohol dehydrogenase
ALT	alanine transaminase
ANOVA	analysis of variance
APML	acute promyelocytic anemia
ATRA	all-trans-retinoic acid
BEX	bexarotene
BM	bone marrow
BMT	bone marrow transplant
CAR T	chimeric antigen receptor T cell
CCL25/TECK	chemokine ligand 25/thymus-expressed chemokine
CCR9	chemokine receptor 9
CCR10	chemokine receptor 10
CD	cluster of differentiation
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CMV	cytomegalovirus
CO_2	carbon dioxide
CRABP1	cellular retinoic acid-binding protein 1

CRABP2	cellular retinoic acid-binding protein 2
CRBP1	cellular retinol binding protein 1
CT	cholera toxin
CTCL	cutaneous T cell lymphoma
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4
CYP1B1	cytochrome 450 1B1
D	diversity
DC	dendritic cell
DMSO	dimethyl sulfoxide
DNC	double-negative control
DP-RAR	dominant positive retinoic acid receptor
DS	differentiation syndrome
FBS	fetal bovine serum
GMuLV	Gross murine leukemia virus
GPCR	G protein-coupled receptor
HAT	histone acetyl transferase
HDAC	histone deacetylase
HeSCs	hepatic stellate cells
HEV	high endothelial venule
HIV-1	human immunodeficiency virus type 1
HIV_{bal}	pMAX-PRO-bal-gp120
HIV_{gag}	WLV-151M
HSC	hematopoietic stem cell

Hxb2	HIV-1 group M subtype B
ICB	immune checkpoint blockade
Ig	immunoglobulin
IL-2	interleukin 2
ILN	inguinal lymph node
i.m.	intramuscular
i.p.	intraperitoneal
J	joining
LBD	ligand binding domain
LCMV	lymphocytic choriomeningitis virus
LN	lymph node
LPL	lamina propria lymphocytes
LSD	least significant difference
LXR	liver X receptor
MadCAM-1	mucosal addressin cell adhesion molecule-1
MHC	major histocompatibility
MIIC	MHC II loading compartment
mOS	metastatic osteosarcoma
MPP	multipotent progenitor
NK	natural killer
OT-1	C57BL/6-Tg(TcraTcrb)1100Mjb/J
OV	oncolytic virotherapy
P14	B6.Cg-*Tcra^{tm1Mom}*Tg(TcrLCMV)327Sdz/TacMmjax

PBS	phosphate buffered saline
PD-1	programmed death receptor 1
PD-L1	programmed death-ligand 1
pDNA	plasmid DNA
p:MHC	peptide and MHC
PP	Peyer's patch
PPARγ	peroxisomal proliferator-activated receptor gamma
PSG	penicillin-stremtomycin-glutamine
RA	retinoic acid
RALDH	retinol dehydrogenase
RALDH2	retinol dehydrogenase 2
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBC	red blood cell
RDH	retinol dehydrogenase
RER	rough endoplasmic reticulum
RPMI	Roswell Park Memorial Institute
RXR	retinoid X receptor
s.c.	subcutaneous
SI	small intestine
SIV	simian immunodeficiency virus
SMARTA	B6.Cg-$Ptprc^aPepc^b$Tg(TcrLCMV)1Aox/PpmJ
STRA6	signaling receptor and transporter of retinol

TCR   T cell receptor

TIL   tumor infiltrating lymphocyte

TME   tumor microenvironment

Tregs   regulatory T cells

UV   ultraviolet

V   variable

VACV   vaccinia virus

VACV$_{gag}$ Western Reserve vaccinia virus expressing HIV-1 subtype B gag

                       protein

VDR   vitamin D receptor

VDRE   vitamin D response element

CHAPTER 1

INTRODUCTION

Discovery of T Cells

"There isn't a single advance in vaccine, immunotherapy or autoimmunity research that doesn't incorporate [Jacques Miller's] thinking." -Nobel Laureate Peter C. Doherty.

To claim 20th century immunologist Jacques Miller deserves a Nobel Prize is almost inarguable. His discovery in the early 1960's that thymus derived cells were essential for proper immune function was groundbreaking, destroying long-held beliefs that the thymus was either a lymphocyte graveyard or simply a vestigial structure. However, like Louis Pasteur's discovery of a chicken cholera vaccine, Wilhelm Röntgen's discovery of X-rays, and Alexander Fleming's discovery of penicillin, Jacques Miller's discovery of thymic function occurred somewhat by accident. Originally hypothesizing that neonatal thymectomy could prevent Gross murine leukemia virus (GMuLV)-induced lymphocytic leukemia due to a lack of the thymic cells needed for viral replication, Miller was surprised to discover that thymectomized mice succumbed to wasting disease within a matter of weeks, regardless of whether they had received GMuLV injection or not (1,2). Postmortem examination showed the mice had reduced numbers of lymphocytes throughout the body, giving Miller an inkling that immune cells could come from the thymus. To check on his suspicions, Miller performed heterologous skin grafts on the neonatally thymectomized mice, knowing that rejection would occur in immunologically-competent mice (1-3). The lack of rejection he saw strengthened Miller's belief that some lymphocytes were produced by the thymus, and was clinched

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following the discovery that rejection was rescued in thymectomized mice who were given either a thymic graft or lymphocyte infusion following skin transplantation (2). The importance of these thymically-derived lymphocytes, now known as T cells, in eliminating pathogens was further demonstrated when Miller performed his experiments in germ-free facilities; mice thymectomized in a pathogen-free environment accepted heterologous skin grafts, however did not succumb to wasting disease, indicative that the wasting disease was caused by their inability to control infection (1,3). Miller's discoveries marked the birth of T cell biology, a field in which research has exploded over the last 60 years. We now recognize the important role T cells play in eliminating infected cells, malignant cells, and providing immunological memory, and their equally controversial role in autoimmunity and immunopathology. Perhaps just as importantly, Miller's findings finally gave the thymus its due as a functionally important body organ.

Immune System Architecture

To understand the role T cells play in a human immune response, it is important to first characterize the parts of an immune system itself. Here I discuss the basic structure of the lymphatic system, the organ system in which a majority of immune cells are contained and where T cell responses are initiated.

Lymphatic system

The immune system is identified in part by specialized cells and soluble factors largely found within the lymphatic system that work together to defend the host (4). Often referred to as the body's drainage system, the lymphatic system primarily acts to remove cellular waste and recapture excess tissue fluid for return to the blood circulation (4,5). Comprised of an interconnected network of vessels, organs, and tissues that overlay

the blood circulatory system (Figure 1.1A), the porous ends of lymphatic capillaries permit a one-way flow of water, ions, gases, proteins, small antigens (<70kDa) and migrating cells from the body into the lymph (4). Lymph flows through progressively larger lymphatic vessels and ducts, and returns to the bloodstream via the left and right subclavian veins (4). To mitigate the risk of pathogens, toxins, and other undesirables from entering the blood, lymph is continuously filtered through lymph nodes (LNs), lymphatic organs found throughout the body in which large numbers of immune cells congregate (Figure 1.1B) (6). It is within these structures that T (and B) cell recognition of antigen occurs, and immune memory can be generated (4-6). Lack of antigen recognition in one LN results in T/B cell migration to the next, thus permitting these lymphocytes to continually circulate through both the lymphatic and blood circulatory system to search for their cognate antigen (7).

Primary and secondary lymphatic tissues

Lymphatic tissues are often divided into primary and secondary fractions, to distinguish sites of immune cell development from sites of T/B cell responses. Primary lymphoid tissues include the bone marrow (BM) and thymus, the main sites of immune cell development (Figure 1.2) (8). Secondary lymphoid tissues include the spleen and numerous LNs scattered throughout the body, serving as the site for T and B cell response initiation (Figure 1.2) (8).

Extra-lymphatic immune cells

It is important to note that not all immune cells exist within the lymphatic system. Certain immune cell subtypes are found enriched in extra-lymphatic epithelial tissues, poised to detect pathogens invading via compromised skin or mucosal barriers (9-12).

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Still others are found within the central nervous system, patrolling for disruptions in brain homeostasis (13,14). In each case, detection of foreign antigen within these regions results in resident immune cells providing an immediate antimicrobial response, and transport of antigen to the nearest draining LN for recognition by the appropriate antigen-specific T/B cell (15).

Innate vs Adaptive Immunity

The immune system is often subdivided into innate and adaptive fractions, with T cell activity falling into the latter category. However, as onset of adaptive responses are dependent on innate forewarning, here I briefly identify distinguishing features of both the innate and adaptive immune system (Figure 1.3).

Innate Immune System

Pathogenic invasion, cellular damage, and malignancy are initially detected by cells of the innate immune system, which include granulocytes, macrophages, and natural killer (NK) cells (Figure 1.3A) (16). Often called the first responders, innate immune cells act to provide an immediate, nonspecific, pro-inflammatory response upon recognition of non-self antigen (16). The production of pro-inflammatory cytokines by innate immune cells helps to slow body-wide disease dissemination, as well as recruit more immune cells to the affected site (16). Furthermore, these cytokines help activate and mature dendritic cells (DCs), an essential immune cell subtype responsible for directly stimulating adaptive immune cell responses (16-18).

Adaptive Immune System

Complete elimination of disease-causing agents is due to the activity of the adaptive immune system, which is comprised of T cells, B cells, and their respective

derivatives (Figure 1.3B) (18,19). In contrast to innate immune responses, adaptive responses are delayed, highly specific, and provide immunological memory towards the intruder should invasion occur again (18). It is significant to note that the activation of adaptive immune cells is extremely regulated and occurs only in secondary lymphoid organs, not at the site of infection as with innate immune cells (18). DCs that have taken up antigen and been activated in peripheral regions migrate from the tissues in which they are enriched towards draining LNs, to present the antigen to the circulating naïve (not yet activated) T cells (Figure 1.4) (17,18,20). Alternatively, DCs that reside within lymphoid tissue can capture and present antigen that has freely diffused to the LN via the lymphatic capillaries to naïve cells circulating through (21,22).

T Cell Development

The ability of T cells to recognize and respond to an extraordinarily diverse array of antigens while minimizing responses to self is tied to their stringent development process. Here we summarize T cell development from hematopoietic stem cell (HSC) to mature naïve T cell, with attention paid to the remarkable mechanism by which T cells generate their unique antigen recognition receptor, V(D)J recombination.

HSC to T cell progenitor

While the thymus is the major organ for T cell development and maturation, T cells originate in the BM (23). T cells arise from HSCs, multipotent cells that have the capability to either self-renew or differentiate into the progenitor for any blood cell (23,24). This progenitor cell, aptly named a multipotential progenitor cell (MPP), can further differentiate into either a common myeloid progenitor (CMP) or common lymphoid progenitor (CLP), specialized multipotent precursors that differentiate into cells

of the myeloid or lymphoid lineage, respectively (Figure 1.5) (24). CLPs destined to become T cells must receive appropriate Notch-1 signals in order to commit to the T cell pathway; those that do not head down the B cell development pathway by default (Figure 1.5) (25).

Following commitment to the T cell fate, T cell progenitors migrate from the BM to the thymus to continue their development (23,25). Exit from the blood circulation into the thymus occurs via the high endothelial venules (HEVs). HEVs are specialized post-capillary structures (Figure 1.1B) found within lymphoid tissue that lymphocytes use to move from the blood into the lymphatics, and, under specific conditions, use to move from the lymphatics into the blood (6,7,26). Once in the thymus, T cell progenitors undergo a series of development stages to produce their functional antigen recognition receptor, the T cell receptor (TCR) (23,27). Structurally, TCRs are plasma membrane-bound, heterodimeric proteins composed of immunoglobulin (Ig) domain chains that form either an αβ or γδ heterodimer (Figure 1.6) (27). The antigen recognition region of the TCR is located at the distal end of the receptor, facing the extracellular environment, and is the region that displays tremendous variability in terms of its specificity (Figure 1.6) (27-29). This variability is due to the fact that TCR chains are formed by V(D)J recombination, a process by which intentional rearrangement of TCR Ig gene segments results in each T cell possessing a unique TCR (27,28).

V(D)J recombination

V(D)J recombination occurs individually for both chains forming a TCR, and involves recombination events that occur between specific germline gene segments termed variable (V) and joining (J) for α and γ chains, and separate variable (V), diversity

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(D), and joining (J) gene segments for β and δ chains (27,28). Each gene segment

possesses multiple different versions in the germline DNA, allowing for a large number

of different TCRs to be generated based on combination alone (27,28). V(D)J

recombination enzymes drastically increase the breadth of the TCR repertoire by adding

or removing nucleotides present at the joining region of recombined gene segments,

boosting possible TCR combinations to above 10^{20} (30). Regular testing occurs during the

process of V(D)J recombination to guarantee that newly generated chains are capable of

transmitting signal intracellularly (23,27-29). As T cells possessing αβ TCRs are

functionally well-characterized and comprise the majority of T cells in the body, they will

therefore remain the focus for this dissertation and γδ TCRs will not be further discussed.

Positive and negative selection

Developing T cells with newly minted TCRs must undergo additional testing to

confirm their TCR is optimally functional before they can leave the thymus. Testing is

divided into two phases, which are spatially and temporally separated in the thymus. The

first phase, positive selection, occurs on DCs in the thymic cortex (23). Positive selection

ensures that T cell TCRs are capable of recognizing major histocompatibility proteins

(MHC), self-proteins on which foreign peptides must be presented for recognition (Figure

1.7) (23). The second phase, negative selection, occurs on DCs in the thymic medulla

(23). Here, T cell TCRs are tested for self-reactivity. Those who possess TCRs that

recognize self-peptide presented on MHC above a threshold value are induced to undergo

apoptosis, to prevent these cells from circulating and inducing autoimmune responses

elsewhere in the body (23).

Lineage commitment

In-between the phases of positive and negative selection lies lineage commitment, the process by which developing T cells commit to becoming one of the two major T cell subtypes: either a CD4+ or CD8+ T cell (23). These cells are structurally differentiated from each other by the presence of either a CD4 or CD8 glycoprotein on their surface (Figure 1.8), and functionally differentiated based on their activity during an immune response; CD4+ T cells are largely categorized as 'helper' T cells due to the assistance they provide immune cells during the response, and CD8+ T cells are referred to as 'cytotoxic' T cells due to their ability of inducing target cell apoptosis (29,31,32). Lineage-committed T cells that have passed negative selection are finally free to leave the thymus and enter the circulation.

T Cell Recognition of Antigen and Activation

Unlike cells of the innate immune system, which provide immediate antimicrobial effects upon recognition of foreign antigen, T cell recognition and subsequent activation are delayed processes under strict control. Here we identify how the two main T cell subtypes, CD4+ and CD8+, recognize antigen, and begin the activation process to becoming a functional cell capable of contributing to an immune response.

Antigen recognition overview

Naïve CD4+ and CD8+ T cells both recognize foreign antigen presented as a linear peptide fragment on the MHC of activated DCs that have either traveled to the draining LN via the lymphatics, or captured antigen in the LN itself (17,18,29). As CD4+ and CD8+ T cells recognize different MHC, DCs are tasked with processing antigen appropriately for both CD4+ and CD8+ T cell recognition (29). Furthermore, most

microbial antigens initially exist in a 3D conformation, therefore DCs must first fragment and linearize these structures before loading them onto MHC.

CD4+ T cell antigen processing

CD4+ T cells recognize antigenic peptides presented on MHC II molecules (29). To accomplish this, DCs that have taken up protein antigen via endocytosis first fuse the antigen-containing endosomes with lysosomes, whose low pH and degradative enzymes facilitate the breakdown of antigenic proteins (33). Vesicles containing fully formed MHC II molecules that were synthesized on the rough endoplasmic reticulum (RER) then fuse with the endosomes containing antigenic peptide fragments, forming the MHC II loading compartment (MIIC) (33). Additional proteins within the MIIC facilitate loading of high affinity antigenic peptide onto the MHC II molecule, which then travels to and fuses with the plasma membrane for presentation to CD4+ T cells (33). Only CD4+ T cells that are capable of recognizing both the MHC II molecule, via their TCR and CD4 glycoprotein, and specific peptide being presented will begin activation; CD4+ T cells specific for other peptides will continue browsing (Figure 1.9) (33,34).

CD8+ T cell antigen processing

In contrast to CD4+ T cells, CD8+ T cells recognize peptides presented in the context of MHC I molecules (Figure 1.10) (29,33). Endocytosed protein antigen can be funneled from the endosomal interior into the cytoplasm prior to lysosomal fusion, where it is then subject to degradation by the immunoproteasome, a multi-subunit protein complex specialized for generating peptide fragments capable of binding MHC I (33). These peptide fragments are moved into the RER near to newly synthesized MHC I molecules, and chaperone proteins assist with loading peptides onto the MHC molecules

(33). Similar to MHC II, loading of high affinity peptide results in MHC relocation to the plasma membrane, where it then presents the peptide to perusing CD8+ T cells. As with CD4+ T cells, CD8+ T cells can only begin their activation process if they recognize both the peptide (via their TCR) and MHC I molecule (via their TCR and CD8 glycoprotein) on which the peptide is hoisted (34).

T cell activation

T cell activation can be briefly defined as the upregulation of genes that promote T cell survival, proliferative ability, and differentiation into effector cells capable of mounting an immune response. Extensive detail of the intracellular signaling cascades needed for gene upregulation will not be discussed here, however it is important to note that, while T cell recognition of both peptide and MHC (p:MHC) initiates the T cell activation process, full T cell activation requires two additional steps.

Along with p:MHC:TCR stimulation, further stimulatory signals are provided by the DC to the T cell via many other receptor-ligand interactions, a notable one being cluster of differentiation protein 80/86 (CD80/86) ligand on the DC binding to CD28 receptor on the T cell (Figure 1.11) (35). These 'costimulatory' signals trigger intracellular pathways that reinforce upregulation of T cell activation genes and therefore represent the second step required for activation; lack of costimulation is associated with severe impairment of T cell activation and subsequent apoptosis (35,36).

Cytokine signals represent the third step required for full T cell activation (Figure 1.11) (37,38). These signals are provided by both the activated T cells themselves and the activating DCs, and assist with T cell expansion and effector cell differentiation (37-39). Successful provision of signals 1 and 2 result in T cell upregulation of Interleukin-2 (IL-

2), a survival cytokine that stimulates both CD4+ and CD8+ T cell proliferation (40). While CD8+ T cells also use IL-2 to differentiate into effector cells with cytotoxic ability, CD4+ T cells require additional cytokines, as they must be able to differentiate into a helper subtype specific for the type of infection at hand (31). Each helper subset is induced by different sets of cytokines, which are provided by DCs engaged with the T cells (31).

T Cell Function

Successful activation results in naïve T cell differentiation into cells capable of contributing to an immune response. The resulting effector cells can influence the action of other immune cells or be directly cytotoxic towards infected or tumorigenic self cells, and are often identified by their expression of CD4 or CD8. Here I summarize the canonical effector function of both T cell subtypes following their activation, as well as the significance of these cells in a classical immune response.

CD4+ T cells – the helpers

The central role of effector CD4+ T cells is to help shape the overall immune response by regulating immune cell function, a task largely accomplished by their production of cytokines (41). Each helper subset produces a distinct array of cytokines that, upon cell-cell engagement, can stimulate innate activity, expand CD8+ T cell populations, regulate DC activation status, and permit newly activated B cells to produce high-affinity antibodies of an appropriate infection-specific isotype (41). While the details of each helper subset will not be discussed, it is important to note that not all subsets promote a pro-inflammatory response. Regulatory T cells (Tregs) are a CD4+

helper subset that act to subdue immune responses via their sequestration of IL-2, suppression of DC activity, and direct inhibition of effector CD8+ T cell activity (31).

CD8+ T cells – the killers

Unlike effector CD4+ T cells, effector CD8+ T cells are not further subdivided, and primarily act to kill target cells by triggering apoptotic pathways (42). Effector CD8+ T cells can induce apoptosis using multiple mechanisms, including cytotoxic granule release, death receptor expression, and cytokine production (42). Regardless of the mechanism used, apoptotic pathway selected, or initiator/executioner caspase activated, the end result is a quiet, non-immunogenic death for the doomed cell.

Protective role of T cells during infection and cancer

Individuals with T cell immunodeficiencies are highly susceptible to pathogenic infection, highlighting the importance of these cells in immune responses (43). While other immune cells can display cytotoxic activity towards infected cells, patients with T cell deficiencies exhibit persistent, recurrent, and opportunistic infections, indicating an inability of other immune subsets to quell pathogenic microbes, provide immunological memory during re-infection, or maintain commensal populations (43). Unsurprisingly, individuals who lack T cell function also display abnormal antibody responses, as a lack of helper T cells inhibits full B cell activation (44).

Furthermore, reduced or absent effector CD8+ T cell presence is positively correlated with tumor progression, indicating the need for these cells to destroy malignant cells (45-48). It has been postulated that people who lack T cells may be at higher risk for developing certain cancers, however this has proven difficult to study; individuals who

do not produce T cells succumb to infections very early in life if the condition is not repaired by a bone marrow transplant (BMT) (49,50).

T Cell Migration Following Activation

Newly activated effector T cells are useless if they cannot move from the draining LN to the body area in which they are needed. While a subpopulation of effector CD4+ T cells stay behind to assist with B cell differentiation, the remainder population and all effector CD8+ T cells must leave the LN and travel the body to reach the diseased site. Here we describe how these effector T cells traffic from the LN to an affected tissue, the cells responsible for giving them directions, and how migratory activity can impact therapeutic efficacy.

Chemokines direct immune cell migration

Overall immune cell migration from one area to another is governed by chemoattractant cytokines called chemokines, small, soluble proteins produced by both immune and non-immune cells that activate intracellular signaling pathways needed for movement (51). Chemokines exert this effect upon binding to chemokine receptors, immune cell surface receptors of the G protein-coupled receptor (GPCR) family (51). Over 40 different chemokines have been identified, and are grouped into sub-families as either C, CC, CXC, or CX3C chemokines, depending on the organization of cysteine residues in a conserved sequence motif (51). Consequently, the 19 identified chemokine receptors are divided based on the chemokine sub-family they bind, and are written as XCR, CCR, CXCR, or CX3CR (51).

Under non-inflammatory conditions, certain constitutively expressed chemokines promote immunosurveillance by directing immune cell traffic through the blood, lymph,

and tissues (51). However, pro-inflammatory conditions result in increased production of tissue-specific and inflammatory chemokines to selectively enhance immune cell recruitment to the affected area (51). In order for newly activated effector T cells to make the trek from the draining LN, they must express chemokine receptors capable of binding the chemokines being released (51). In brief, initial egress from the draining LN results in effector T cell entry into the blood circulation. Cells are then prompted to slow down and roll along the blood vessel wall via low affinity interactions between adhesive selectin and addressin proteins, expressed on both the effector and endothelial cell surface (Figure 1.12A) (52). This slowing enables chemokine receptor-chemokine ligand binding, which activates surface integrin receptors expressed by the T cell to a high affinity form (Figure 1.12B) (52). Activated integrin-ligand binding results in stable adhesion, allowing the effector cell to then migrate through inter-endothelial junctions into the target tissue (Figure 1.12C) (52).

DCs imprint effector T cells with tissue-specific homing phenotypes

Effective migration and tissue entry requires effector T cells to express both the appropriate chemokine and integrin receptors prior to exit from the draining LN. This imprinting effect is mediated by DCs during T cell activation; DCs that have migrated from specific body regions program T cells to express the combination of receptors that will send them to the same location (53). While the precise moment of imprinting is not well elucidated, it has been shown that DC secretion of select vitamins influences T cell expression of tissue-specific homing receptors (53,54).

Clinical relevance of migration

Modulating effector T cell migration could improve the effectiveness of several different immunotherapies, including cancer treatments and vaccines towards infectious diseases. The recent introduction of immune checkpoint blockade (ICB) therapy, a cancer therapeutic that uses antibodies to block inhibitory interactions between malignant cells and effector T cells within the tumor and promote the activation of new tumor-specific T cells, has shown success in treating a variety of cancers, however this efficacy is currently limited to a subset of patients (55-57). It is hypothesized that this may be due to the newly activated tumor-specific T cells inefficiently migrating to the tumor site, which would result in effector cells already within the tumor becoming quickly overwhelmed and losing function. Identifying ways to improve the migration of these newly activated cells could enhance T cell effector function in the tumor microenvironment (TME), resulting in better control and patient survival.

Similarly, manipulating the migration of effector T cells during vaccination could result in better immune protection following infection. It has been experimentally shown that treating mice with vitamin A during vaccination results in better T cell migration to mucosal surfaces and reduced viral load following subsequent virus challenge at the vaginal mucosa (58). Therefore coupling low efficacy vaccines with adjuvants that induce tissue-specific migration may boost vaccine potency.

Vitamin A and T Cell Migration

It is well established that T cells activated in the presence of the biologically active vitamin A derivative all-trans-retinoic acid (ATRA) enhance their expression of homing receptors that target them to mucosal regions (59). Here we summarize how ATRA is generated, and highlight how ATRA signaling results in receptor upregulation.

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ATRA generation

Vitamin A is an umbrella term used to encompass several structurally related, fat-soluble compounds that play a significant role in numerous physiological processes, including immune function (60-63). Vitamin A is obtained from the diet, found in both animal and plant food sources. Major dietary forms of vitamin A include retinol and β-carotene, both of which can serve as precursors to the biologically relevant ATRA (60). In brief, retinol taken up from the diet is transported throughout the body on the back of retinol binding protein 4 (RBP4), a hepatically-produced carrier protein responsible for delivering vitamin A to cells in need (64). Intake of β-carotene, the predominant vitamin A found in plant sources, often requires conversion to retinol before it can be transported (65,66). Docking of RBP4 to the extracellular signaling receptor and transporter of retinol (STRA6) protein permits retinol entry into the cell, where cellular retinol binding protein 1 (CRBP1) can bind the hydrophobic compound (Figure 1.13) (64). Retinol conversion to ATRA occurs in the cytosol; retinol is first oxidized to the intermediate retinaldehyde via the catalytic activity of several alcohol and retinol dehydrogenases (ADHs and RDHs, respectively), and then further oxidized to ATRA by the action of multiple retinaldehyde dehydrogenases (RALDHs) (Figure 1.13) (67). It has been observed that mucosally-derived DCs preferentially express ADH-1, -4, and -5 and RALDH-1 and -2 to facilitate their production of ATRA (68).

It should be noted that retinol can alternatively be converted to an isomeric form of ATRA called 9-cis-retinoic acid (9cRA), which is classified as a biologically active vitamin A metabolite (69). This thesis work primarily focused on using the ATRA metabolite, as the function of 9cRA on immune cell migration is not well elucidated.

ATRA signaling

ATRA is picked up by cellular retinoic acid-binding protein 1 and 2 (CRABP1 and CRABP2, respectively) and transported into the nucleus, where ATRA then acts like a hormone upon binding its receptor, the retinoic acid receptor (RAR) (Figure 1.14) (59,63,70). The RAR is a type II nuclear receptor, meaning it exists within the nucleus as a heterodimer with the retinoid X receptor (RXR) (Figure 1.14A) (59,63,71). The RAR/RXR heterodimer binds to retinoic acid responses elements (RAREs), specific DNA sequences found within the promoter region of retinoic acid (RA) responsive genes (72). In the absence of ATRA, the heterodimer is bound to corepressor complex proteins that maintain chromatin condensation and transcriptional silence by promoting histone deacetylase (HDAC) activity (72). ATRA binding to the RAR induces conformational changes to the heterodimer, resulting in corepressor protein dissociation and coactivator complex protein recruitment (Figure 1.14B) (72). Binding of coactivator proteins stimulates recruitment of histone acetyl transferases (HATs) and transcriptional machinery proteins, which ultimately promote chromatin relaxation and gene transcription (72).

ATRA and mucosal homing receptor upregulation

ATRA signaling through the RAR/RXR heterodimer has been shown to induce effector T cell expression of chemokine receptor 9 (CCR9) and integrin alpha 4 beta 7 ($\alpha 4 \beta 7$), both of whose ligands, chemokine ligand 25 (CCL25) and mucosal addressin cell adhesion molecule-1 (MadCAM-1), respectively, are predominantly expressed by gut-mucosal endothelial cells (59,73,74). Interestingly, high numbers of CCR9+ effector T cells have also been seen in the lungs and vaginal tract following vaccination coupled

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with ATRA treatment, which suggests that CCL25 expression is inducible by other mucosal regions during inflammation (58).

Limitations to Exogenous Vitamin A as a Therapeutic Modality

The potential of ATRA treatment to enhance mucosal immune protection during infection has been documented in murine models (58,75). Additionally, the non-immune ability of ATRA signaling to encourage dedifferentiated malignant cell maturation into a non-malignant phenotype has led to its successful use as a cancer treatment for individuals with acute promyelocytic leukemia (APML), a disease specifically marked by RAR translocation (76-78). The further discovery that ATRA treatment can upregulate pro-apoptotic genes has stimulated interest in its overall ability to impede cancer progression, however the general use of ATRA will likely remain limited due to side effects associated with treatment (79-81). Here we discuss characteristics of ATRA that preclude its widespread administration, as well as patient side effects reported following treatment.

Chemical instability

Reconstituted ATRA has been shown to be highly susceptible to photoisomerization when exposed to visible light, with degradation occurring as soon as 30 minutes post-exposure (82,83). Exposure to other ubiquitous elements such as heat and oxygen also result in ATRA oxidation and degradation, which together indicate that the sensitivity of ATRA to the elements may impede treatment transport and long-term storage (84).

High toxicity potential

The fat-soluble nature of vitamin A slows its excretion from the body, which can quickly result in toxic buildup (85-87). Excess vitamin A is stored in hepatic stellate cells (HeSCs) as retinyl palmitate, which can readily be converted to retinol based on body needs (88). However, high accumulation within HeSCs has been reported to trigger their activation, resulting in liver inflammation, injury, and fibrosis (89).

Adverse side effects

Differentiation syndrome (DS) is a life-threatening complication seen in APML patients treated with exogenous ATRA, occurring in an estimated 25% of individuals (90-93). Characterized by idiopathic fever, peripheral edema, pulmonary hemorrhage, and renal failure, DS is often fatal if swift diagnosis and interventions are not taken (90-93). Cessation of ATRA and use of corticosteroids such as dexamethasone have shown success in reducing mortality associated with DS, however this treatment efficacy is reduced in patients who present with more severe forms of DS (90-93).

Rexinoids and T Cell Migration

The RXR partner of the RAR also contains a ligand binding domain (LBD) (Figure 1.14), and it has been demonstrated that ligand bound to both the RAR and RXR can enhance transcription of RA-responsive genes (94,95). Surprisingly, known endogenously occurring ligands for the RXR remain functionally elusive. Here we characterize the RXR and discuss artificial rexinoids, synthetically created agonists for the RXR. We further examine the immunomodulatory potential of rexinoid-mediated signaling to influence effector T cell migration.

RXR signaling

Like the RAR, the RXR exists within the nucleus as a dimeric protein capable of binding ligand, with its activity also regulated by the dissociation and association of corepressor/coactivator complexes, respectively (94,95). A highly promiscuous receptor, the RXR has been shown to be the functional binding partner of a myriad of other nuclear receptors besides the RAR, indicating its critical role in regulating hormonal activity (96). The RAR/RXR heterodimer has been termed a "non-permissive" heterodimer, meaning that ligand bound to the RXR only is insufficient to induce transcription (72,94). Indeed, the RXR is physically unable to bind its ligand in the absence of an RAR ligand, as corepressor complex proteins remain associated with the heterodimer when only RXR ligand is present (94). As previously mentioned, RAR activation triggers corepressor dissociation, thus permitting the RXR to bind its ligand and synergistically affect RAR-mediated transcription. Contrastingly, some RXR heterodimers, such as the RXR/peroxisomal proliferator-activated receptor gamma (PPARγ) and RXR/liver X receptor (LXR), are "permissive", meaning ligand binding to either receptor enables transcriptional progression (94).

Rexinoids

A small handful of endogenously occurring molecules, including 9cRA and some fatty acids, have been shown to bind the RXR, however their *in vivo* efficacy to activate the receptor and influence transcription remains controversial (97-99). This has led many groups to create synthetic RXR agonists, which are collectively referred to as 'rexinoids'. Structurally similar to ATRA and 9cRA, rexinoids display an improved stability and lower toxicity profile compared to ATRA (100-102). Rexinoid use has shown promising results in a variety of preclinical models of disease, including Alzheimer's, diabetes, and

cancer, due to their ability to modulate RXR activity (103,104). In 1999, clinical approval was given to the rexinoid bexarotene (Targretin®) for the treatment of cutaneous T cell lymphoma (CTCL), as it was shown that use of this rexinoid induced malignant cell apoptosis (101,102). RAR signaling is associated with the upregulation of pro-apoptotic and anti-proliferative genes, therefore it may be that bexarotene signaling through the RXR enhances RAR anti-cancer activity.

Rexinoid modulation of T cell migration

While signaling via the RAR is shown to upregulate T cell expression of mucosal homing receptors CCR9 and α4β7, it is unclear how rexinoid use modulates this activity. The ability of RXR signaling to enhance RAR activity suggests that rexinoids may improve mucosal receptor upregulation and subsequent T cell migration upon binding the RXR, indicating a potential immunotherapeutic role. Furthermore, the discovery that some rexinoids can cross-bind and activate the RAR indicates they may be able to mimic the activity of ATRA, therefore eliminating risks associated with exogenous ATRA use (105).

Thesis Objective

This dissertation work focused on identifying ways to manipulate effector T cell migration to mucosal surfaces, a major site of pathogen access and cancer development. By enhancing effector cell presence within these regions, better immune protection against mucosal diseases can be achieved. Multiple approaches were used to address this objective, and are the subject of Chapters 2 and 3. In brief, Chapter 2 discusses the ability of several novel rexinoids to modify effector T cell expression of CCR9 and α4β7 and subsequent cellular migration. Chapter 3 considers the use of pro-synthesis ATRA

enzymes as mucosal homing adjuvants to enhance vaccine efficacy towards human immunodeficiency virus type 1 (HIV-1), a pathogen largely transmitted across mucosal surfaces (106). While brief discussions of findings are included in these chapters, an in-depth discussion regarding significance and future potential is provided in Chapter 4.
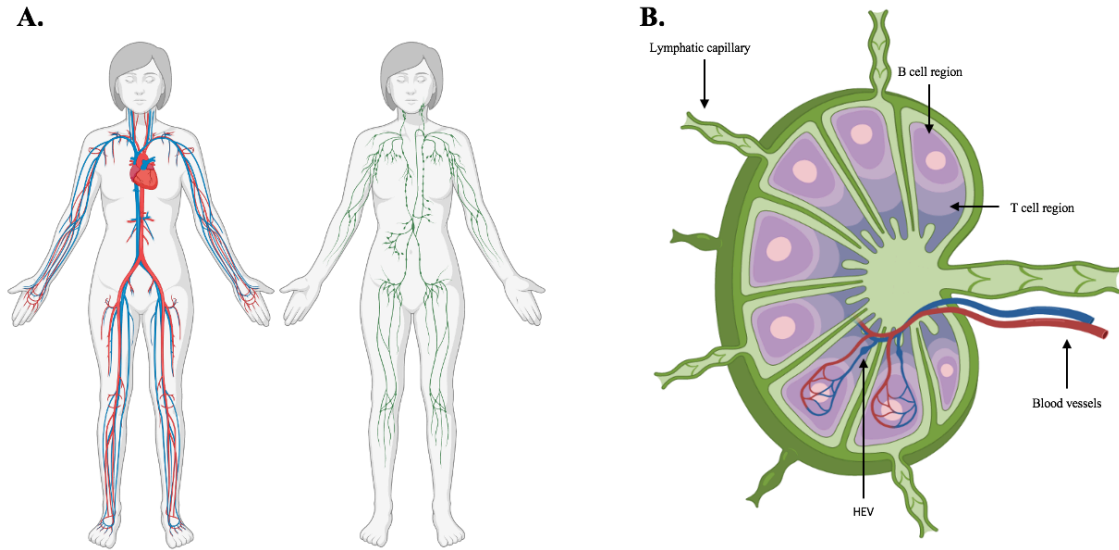
Figure 1.1 Lymphatic System and Lymph Node Structure. (A) Leftmost image shows body layout of the blood circulatory system, rightmost image shows mirror image layout of lymphatic system (primary and mucosal lymphoid tissues not shown). (B) shows basic structure and discrete regions of a lymph node. Images created using BioRender software.
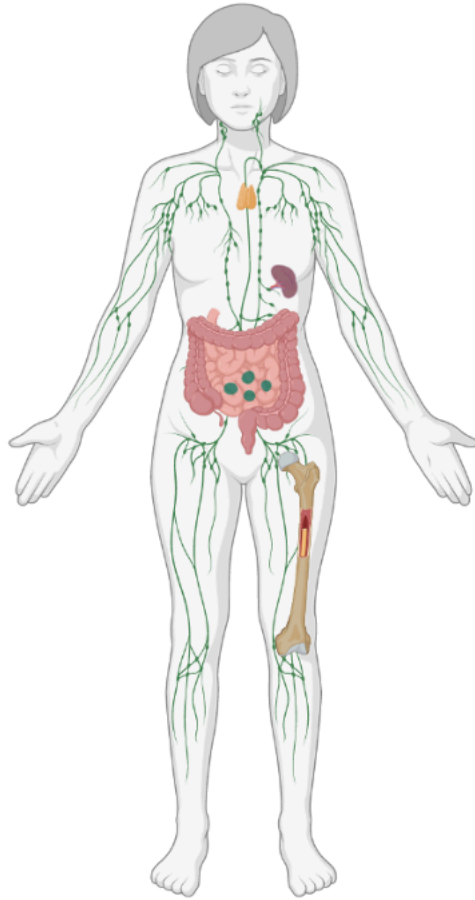
Figure 1.2 Primary and Secondary Lymphoid Tissues. Immune cell development occurs in the primary lymphoid organs, which include the red bone marrow (femur shown) and thymus (peach-colored structure shown in chest cavity). T and B cell responses occur in secondary lymphoid tissues, which include the spleen (dark red organ) and lymph nodes (smaller green bean-shaped structures). Mucosal regions such as the gut also contain lymphatic tissues, represented here by the larger green circles shown in the small intestine. Image created using BioRender software.

A.
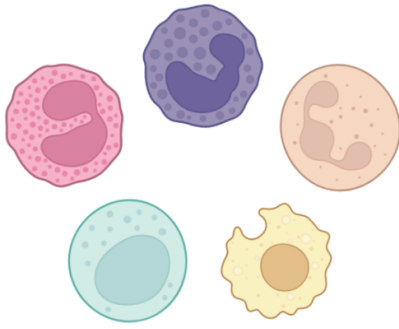
B.
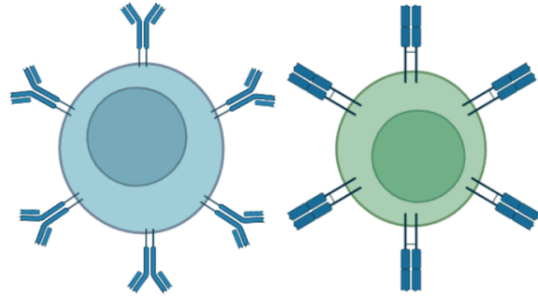
Figure 1.3 Innate and Adaptive Immune Cells. (A) shows cells of the innate immune system, which include (clockwise) basophils, neutrophils, macrophages, NK cells, and eosinophils. The granular illustration represents preformed proinflammatory molecules used by innate immune cells to respond to infection. (B) shows a simplified structure of the two major adaptive immune cells, B cells (left) and T cells (right), differentiated by their antigen-receptors (BCR and TCR, respectively). Image created using BioRender software.
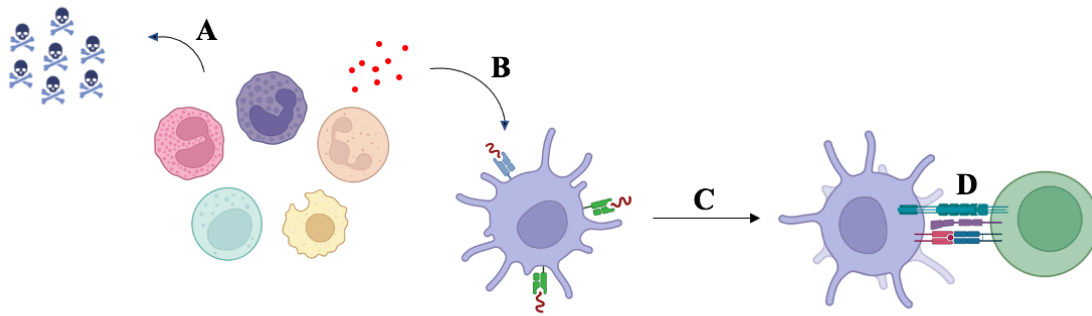
Figure 1.4 Dendritic Cells Link Innate and Adaptive Immunity. Innate cell recognition of pathogen in the periphery (A) results in an initial proinflammatory response. Release of proinflammatory cytokines helps activate and mature DCs that have acquired pathogen antigen (B). Maturation stimulates DC migration from the periphery to the draining lymph node (C), where it then presents the antigen to naïve T cells (D). Image created using BioRender software.
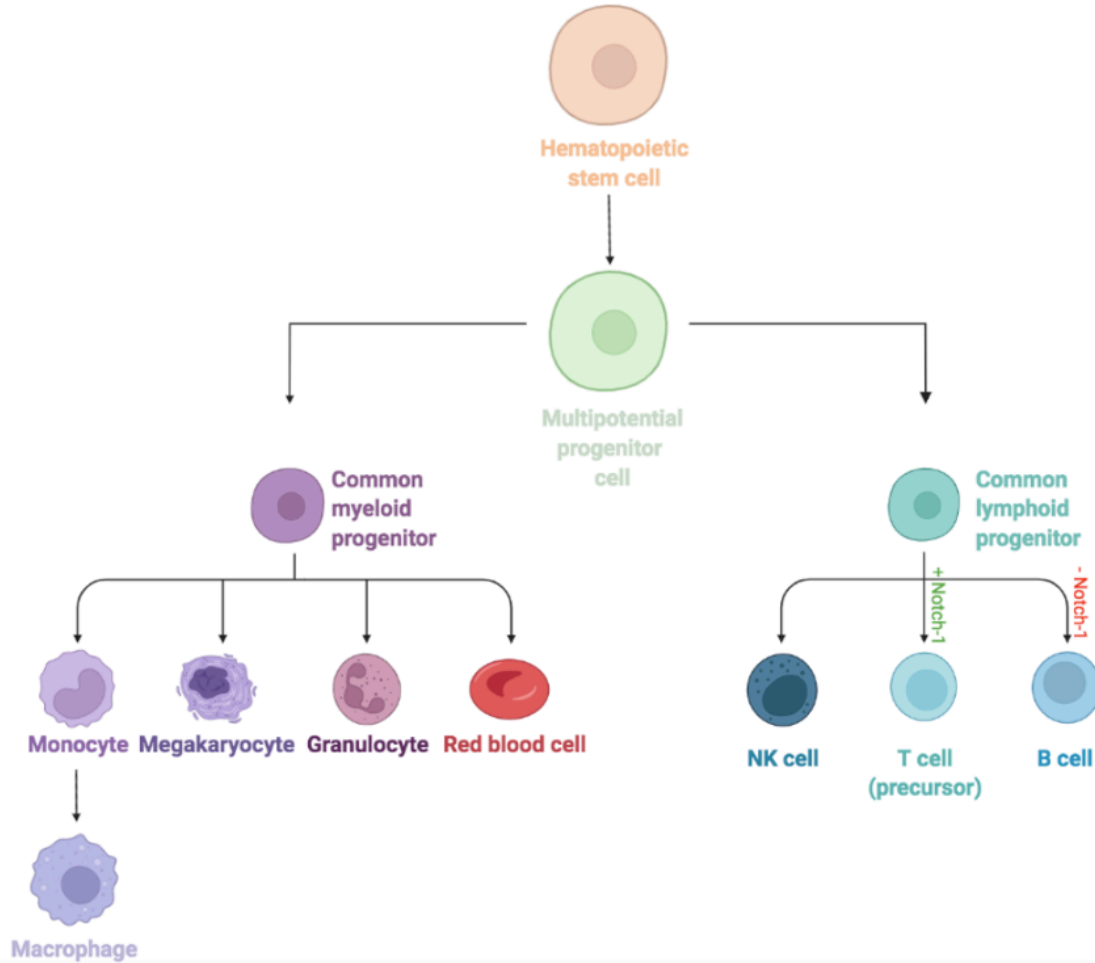
Figure 1.5 Immune Cell Hematopoiesis. Hematopoietic stem cells can differentiate into multipotential progenitor cells, which can further differentiate into myeloid and lymphoid lineage cell precursors (common myeloid progenitor and common lymphoid progenitor, respectively). Common lymphoid progenitor cells that receive Notch-1 signals continue down the T cell development pathway, while lack of Notch-1 signaling results in the B cell development pathway. Image created using BioRender software.
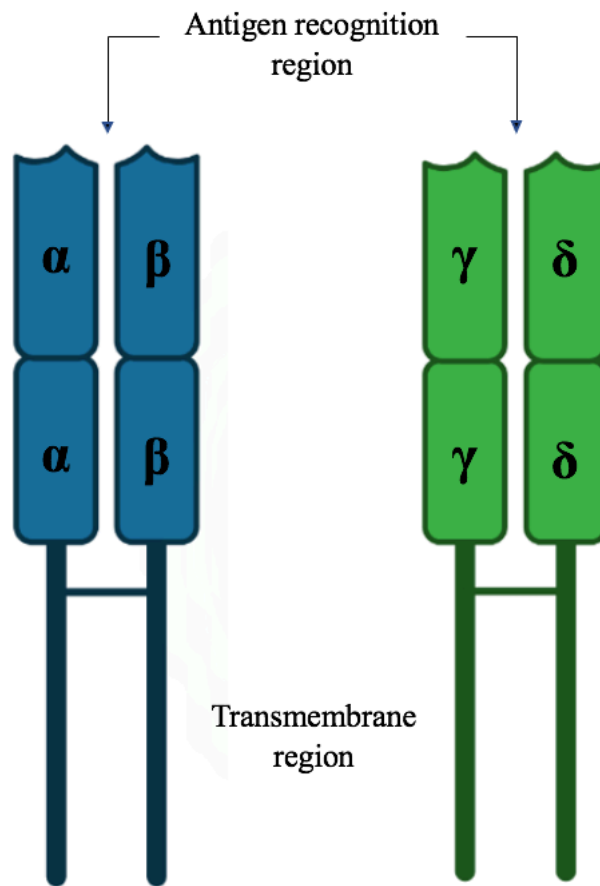
Figure 1.6 TCR Structure. Leftmost image shows basic Ig domain organization for T cells expressing an αβ TCR, while rightmost image shows γδ TCR. Antigen recognition region is located distal to the cell membrane. Image created using BioRender software.
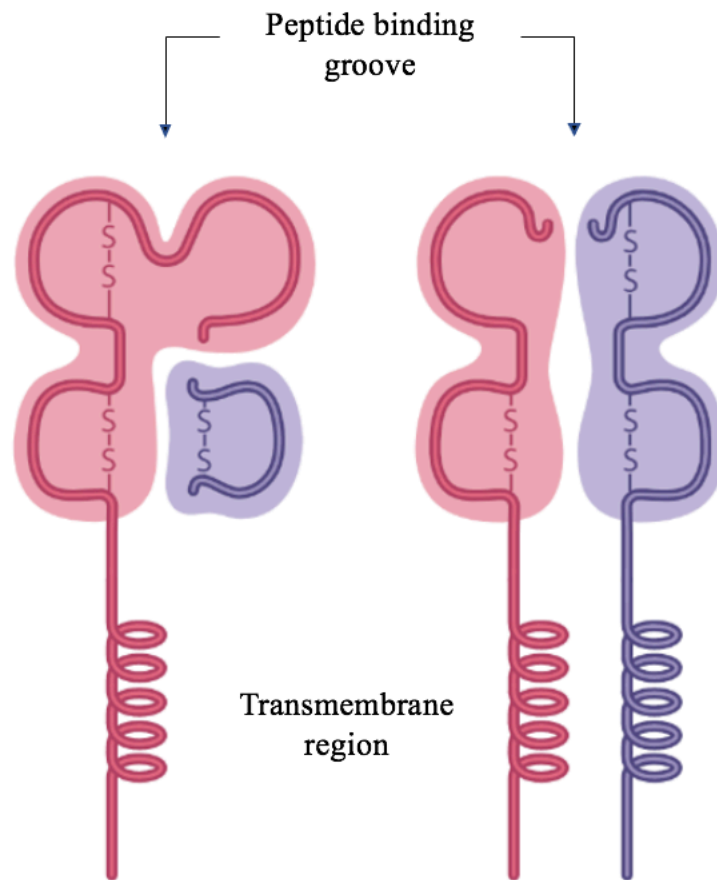
Figure 1.7 MHC Structure. T cell recognition of antigen occurs via peptide presentation on MHC molecules. Leftmost image shows the basic structure of an MHC class I molecule, comprised of a 3 domain α chain (pink) noncovalently associated with the β2 microglobulin protein (purple). Rightmost image shows the structure of an MHC II molecule, comprised of noncovalently associated α (pink) and β (purple) chains. Image created using BioRender software.
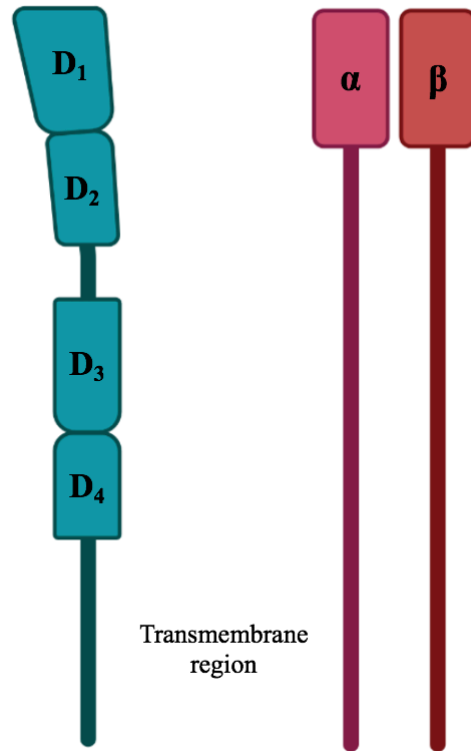
Figure 1.8 CD4 and CD8 Structure. CD4 and CD8 glycoproteins bind to conserved regions on MHC molecules and enhance the T cell activation process. CD4 (leftmost image) is a monomeric protein consisting of 4 Ig-like domains, with domains labeled membrane distal to proximal. CD8 (rightmost image) is a dimeric protein, consisting of an α and β chain. Image created using BioRender software.
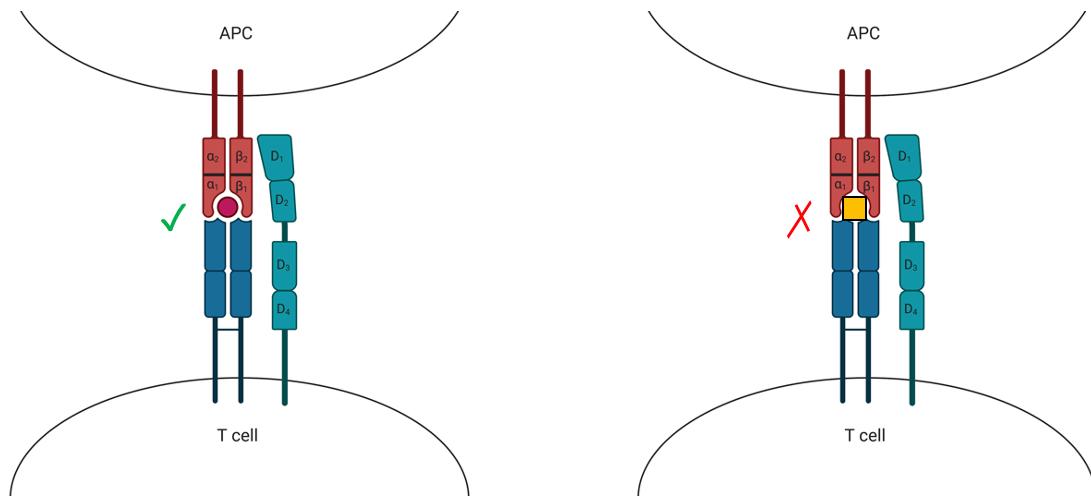
Figure 1.9 CD4+ T Cell Recognition of Peptide Presented on MHC II. T cells must recognize both the presented peptide and MHC molecule for activation to begin. Leftmost image illustrates a CD4+ T cell recognizing presented peptide via its TCR, and MHC molecule by both its TCR and CD4 glycoprotein. Rightmost image shows an unrecognized peptide, inhibiting T cell activation. APC = dendritic cell. Image created using BioRender software.
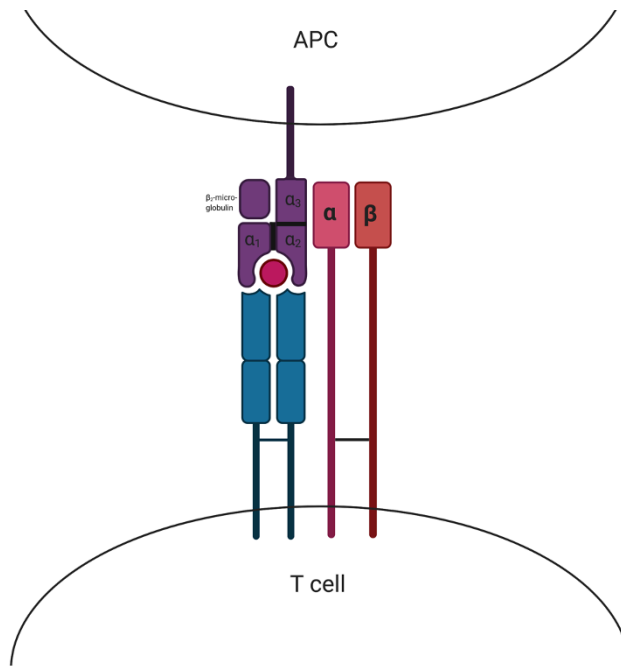
Figure 1.10 CD8+ T Cell Recognition of Peptide Presented on MHC I. Image shows CD8+ T cell recognizing peptide presented on MHC I via its TCR, while MHC I is recognized by the TCR and CD8 glycoprotein. APC = dendritic cell. Image created using BioRender software.
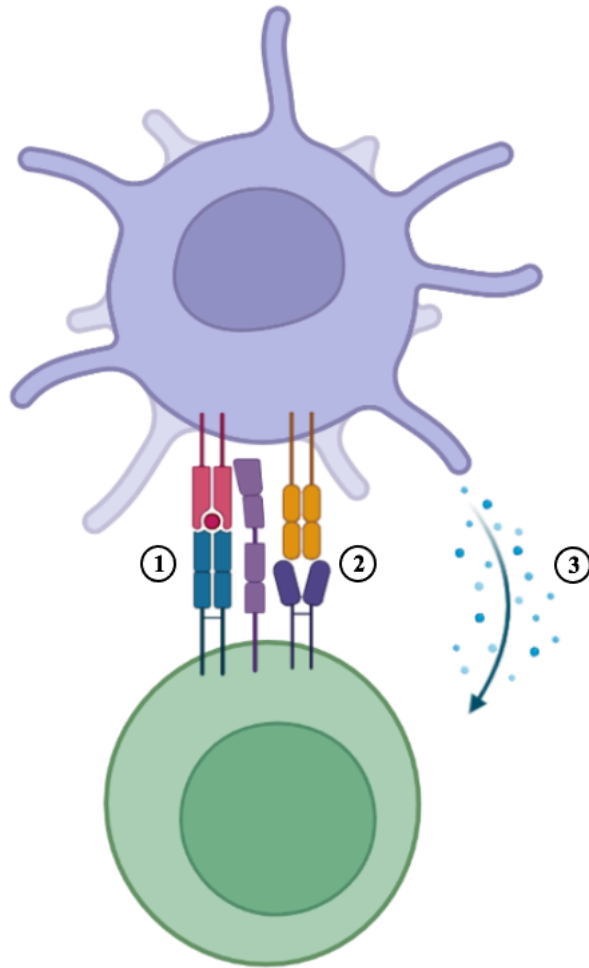
Figure 1.11 3 Signals for T Cell Activation. Full T cell activation occurs following TCR recognition of peptide and MHC (1), costimulation to reinforce intracellular activation pathways (2), and cytokine signals that promote proliferation and differentiation (3). Image created using BioRender software.
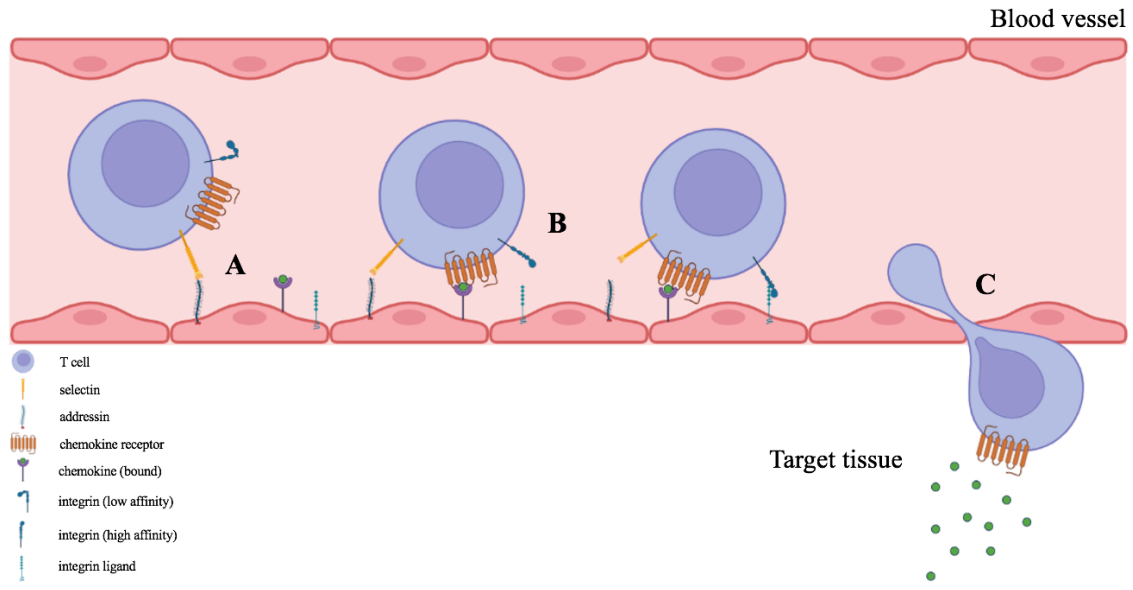
Figure 1.12 T Cell Migration into Target Tissue. Interactions between adhesion proteins on T cells and endothelial cells promotes T cell rolling (A). Tissue-specific chemokines expressed by endothelial cells bind to chemokine receptors expressed on T cells, prompting activation of integrin receptors on the T cell to a high affinity form (B). Stable binding between integrin receptors and ligand stimulates T cell migration from the circulation into target tissue (C), where an increased chemokine gradient continues to direct T cell migration. Image created using BioRender software.
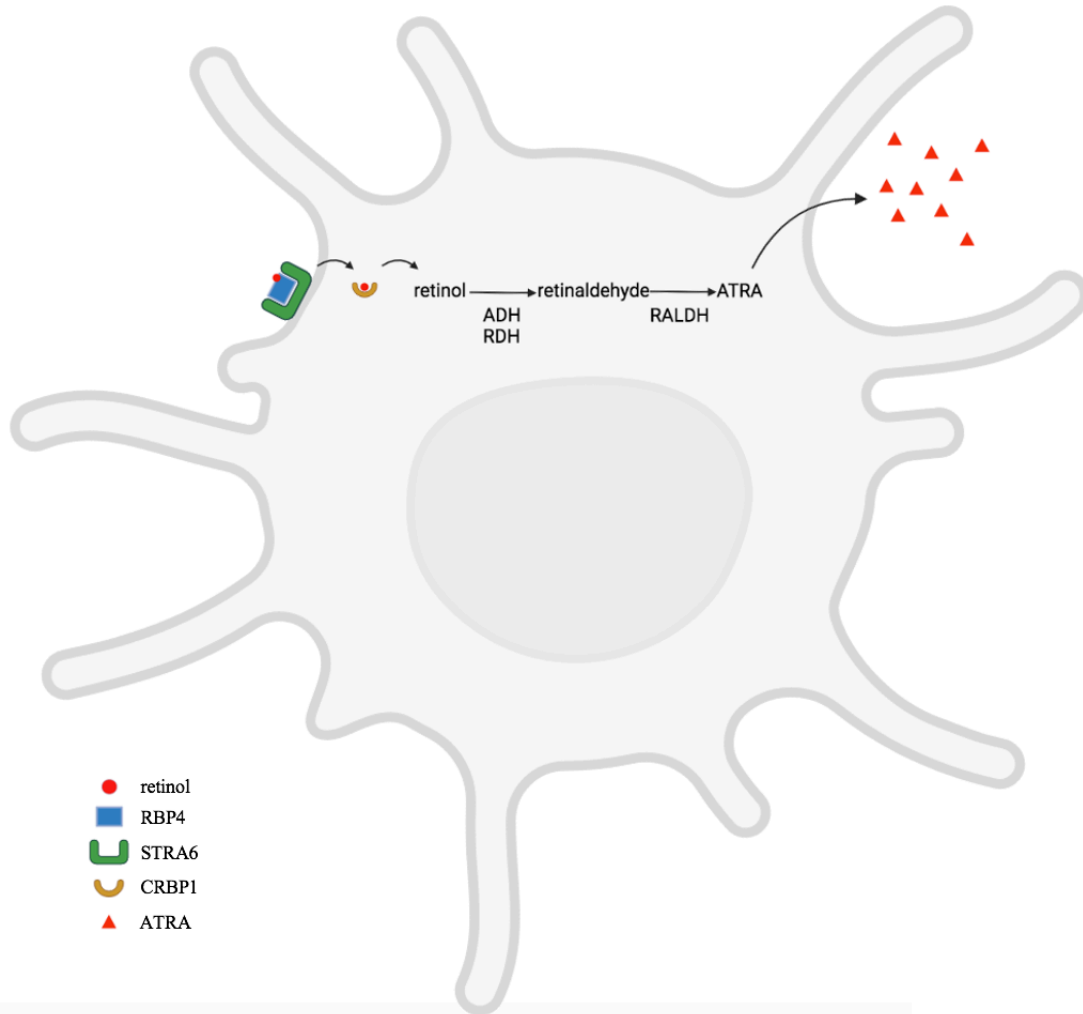
Figure 1.13 Retinol Conversion to ATRA in Dendritic Cells. Dietary retinol is able to travel through the blood circulation bound to RBP4. Docking of RBP4 to extracellular STRA6 permits entry of retinol into the cell, where it is picked up by CRBP1. Multiple ADH and RDH enzymes facilitate conversion of retinol to retinaldehyde, while RALDH enzymes oxidize retinaldehyde to active ATRA. ATRA can then be used intracellularly, or released to act on other target cells. Image created using BioRender software.
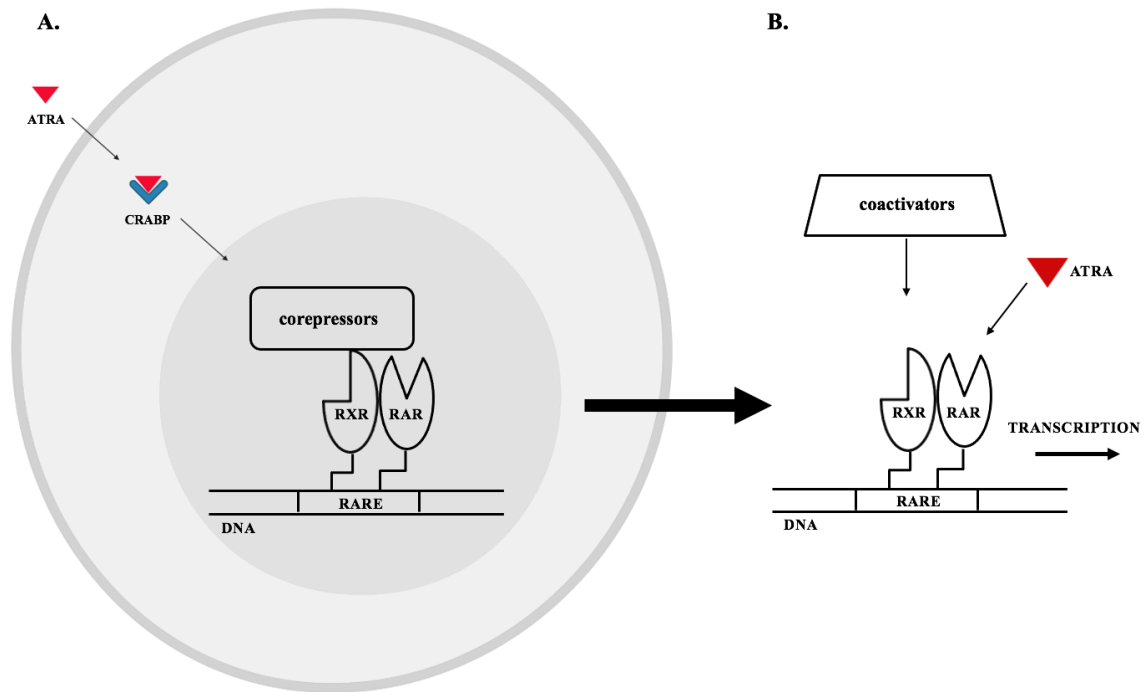
Figure 1.14 ATRA Signaling Within the Cell. Cytosolic CRABP binds ATRA and transports it into the nucleus where its receptor, the RAR, is found in heterodimeric form bound to RAREs (1). ATRA binding to the RAR results in a conformational change to the heterodimer, causing previously bound corepressor proteins to dissociate, coactivator proteins to associate, and ultimately resulting in gene transcription (2). Image created using BioRender and PowerPoint software.

CHAPTER 2

Rexinoids modulate effector T cell expression of mucosal homing markers CCR9 and α4β7 integrin and direct their migration *in vitro*

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Abstract:

Altering T cell trafficking to mucosal regions can enhance immune responses towards pathogenic infections and cancers at these sites, leading to better outcomes. All-trans-retinoic acid (ATRA) promotes T cell migration to mucosal surfaces by inducing transcription of the mucosal-homing receptors CCR9 and α4β7 via binding to retinoic acid receptors (RARs), which heterodimerize with retinoid X receptors (RXRs) to function. However, the unstable nature and toxicity of ATRA limit its use as a widespread treatment modality for mucosal diseases. Therefore, identifying alternatives that could reduce or eliminate the use of ATRA are needed. Rexinoids are synthetically derived compounds structurally similar to ATRA. Originally named for their ability to bind RXRs, rexinoids can enhance RAR-mediated gene transcription. Furthermore, rexinoids are more stable than ATRA and possess an improved safety profile, making them attractive candidates for use in clinical settings. Here we show that select novel rexinoids act as ATRA mimics, as they cause increased CCR9 and α4β7 expression and

enhanced migration to the CCR9 ligand, CCL25 *in vitro*, even in the absence of ATRA. Conversely, other rexinoids act synergistically with ATRA, as culturing cells with suboptimal doses of both compounds resulted in CCR9 expression and migration to CCL25. Overall, our findings show that rexinoids can be used independently or synergistically with ATRA to promote mucosal homing of T cells *in vitro*, and lends support for the prospective clinical use of these compounds in immunotherapeutic approaches for pathogenic infections or cancers at mucosal surfaces.

Introduction:

Mucosal surfaces represent a main entryway for pathogens to anatomic access and are common sites for cancer development. Enhancing immunity at these regions can provide better protection and improve strategies for treating these diseases. Our previous work in mouse models has shown that increasing the migration of vaccinia virus (VACV)-specific T cells to mucosal regions during vaccination boosts protection at these sites during VACV challenge (58). Correlative evidence also exists in non-human primate models; in rhesus macaques, the use of an attenuated cytomegalovirus (CMV) vaccine vector for simian immunodeficiency virus (SIV) increases effector T cell numbers at mucosal regions, resulting in vastly improved control and clearance of SIV following viral challenge (107,108). In humans, clinical evidence further suggests that enhancing immune presence at mucosal sites corresponds positively with protection (109-114). Individuals with vitamin A deficiencies exhibit severely impaired mucosal immunity, resulting in increased susceptibility to infections (61-63,115). As the heightened immune protection seen is predominantly a result of increased effector T cell

presence in the mucosal regions, identifying ways to promote T cell migration to these

areas is likely to improve resistance to diseases affecting these areas (58,59,75).

Effector T cell trafficking to and entry into mucosal regions is governed by their

expression of receptors that instigate mucosal homing, including C-C chemokine receptor

type 9 (CCR9) and $\alpha_4\beta_7$ integrin ($\alpha_4\beta_7$) (58,59,116,117). Upregulation of these mucosal

homing receptors during T cell activation is dependent on signaling via the retinoic acid

receptor (RAR), a type II nuclear receptor that heterodimerizes with another nuclear

receptor, the retinoid X receptor (RXR), to mediate transcription (59,63,117). Both the

RAR and RXR possess α, β, and γ isotypes, with activation of the RARα/RXRα

heterodimer implicated in transcription of CCR9 and the α_4 subunit of $\alpha_4\beta_7$, via

cooperation NFATc2 (59,63,116,118-121). Binding of all-trans-retinoic acid (ATRA), a

biologically active vitamin A metabolite and the most abundant naturally occurring pan-

RAR ligand, to the ligand-binding domain (LBD) of the RAR triggers activation of the

heterodimer, ultimately resulting in RAR-mediated transcription (94,122,123).

Like the RAR, the RXR also possesses an LBD, and ligand bound to both the

RAR and RXR has been shown to enhance transcription of retinoid-dependent genes

(94,95,118,124). However, identification of endogenously occurring RXR ligands has

remained limited. 9-cis-retinoic acid (9cRA), a naturally occurring stereoisomer of

ATRA, has been reported as a high affinity RXR ligand, however its detection *in vivo*

remains elusive (97-99). Fatty acids such as docosahexaenoic acid and phytanic acid are

also capable of binding the RXR, however endogenously occurring levels are likely too

low to activate the receptor under most physiologic scenarios (98,99). The challenge to

conclusively identify naturally occurring RXR ligands has led many groups to utilize synthetic agonists, which have since been coined 'rexinoids'.

Despite studies showing that ATRA can promote the expression of mucosal T cell homing proteins and subsequent migration to mucosal sites *in vivo*, resulting in better protection against mucosal infection, little is known about the effect of rexinoids on effector T cells. The functional similarity seen between rexinoids and ATRA in experimental models indicates that these synthetic small molecules may be able to exert similar effects as ATRA on effector T cells, by influencing their migration to mucosal-associated regions (125,126). The ability of rexinoids to bind the RXR suggests that they may improve the impact of endogenous ATRA on T cell mucosal-related function. Additionally, reports that some rexinoids bind to the RAR indicates they may be able to mimic the effect ATRA has on T cell activity (105,125).

Here we assessed the ability of a panel of rexinoids (Fig. 1) to induce expression of CCR9 and $\alpha_4\beta_7$ and to promote T cell migration *in vitro*. These rexinoids include a fluorobexarotene analog, A18, halogenated bexarotene analogs A20-A22, rexinoids A30-A41 which are described in our previous work and references therein, and rexinoids A52-A63 which are again described in our prior work and citations therein (127-130). A subset of rexinoids (A18, A20, and A41) were capable of exerting this effect independently of ATRA, suggesting these compounds can act as ATRA mimics while retaining lower toxicity and enhanced stability. Conversely, other rexinoids (A55, A56, and A57) displayed synergy with suboptimal doses of ATRA to enhance CCR9 expression. Moreover, treatment with ATRA mimics induced T cell migration *in vitro* towards the CCR9 ligand CCL25, while treatment with the ATRA cooperating rexinoids

also resulted in improved migration. Furthermore, preliminary *in vivo* data suggest

rexinoid treatment is accompanied by reduced toxicity compared to ATRA. Together,

these data suggest that rexinoids may have potential to be used as a novel

immunotherapeutic treatment modality for mucosal diseases by either replacing ATRA-

based strategies or by being used in conjunction with non-toxic ATRA levels to bolster

efficacy.

Materials and Methods:

Rexinoid and ATRA preparation

A panel of novel rexinoids and bexarotene (BEX) were generously donated by the

Wagner, Marshall, and Jurutka labs at 1mM and diluted in 95% ethanol (Koptec) or

DMSO (Sigma-Aldrich) to $2x10^5$ nM. Powdered ATRA (Sigma-Aldrich) was dissolved

in DMSO and stored in the dark at -20C.

Lymphocyte isolation and culture

Spleens were harvested from B6.Cg-*Tcra^{tm1Mom}*Tg(TcrLCMV)327Sdz/TacMmjax

(P14), B6.Cg-*Ptprc^{a}Pepc^b*Tg(TcrLCMV)1Aox/PpmJ (SMARTA), or C57BL/6-

Tg(TcraTcrb)1100Mjb/J (OT-1) transgenic mice (Jackson Labs) and mechanically

dissociated into a single cell suspension using a 70 m nylon mesh strainer (Fisherbrand).

Splenic red blood cells (RBCs) were lysed using Ammonium-Chloride-Potassium (ACK)

lysing buffer (ThermoFisher). T cells were stimulated with 1 g/mL of appropriate viral

peptide (LCMV gp$_{33-41}$, LCMV gp$_{61-80}$, or OVA$_{257-264}$, respectively) (GenScript;

Anaspec). Cells were cultured in a 96-well plate for 8 days using RPMI complete

medium (10% FBS, 1% PSG 100X) supplemented with $2.5x10^{-5}$ g/ L IL-2 (Gibco)

and 100nM of indicated rexinoid treatment or ATRA in a final volume of 200 L. 8 day

treatment timeframe was determined using a time course assay (Supplementary Figure S2.1). Fresh culture medium with IL-2 and rexinoid or ATRA was replaced every 48 hours. Vitamin A deficient media was made using charcoal-stripped FBS (ThermoFisher).

Flow cytometry

Expression of mucosal homing receptors was determined using flow cytometry. Cells were stained with a 1:100 dilution of the following fluorochrome-conjugated anti-mouse monoclonal antibodies: CCR9 (CW-1.2) and α4β7 (DATK32). P14 cells were further stained with Thy1.1 (HIS51) and CD8α (53-6.7); SMARTA cells were further stained with CD4 (GK1.5) and Vα2 (B20.1); OT-1 cells were further stained with CD8α (53-6.7)and Vα2 (B20.1). All antibodies were purchased from ThermoFisher. Flow cytometry data were collected using a BD LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo 8.8.7 software. Graphs were created using Prism 8 software (GraphPad). Error bars indicate SD from the mean. Data from SMARTA mice included in Supporting Information.

In vitro migration assay

P14 or SMARTA splenocytes, processed and cultured as described above for 7 days, were plated into the upper chamber of a 96 well HTS Transwell plate insert with 3.0um pore size (Corning) at a concentration of $5x10^5$ cells in 75 L of chemotaxis buffer (RPMI medium containing 0.1% FBS). Recombinant mouse CCL25/TECK protein (R&D Systems) was reconstituted to 10 g/mL in 1X PBS (GenClone) containing 0.1% FBS, resuspended in 235 L chemotaxis buffer at a concentration of 250nM, and plated into the lower chamber. Control wells received no chemokine. Cells

were incubated for 6 hours at 37C in 5% CO_2. Live cells that migrated into the lower chamber were subjected to a 1:2 trypan blue stain and manually quantified using a Neubauer improved C-Chip hemocytometer (INCYTO). Assays using P14 splenocytes were performed in triplicate, while those using SMARTA splenocytes were performed in duplicate. Graphs were created using Prism 8 software. Statistical significance calculated using 2-way analysis of variance (ANOVA). Data from SMARTA mice included in Supporting Information.

In vivo toxicity

6-12 week old female Balb/cJ mice (Jackson Labs) were inoculated via the tail vein with $1x10^6$ K7M2 cells (ATCC; cells not tested for mycoplasma) at day 0, and treated daily for 14 days with either 40mg/kg of vehicle control (n=4), ATRA (n=5), or rexinoid A55 (n=5) delivered via intraperitoneal (i.p.) injection, or 100mg/kg vehicle control (n=5), ATRA (n=5), or rexinoid A41 (n=6) delivered via oral gavage. Treatment timeframe was determined using previous unpublished data showing lung tumor establishment by Day 14 (data not shown). K7M2 cells were cultured using DMEM complete medium (10% FBS, 1% PSG 100X) under sterile conditions. Vehicle control, ATRA, and rexinoids were dissolved using DMSO and diluted to working concentrations using soybean oil (Sigma-Aldrich). Mouse weights were taken every 24 hours during the course of treatment. For liver toxicity, serum used to measure alanine transaminase (ALT) levels was obtained following cardiac puncture at Day 14, and analyzed using liquid ALT reagent kits (Pointe Scientific). Graphs created using Prism 8 software.

Statistical analyses

One-way and two-way ANOVA were used for data analysis to establish the impacts of rexinoid and/or ATRA on the percentage of CCR9 and α4β7 expression. Follow-up tests for pairwise comparisons among groups were also performed post-ANOVA using Fisher's Least Significant Difference (LSD) test. All tests were performed at the $\alpha = 0.05$ significance level in JMP Pro 16, a statistical software package.

Results:

Effector CD8+ T cells increase expression of CCR9 and $\alpha_4\beta_7$ *in vitro* following rexinoid treatment

ATRA is capable of modifying T cell expression of the mucosal homing markers CCR9 and $\alpha_4\beta_7$. As rexinoids have displayed functional similarity to ATRA in other studies, we sought to determine if our panel of novel rexinoids could also modulate T cell expression of CCR9 and $\alpha_4\beta_7$. To do this, splenocytes isolated from naïve P14 mice, expressing a transgenic TCR specific for the H-2D^b restricted GP$_{33-41}$ peptide of lymphocytic choriomeningitis virus (LCMV), were activated in vitro and cultured with a panel of 40 rexinoids for 8 days. Many rexinoids administered at 100nM were able to significantly enhance CD8+ T cell expression of CCR9, compared to negative controls (Fig. 2.2A-C). Culture with the FDA approved rexinoid BEX also significantly enhanced CCR9 expression on responding T cells compared to negative controls (Fig. 2.2C). Interestingly, rexinoid A41 improved T cell expression of CCR9 better than BEX, identifying a candidate that may possess improved functional efficacy compared to a current existing treatment. Rexinoid treatment also significantly enhanced $\alpha_4\beta_7$ expression at day 8 of activation compared to negative controls, with A41 again outperforming BEX (Fig. 2.2D).

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The ability of rexinoids to enhance CCR9 expression on effector T cells is independent of antigen and MHC specificity

As rexinoids had a pronounced effect on CCR9 expression, our subsequent experiments focused primarily on the expression of this chemokine receptor as an indicator of mucosal homing protein expression. To determine if the change in T cell expression of CCR9 was antigen or MHC specific, we cultured T cells from either SMARTA and OT-1 mice, TCR transgenic mice expressing TCR specific for different peptide (LCMV GP_{61-80} and $OVA_{257-264}$, respectively) presented in the context of a different MHC (H2-IA^b and H-2K^b, respectively). Rexinoid treatment of T cells from these other TCR transgenic mouse strains also resulted in increased CCR9 expression (Fig. 2.3A,B). Moreover, the patterns of increased expression were similar to that obtained for T cells from P14 mice, with no significant differences seen between CD8 and CD4 T cells (p= >0.05). These data suggest that the ability of rexinoids to modulate T cell expression of CCR9 is not limited by antigen specificity or MHC. Moreover, both CD4 and CD8 T cells are able to increase expression of mucosal homing proteins.

Some rexinoids act independently of ATRA to enhance T cell expression of CCR9

We next sought to determine which rexinoids were capable of altering CCR9 expression independently of ATRA. As charcoal stripping FBS removes lipophilic substances from the serum, including ATRA and other vitamin A derivatives, we supplemented RPMI medium with charcoal stripped FBS in place of standard FBS to create appropriate ATRA deficient culture conditions. P14 T cells were cultured as described above with the indicated rexinoids but without ATRA. The ability of a majority of the rexinoids to alter T cell expression of CCR9 declined to background levels when

vitamin A/ATRA was removed from the medium, indicating their dependence on ATRA for increased expression of mucosal homing proteins (Fig. 2.4A, Supplementary Figure S2.2). However, some rexinoids (A18, A20, A41) retained their ability to enhance CCR9 expression, despite the lack of vitamin A/ATRA in the culture medium. Overall, these findings demonstrate that select rexinoids can mimic the effects of ATRA in enhancing T cell expression of CCR9, while retaining enhanced safety and stability profiles.

Some rexinoids act synergistically with ATRA to enhance T cell expression of CCR9

In order to test whether other rexinoids may act synergistically with ATRA, we cultured T cells from P14 mice as described above in charcoal-stripped media that had been supplemented with suboptimal amounts of ATRA (0.1nM) and rexinoids (1nM); neither ATRA nor rexinoids at these concentrations caused expression of CCR9 above background levels (rexinoids only Fig. 2.4A, ATRA only Fig. 2.4B). T cells cultured with selected rexinoids and ATRA at suboptimal concentrations showed significantly improved expression of CCR9 (boxed region Fig. 2.4B, 2.4C) compared to treatment with an equivalent dose of ATRA alone (Fig. 2.4B) or rexinoid alone (Fig. 2.4A). These data suggest that select rexinoids act synergistically with ATRA to promote CCR9 expression. This is supported by previously published in vitro data that shows low activation of RAR (Table 2.1). The ability of these rexinoids to cooperate with lower concentrations of ATRA *in vitro* suggests a potential strategy to use these compounds in cooperation with physiological levels of vitamin A levels *in vivo* to promote T cell migration to mucosal regions.

Rexinoid treatment improves chemokine-mediated migration of effector T cells

To evaluate the ability of rexinoid-treated T cells to migrate towards chemokine, we performed an *in vitro* transwell migration assay using the CCR9 ligand CCL25. P14 or SMARTA splenocytes were cultured as described above with selected rexinoids that showed the potential to act as an ATRA mimic (A18, A20, A41), or rexinoids that showed synergistic activity with ATRA (A55, A56, A57). Following culture, cells were seeded into the upper well of a transwell plate and incubated to allow for migration through the cell-permeable membrane towards CCL25 in the lower chamber. CD8+ T cells treated with the ATRA mimicking rexinoids A18 and A41 displayed significant migration towards CCL25 (Fig. 2.5A). Notably, A41 treatment significantly increased CD8+ T cell migration compared to ATRA treatment (Fig. 2.5A). CD8+ T cells treated with the ATRA cooperating rexinoids A55, A56, or A57 also showed significantly better migration towards CCL25 (Fig. 2.5B). These results indicate that treatment with ATRA mimicking or ATRA cooperating rexinoids induces effector T cell migration, with some rexinoids outperforming ATRA and BEX. Rexinoid-treated CD4+ T cells displayed increased migration when treated with A41 and A56 (Supplementary Figure S2.3).

Rexinoid treatment displays lower toxicity potential *in vivo* compared to ATRA

To measure the *in vivo* toxicity of rexinoid treatment, we used an established metastatic osteosarcoma (mOS) mouse model for which ATRA has previously been used. Briefly, Balb/cJ mice were inoculated with K7M2 cells via tail vein injection prior to daily i.p. treatment with vehicle control or a previously established effective dose of 40mg/kg ATRA or 40mg/kg rexinoid A55. As a measure of toxicity, mouse weights were taken every 24 hours over the course of treatment. Mice that were treated with vehicle control or rexinoid A55 displayed minimal weight changes during the course of

treatment, while mice treated with ATRA had significantly higher weight loss (Fig. 2.6A), skin erythema, and fur loss (images not shown). *In vivo* toxicity was further examined using a high concentration of treatment delivered orally. Balb/cJ mice were similarly inoculated with K7M2 cells, and treated daily with a predetermined dose of 100mg/kg vehicle control, ATRA, or rexinoid A41, delivered via oral gavage. Mice treated with vehicle control or rexinoid A41 displayed minimal weight changes, while ATRA-treated mice displayed significant losses following treatment onset (Fig. 2.6B). ATRA-treated mice were removed from study after 5 days treatment, due to rapid physical decompensation. Balb/cJ mice treated with an oral dose of 40mg/kg ATRA also showed greater elevation of the liver enzyme ALT at day 14 compared to mice treated with 40mg/kg vehicle control, A55, or A41 (Supplementary Figure S2.4). Together, these findings suggest that rexinoids are better tolerated and less toxic than ATRA when delivered as a therapeutic modality.

Discussion:

Identifying compounds that can favorably alter T cell migration to mucosal surfaces has the potential to improve immune responses towards diseases at these surfaces. Here we tested a panel of novel rexinoids for their ability to both influence effector T cell expression of mucosal homing markers CCR9 and $\alpha4\beta7$ and to affect their migration towards a mucosally expressed chemokine *in vitro*. Our results show that many rexinoids are capable of enhancing CCR9 and $\alpha4\beta7$ expression on responding T cells. Several rexinoids induced T cell expression of CCR9 independently, mimicking the naturally occurring biologic ATRA, while others worked synergistically with subtoxic doses of ATRA to enhance expression, indicating a potential to cooperate with vitamin A

present *in vivo*. Furthermore, both ATRA mimicking and ATRA cooperating rexinoids were seen to improve T cell migration towards the CCR9 ligand CCL25, with some outperforming bexarotene and ATRA. These findings introduce several rexinoids that can imprint T cells with a mucosal homing phenotype and influence their migration, and may have clinical relevance in treating mucosal diseases.

In addition to CCR9 and $\alpha_4\beta_7$, the expression of a myriad of other genes have also been shown to be under the control of RAR signaling, including those that inhibit cell cycle progression and promote apoptosis (131-136). These discoveries have led to ATRA being used clinically as an anti-cancer drug; combination treatments that include ATRA have been successful in inducing cancer remission, most notably with acute promyelocytic leukemia (APML), a disease marked by an RARα translocation (76-78). Unfortunately, these favorable results are dampened by adverse side effects attributed to ATRA usage. Various toxicities, including hepatotoxicity due to retinyl ester buildup in hepatic stellate cells (HeSCs), and mucocutaneous toxicity, have been reported in cancer patients receiving ATRA treatment (84-87). Here we confirmed that mice treated with ATRA fare poorly, as evidenced by their severe weight loss, physical appearance, and higher ALT levels. An additional complication seen with ATRA use is differentiation syndrome (DS), which can be life-threatening (84,90-92). Surprisingly, similar adverse health effects have also been reported following the use of synthetic vitamin A derivatives such as isotretinoin and acitretin, which has led us to postulate that the toxicities seen may be due to aberrant activation of the other RAR isoforms (137-139). This is supported by the finding that RARγ deficient mice show resistance to ATRA-mediated toxicity (140). Additionally, activation of all three RAR isoforms have been

shown to display teratogenic potential (141). As the ATRA cooperating rexinoids demonstrate high selectivity for the RXR, their use could avoid such toxicity. Furthermore, the widespread use of ATRA is limited due to its instability when exposed to ubiquitous elements such as ultraviolet (UV) light, ambient temperatures, and oxygen (83,84). The improved stability of rexinoids compared to ATRA is another attractive characteristic; their long shelf life and resistance to fluctuations in temperature, UV light, or oxygen presence makes them more durable treatment options.

In animal models of lung cancer, rexinoid use has been seen to mediate similar antiproliferative and proapoptotic effects on cancer cells as is observed with ATRA (142,143). Importantly, rexinoid treatment has been shown to be better tolerated than ATRA in both animal and human models. Clinical trial results show that bexarotene, which is currently used as a treatment modality for patients with cutaneous T cell lymphoma (CTCL), can be safely administered at dosages of $300mg/m^2/day$, while side effects are seen with ATRA dosages higher than $45mg/m^2/day$ (84,101,102,144-148). However, it is currently unknown for most rexinoids whether they are behaving as ATRA mimics or acting in synergy with ATRA. Here we have not only identified RXR ligands that act similarly to ATRA in altering mucosal homing capabilities, but we have further determined if this effect is dependent on ATRA or not. The rexinoids capable of exerting their effect independently of ATRA have the potential to replace ATRA in therapeutic settings, as they could provide a similar efficacy with a considerably reduced ability to induce toxicity. ATRA cooperating rexinoids also have potential for use in treatment settings; combining these compounds with a much lower dose of ATRA may enhance ATRA mediated effects while minimizing toxicity side effects.

It is well-established that effector T cell infiltration into affected tissues positively correlates with protection from viral infection and tumor regression, therefore identifying methods that can specifically impact their migratory ability may improve immune responses in these microenvironments (55,58,75,149). Our discovery of several rexinoids that favorably modulate T cell mucosal homing abilities *in vitro* indicates that they may be useful as an adjuvant during vaccination towards viruses that infect mucosal surfaces, and in immunotherapies targeting tumors that form at mucosal sites. We have previously shown in mouse models that ATRA has the potential to function as an adjuvant; i.p. injection of ATRA during vaccination increases the number of virus specific T cells to mucosal regions and boosts protection during viral challenge (58). However, this treatment is physically taxing to the mice, resulting in weight loss and inflammation at the injection site. Changing the route of delivery may improve tolerability, however the tradeoff is a reduction in ATRA bioavailability. Our preliminary *in vivo* work has shown that mice are not subject to the same physical discomforts following rexinoid treatment delivered via i.p. injection, as observed by their minimal weight loss during treatment (Fig. 2.6A). Furthermore, high dose rexinoid delivered orally was well-tolerated, which could compensate for reduced bioavailability when delivered a more preferable route, unlike high dose ATRA (Fig. 2.6B). Thus, administering either the ATRA mimicking or ATRA cooperating rexinoids via the same route as ATRA may result in similar immune modulating activity, without the associated pathology.

Adoptive cell transfer (ACT) and immune checkpoint blockade (ICB) are immunotherapies currently showing great promise as cancer treatment modalities (150-153). The ability of our rexinoids to modulate T cell migration suggests that their use in

tandem with either ACT or ICB therapy may enhance the efficacy of these treatments by directing more effector T cells to tumors at mucosal sites. With ACT, the treatment of *ex vivo* expanded tumor-specific T cells with rexinoids prior to re-infusion can result in more T cells effectively homing to the mucosal tumor, which would result in tumor reduction and possible elimination while avoiding the majority of toxicity issues associated with ATRA use *in vivo*. ICB therapy using a combination of PD-L1 and CTLA-4 blocking antibodies has been shown to reverse tumor-specific effector T cell exhaustion and increase the number of tumor-infiltrating lymphocytes (TILs) present, resulting in improved anti-tumor immune responses (149,152,154). Inhibitory interactions between TILs and tumor cells are blocked by anti-PD-L1, while the use of anti-CTLA-4 likely both promotes the activation of new tumor-specific T cells and overcomes regulatory T cell inhibitory pathways. Although promising, this approach currently displays limited efficacy in a subset of patients (55-57). This may be due to the newly activated T cells ineffectively migrating to the tumor site, resulting in the current TILs becoming overwhelmed, and subsequent re-loss of function. Coupling this ICB approach with our identified rexinoids may ameliorate treatment efficacy towards mucosal cancers by better directing the migration of newly activated tumor-specific T cells to these sites. This would result in larger numbers of functional effector T cells present in the mucosal tumors, resulting in improved cancer control and patient survival.

While this work focuses on immune function resulting from interactions between the RXR and RAR, it should be noted that the RXR is promiscuous. It is an essential partner for a multitude of other receptors, all of which require heterodimeric formation with the RXR to exert their function (96,124). Rexinoids that did not affect RAR/RXR

mediated transcription in terms of CCR9 and $\alpha_4\beta_7$ expression may play a role in

mediating expression of non-immune RAR/RXR dependent genes, or may influence the

expression of genes under the control of other RXR heterodimers. The potential of

rexinoid treatment to beneficially regulate a variety of biological processes is an exciting

and growing research area.

Compound	RXR EC$_{50}$ Value (nM) +/- (SD)	RAR % Activation at 100 nM +/- (SD)
A18	43 (5)	25 (6)
A20	90 (14)	13 (2)
A41	71 (10)	48 (10)
A55	13.8 (1.5)	19 (9)
A56	40.9 (0.6)	21 (8)
A57	18.2 (0.4)	16 (6)
BEX	53(6)	23(5)

Table 2.1 RXR EC$_{50}$ Values in nM and % RAR Activation at 100nM Selected Rexinoids. Values obtained from previously published data (105, 128-130). % RAR activation determined from measurements of RAR/RARE reporter activity in transfected cells, rexinoid activity divided by ATRA activity (see ref). Rexinoids included in table were found in this study to either mimic ATRA activity (A18, A20, A41) or cooperate with subtoxic dosages of ATRA (A55, A56, A57) to enhance T cell activity.
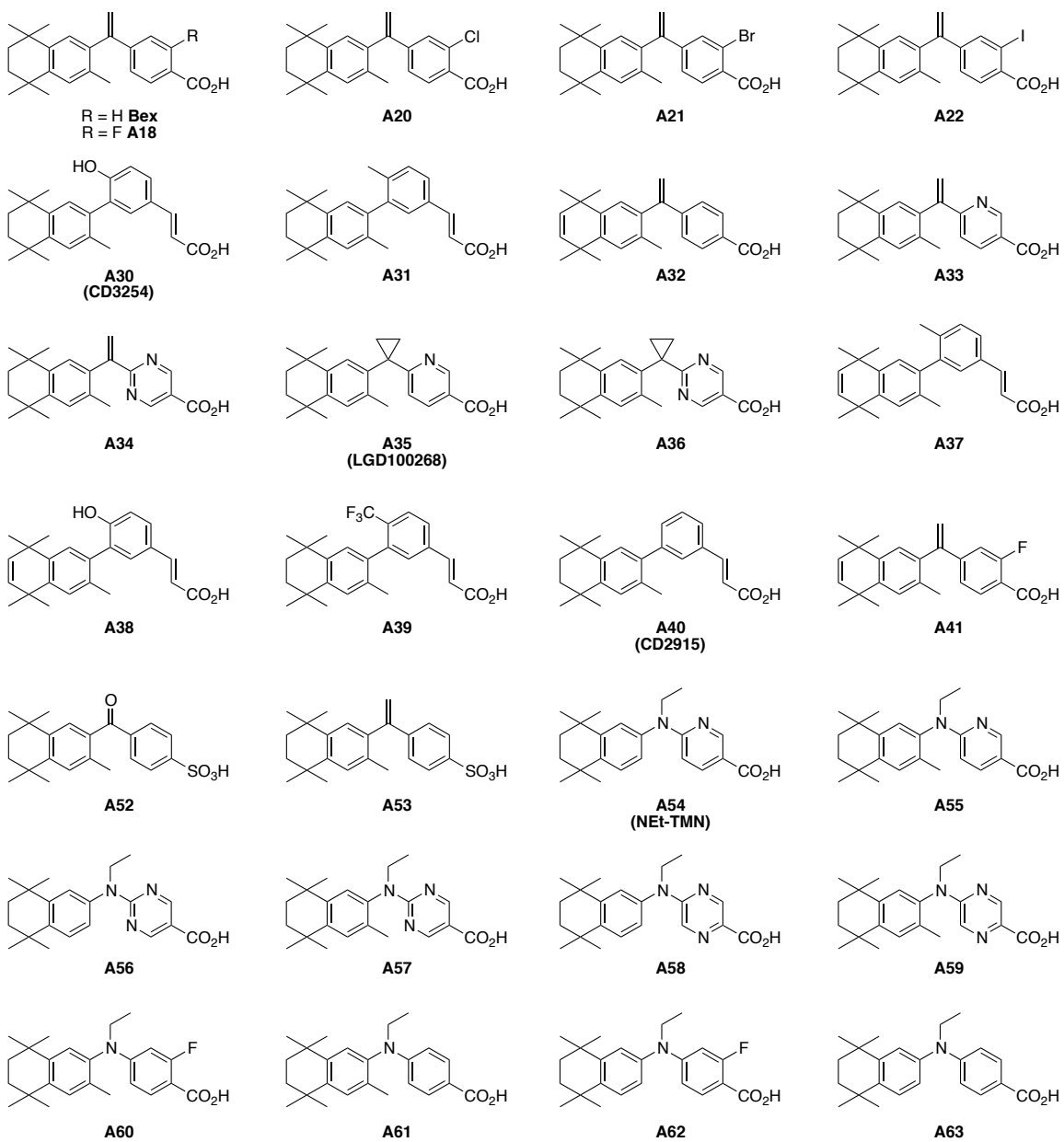
Figure 2.1 Structures of Select Rexinoids from the Tested Panel of Rexinoids.
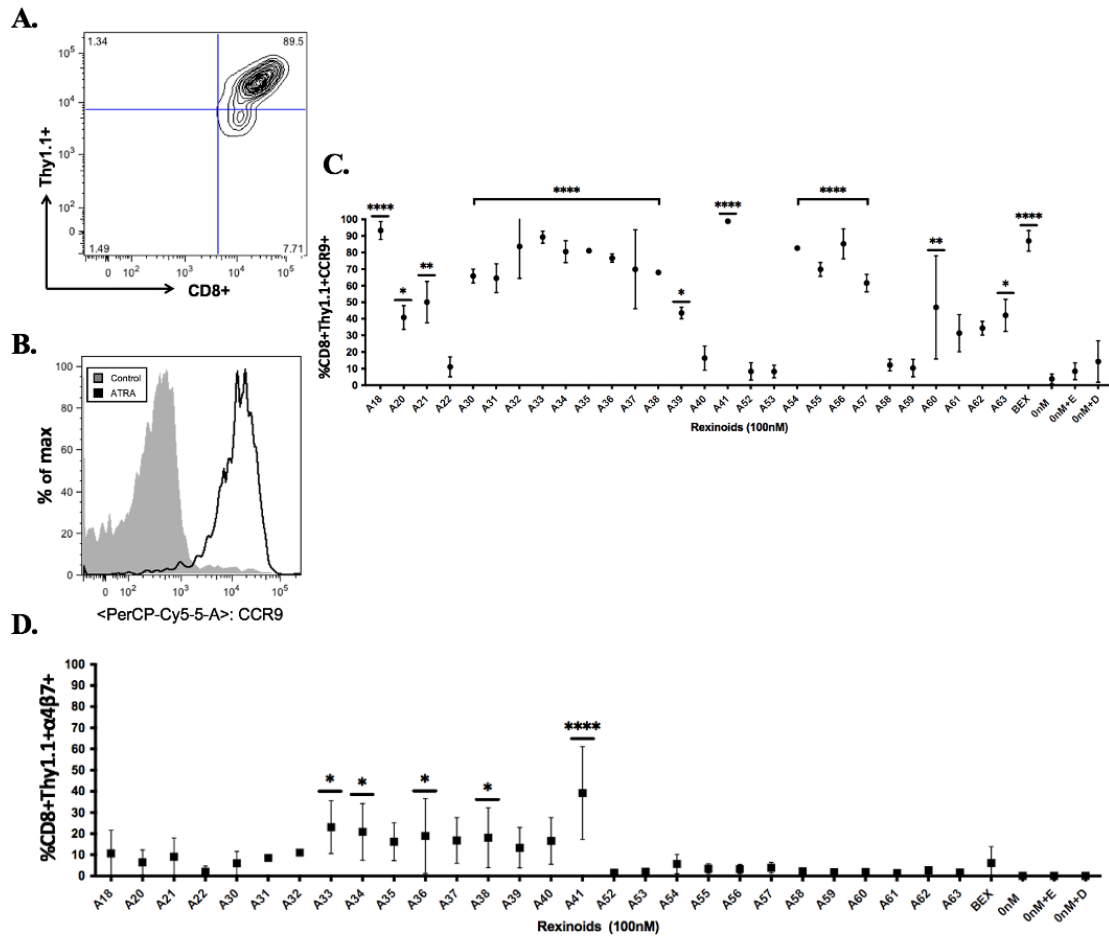
Figure 2.2 Rexinoid Treatment Enhances CCR9 and α4β7 Expression on Effector CD8+ T Cells *In Vitro*. Splenocytes obtained from a naïve P14 mouse were stimulated with GP_{33-41} peptide and cultured for 8 days with a large panel of novel rexinoids delivered at a 100nM concentration (treatment every 48 hours). Cells were then analyzed for changes in expression of CCR9 and a4b7. Analysis was performed using flow cytometry. **(A)** Antigen-specific effector CD8+ T cells gated using appropriate markers. **(B)** CCR9 expression is upregulated on antigen-specific effector CD8+ T cells following ATRA treatment given over an 8 day time course. **(C)** shows % positive CCR9 expression on antigen-specific effector CD8+ T cells following 8 day rexinoid treatment. Experiment performed in triplicate. **(D)** α4β7 expression on antigen-specific effector CD8+ T cells following 8 day rexinoid treatment. Experiment performed in duplicate. Connecting letters report used to determine statistical significance, with ordered differences report used to compare p-values between groups (* = $p<0.05$, ** = $p<0.005$, *** = $p<0.0005$, **** = $p<0.0001$). All error bars represent SD from the mean.
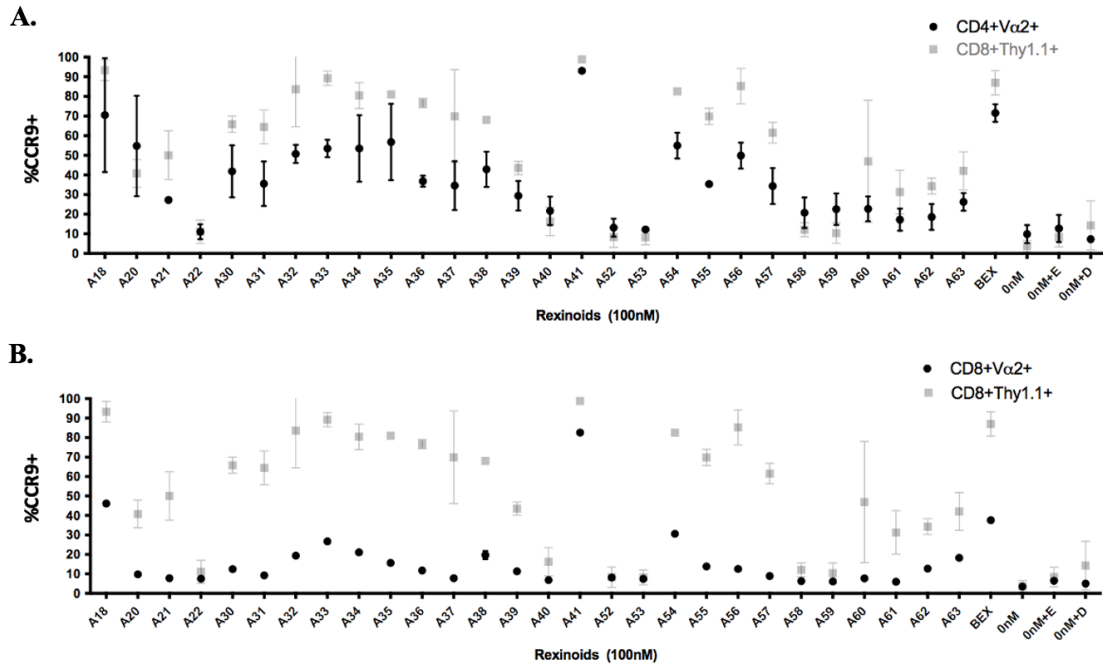
Figure 2.3 Rexinoid Treatment Enhances CCR9 Expression on Effector T cells of Different Antigen Specificity *In Vitro*. Splenocytes obtained from naïve SMARTA and OT-1 mice were stimulated with GP_{61-80} peptide and $OVA_{257-264}$, respectively, and cultured for 8 days with the same panel of novel rexinoids delivered at a 100nM concentration (treatment every 48 hours). Cells were then analyzed for changes in expression of CCR9. Analysis was performed using flow cytometry. **(A)** CCR9 expression on antigen-specific effector CD4+ T cells from SMARTA mice following rexinoid treatment (black circles) superimposed onto results from Fig 2C (gray squares). SMARTA experiment performed in duplicate. **(B)** CCR9 expression on antigen-specific effector CD8+ T cells from OT-1 mice following rexinoid treatment (black circles) superimposed onto results from Fig 2C (gray squares). OT-1 experiment performed in duplicate. All error bars represent SD from the mean.
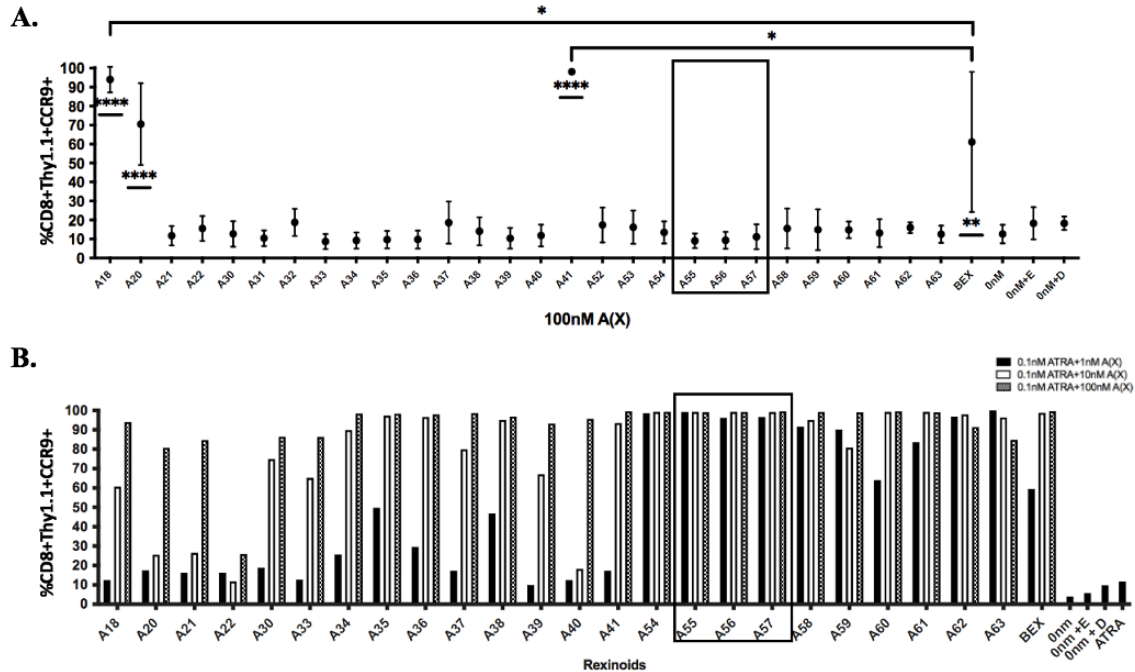
Figure 2.4 Rexinoids can Enhance Effector CD8+ T Cell Expression of CCR9 Independently or in Combination with ATRA. Splenocytes obtained from P14 mice were stimulated with GP$_{33-41}$ peptide and cultured either with 100nM rexinoids in vitamin A deficient media (top), or in vitamin A deficient media supplemented with suboptimal doses of rexinoids and ATRA (bottom). After 8 day culture, effector CD8+ T cells were analyzed for expression of CCR9 using flow cytometry. **(A)** Rexinoids A18, A20, A41 and BEX are able to significantly enhance CCR9 expression independent of ATRA presence, compared to no treatment. Rexinoids A18 and A41 also significantly enhance CCR9 expression, compared to BEX. Experiment performed in triplicate. Connecting letters report used to determine statistical significance, with ordered differences report used to compare p-values between groups (* = $p<0.05$, ** = $p<0.005$, **** = $p<0.0001$). **(B)** Suboptimal doses of several rexinoids cooperate with suboptimal doses of ATRA to enhance CCR9 expression. Boxed region identifies rexinoids that had no effect on CCR9 expression when previously delivered at 100nM. (boxed region 3A; graph representative of one experiment).

C.
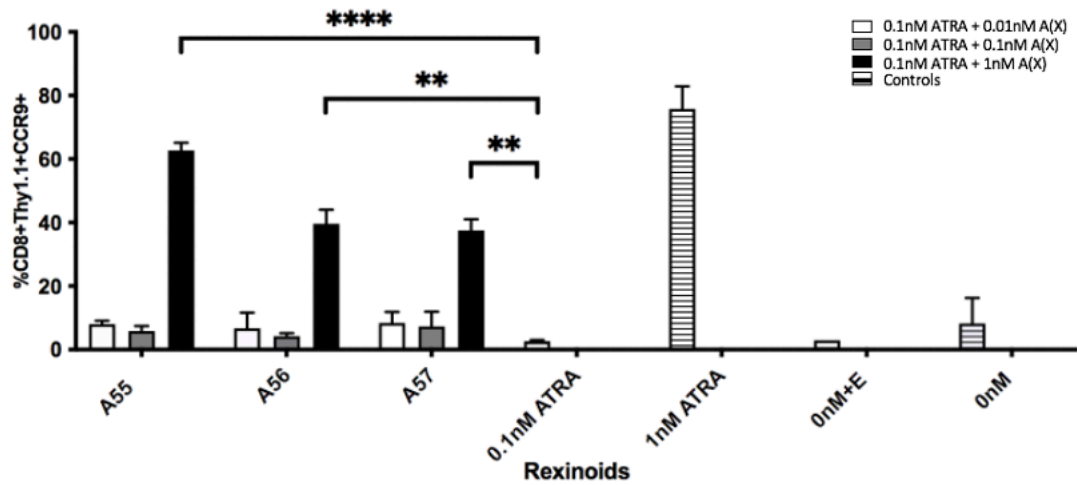
Figure 2.4 cont. (C) Replicate data obtained from culturing cells with suboptimal doses of rexinoid and ATRA. Rexinoids selected were those that showed high cooperativity with ATRA from 3B (boxed region). Suboptimal doses of selected rexinoid combined with suboptimal ATRA significantly improved CCR9 expression, compared to suboptimal ATRA alone (** = p<0.005, **** = p<0.0001). Rexinoid dosages lower than 1nM did not result in enhanced CCR9 expression. Experiment performed in triplicate. All error bars represent SD from the mean.
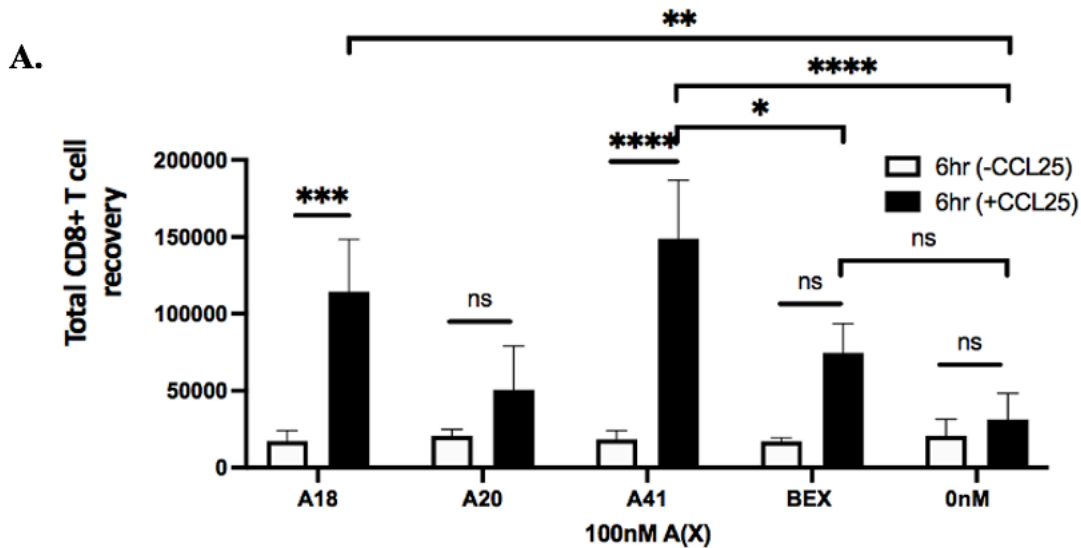
Figure 2.5 Treatment with ATRA Independent and ATRA Cooperating Rexinoids Enhances Effector CD8+ T Cell Migration Towards the Mucosally Expressed Chemokine CCL25 *In Vitro*. Splenocytes obtained from P14 mice were stimulated with GP$_{33-41}$ peptide and cultured for 7 days with 100nM selected rexinoids or 10nM ATRA. Cells were then subjected to a Boyden chamber assay. $5x10^5$ splenocytes resuspended in chemotaxis buffer were seeded into the top insert of a 96 well HTS Transwell plate and allowed 6 hours to migrate through a membrane (3.0um pore size) towards CCL25 (250nM concentration) plated in the lower chamber. Cells were then isolated from the bottom chamber and manually counted using a hemocytometer. **(A)** Migration following cell culture with ATRA independent rexinoids or ATRA. T cell migration towards CCL25 was significantly improved when cells were cultured with A18 and A41 (adjusted p values = 0.0009 and <0.0001, respectively). Treatment with A18 or A41 also significantly improved migration towards CCL25 compared to no treatment given (adjusted p values = 0.004 and 0.0001, respectively). Treatment with A41 also significantly improved migration compared to treatment with ATRA (adjusted p value = 0.0008). All ATRA independent rexinoids and ATRA tested in triplicate.
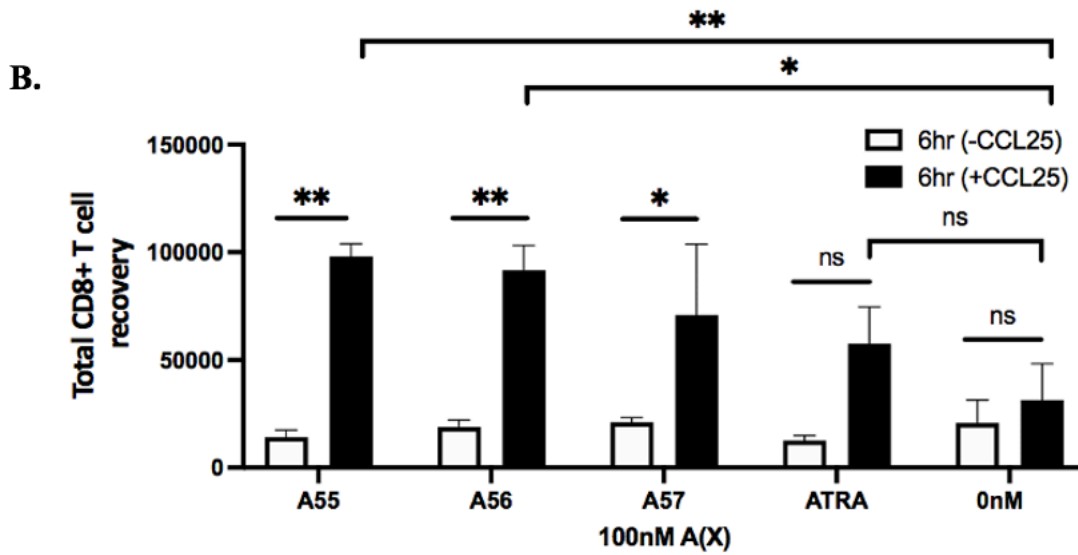
Figure 2.5 cont. (B) Migration following cell culture with ATRA cooperating rexinoids or BEX. Migration towards CCL25 was significantly improved when cells were cultured with A55, A56, and A57 (adjusted p values = 0.001, 0.01, and 0.02, respectively). Treatment with A55 or A56 significantly improved migration towards CCL25 compared to no treatment given (adjusted p values = 0.01 and 0.02, respectively). A55, A56, and BEX rexinoids tested in duplicate, A57 rexinoid tested in triplicate. Statistics were calculated using a two-way ANOVA (* = p <0.05, ** = p<0.005, *** = p<0.0005, **** = p<0.0001) . All error bars represent SD from the mean.
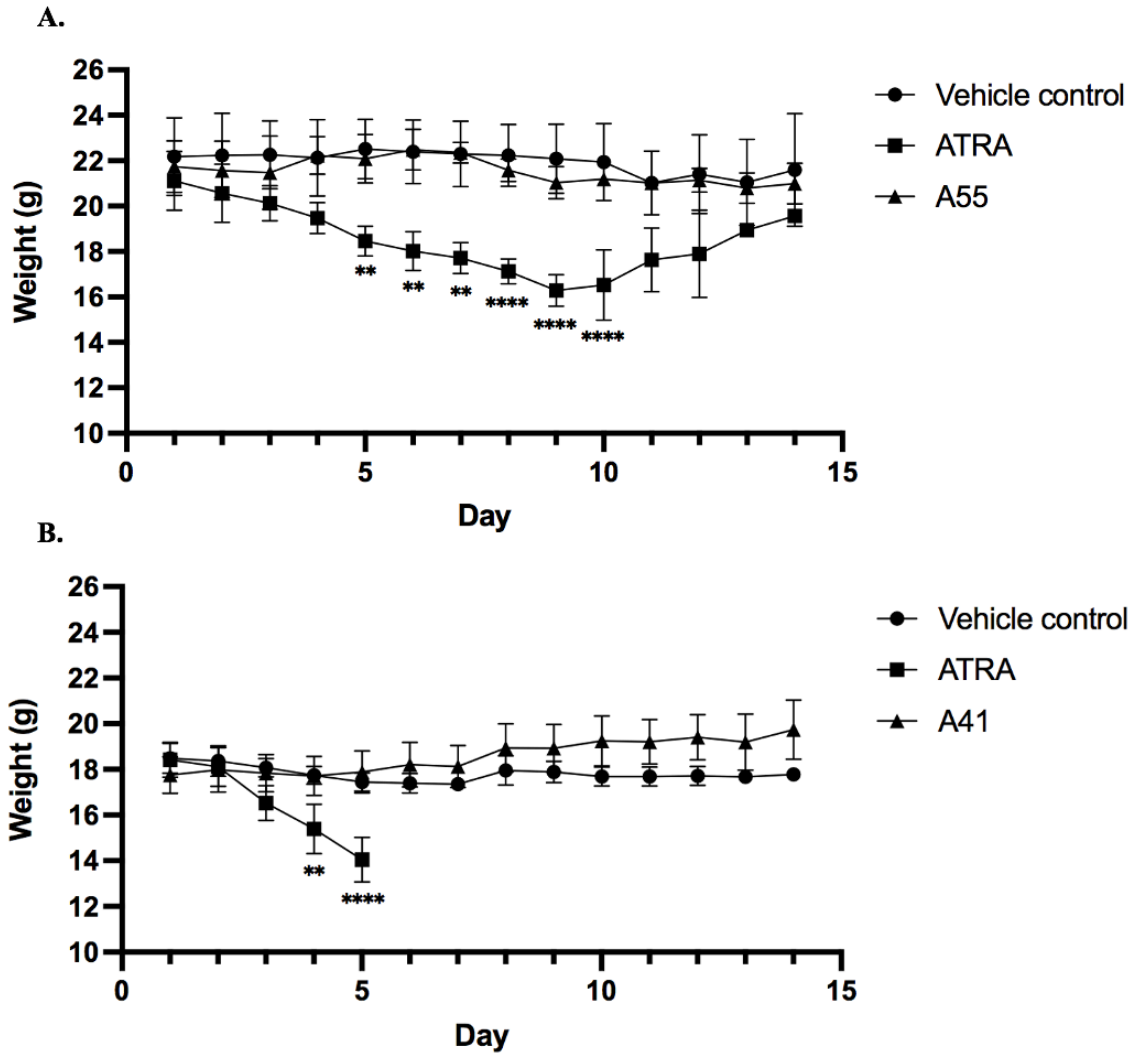
Figure 2.6 Minimal Weight Changes Seen with Rexinoid A55 and A41 Treatment Delivered *In Vivo*. Mice inoculated with K7M2 cells were treated daily with either vehicle control (n=4), rexinoid A55 (n=5), or ATRA (n=4), delivered i.p. at a previously determined concentration of 40mg/kg for 14 days, or with vehicle control (n=5), rexinoid A41 (n=6), or ATRA (n=5) delivered orally at 100mg/kg for 14 days. **(A)** del Mice that received A55 treatment i.p. had weight loss similar to negative control mice, while mice that received ATRA treatment displayed significantly larger weight losses during treatment (** =$p<0.005$, **** = $p<0.0001$). **(B)** Mice that received high dose oral A41 treatment showed slight weight gain, while ATRA-treated mice displayed significant weight loss that necessitated removal from study (** = $p<0.005$, **** = $p<0.001$). All error bars represent SD from the mean.

CHAPTER 3

Identifying Novel Mucosal Adjuvants for an HIV DNA Vaccine

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Abstract:

Adjuvant use during vaccination has been shown to enhance the immunogenicity of highly safe yet poorly efficacious vaccines, resulting in improved T cell-mediated protection. Despite this knowledge, few adjuvants are currently licensed for clinical use, with limited compounds known to enhance mucosal vaccine efficacy. We have previously shown in mouse models that administration of exogenous all-trans-retinoic acid (ATRA) during vaccination enhances CD8+ T cell migration to mucosal surfaces and improves protection at these regions following viral challenge, indicating its potential to function as a mucosal adjuvant. We have further demonstrated that delivery of adjuvant plasmids containing retinaldehyde dehydrogenase 2 (RALDH2), a rate-limiting enzyme in ATRA synthesis, during DNA vaccination also enhances T cell mucosal presence and subsequent immune protection following viral challenge, without exogenous ATRA-associated side effects. Here we built upon this work and assessed the potential of alternative adjuvant plasmids to improve the efficacy of a DNA vaccine for human immunodeficiency virus type 1 (HIV-1), a pathogen primarily transmitted across

mucosal membranes. Candidate plasmids contained sequences for dominant-positive retinoic acid receptor (DP-RAR) or cytochrome 450 1B1 (CYP1B1), which have been implicated in stimulating ATRA synthesis. Both were assessed for their ability to stimulate effector CD8+ T cell migration to mucosal and systemic regions during vaccination, either with or without electroporation, and enhance immune protection following viral challenge. Our findings suggest that DP-RAR and CYP1B1 may encourage effector CD8+ T cell migration to mucosal sites following vaccination, however these results were largely determined to lack significance. Additionally, neither DP-RAR or CYP1B1 treatment resulted in significantly lowered titers following viral challenge. Ultimately, further optimization is needed to fully assess the potential of DP-RAR and CYP1B1 as mucosal adjuvants.

Introduction:

Mucosal body surfaces represent a major site of pathogen transmission (155). Increasing T cell immunity at these regions can prevent disease progression and limit pathogen spread to vulnerable populations, therefore improving treatments that enhance T cell-mediated immune protection is of critical importance. Vaccines act to increase pathogen-specific memory T cell populations, and currently remain the most promising defense against mucosal-transmitted pathogens (156,157). However, significant hurdles remain in developing efficacious mucosal vaccines, as negative correlations are often seen when comparing vaccine safety and potency (158,159). Identifying adjuvant compounds that stimulate pathogen-specific CD8+ T cell migration to mucosal regions during vaccination could enhance the immunogenicity of low-risk mucosal vaccines showing substandard effectiveness, resulting in long-term immune protection.

We have previously shown in mouse models that provision of biologically active all-trans-retinoic acid (ATRA) during subunit vaccination improves vaccine-specific effector CD8+ T cell migration to mucosal regions and enhances immune resistance to viral challenge at mucosal sites, indicating its potential to act as a mucosal vaccine adjuvant (58). We have further shown that administration of plasmids containing retinaldehyde dehydrogenase 2 (RALDH2), an alcohol dehydrogenase family member responsible for catalyzing the synthesis of ATRA from retinaldehyde precursors, during DNA vaccination also improves vaccine-specific effector CD8+ T cell migration and subsequent resistance to viral challenge (75). This latter discovery showing enhancement of *in vivo* ATRA synthesis resulting in similar immune efficacy as exogenous ATRA treatment is attractive from a clinical standpoint, as ATRA-associated toxicity is reduced, and plasmid preparations show higher stability (160).

Multiple other proteins have been implicated in stimulating ATRA synthesis. Work previously performed by Profectus Biosciences showed that cells transfected *in vitro* with a mutated dominant-positive retinoic acid receptor (DP-RAR) produced higher levels of ATRA compared to cells transfected with RALDH2 (*unpublished*). Furthermore, several studies have reported that the cytochrome P450 family member 1B1 (CYP1B1) can generate ATRA from precursor compounds independent of RALDH2 (161-163). The ability of DP-RAR and CYP1B1 to generate ATRA indicates they may be able to preferentially stimulate CD8+ T cell migration to mucosal regions during vaccination, which could bolster the protective efficacy of low-risk mucosal vaccines. Additionally, the higher *in vitro* potency seen with DP-RAR suggests it may outperform RALDH2 in modulating mucosal immune protection.

Here we used mouse models to assess the ability of DP-RAR and CYP1B1 to act as mucosal adjuvants and enhance effector CD8+ T cell migration during DNA vaccination towards human immunodeficiency virus type 1 (HIV-1), a pathogen predominantly spread across mucosal surfaces (106). We further measured the ability of these alternative adjuvants to stimulate long-term mucosal immune protection by intravaginally challenging vaccinated mice with recombinant virus containing the HIV-1 vaccine epitopes. Our study design largely mimicked our previous work with RALDH2, with several groups of mice receiving vaccine and adjuvant plasmids coupled with electroporation to enhance plasmid uptake (Table 3.1) (75). Our findings indicate that use of DP-RAR and CYP1B1 stimulates CD8+ T cell migration to mucosal regions, however the increased migration is not significant, nor do the alternative adjuvants consistently outperform control groups. Additionally, high dose DP-RAR treatment resulted in lowered ovarian viral titers following challenge, however this finding was not significant compared to vaccine-only controls. Together, these data suggest that DP-RAR and CYP1B1 have weak potential to function as mucosal adjuvants when delivered i.m. with DNA vaccination, compared to RALDH2.

Materials and Methods:

Vaccine plasmids

Two plasmid DNA (pDNA) expression vectors containing HIV-1 subtype B antigens were used to vaccinate recipient mice. In brief, plasmid WLV-151M (HIV$_{gag}$) expresses a gag/pol fusion protein from the HIV-1 group M subtype B isolate (HXb2), and plasmid pMAX-PRO-bal-gp120 (HIV$_{bal}$) contains the glycoprotein 120 subunit from the laboratory adapted HIV-1 subtype B BaL strain. Purified vaccine plasmids were

provided by Profectus Biosciences and stored at 4C. Vaccine plasmids were diluted to appropriate concentrations (10μg and 30μg) in 1X phosphate-buffered saline (1X PBS) one day prior to injection.

Adjuvant plasmids

pDNA expression vectors containing adjuvant protein sequences were administered in tandem with vaccine plasmids. In brief, pMAX-PRO-hRALDH2 contained human RALDH2, pMAX-PRO-hDP-RAR contained human DP-RAR, and WLV-001NM-MCS-mCYP1B1 contained mouse CYP1B1. Purified adjuvant plasmids were provided by Profectus Biosciences and stored at 4C. Adjuvant plasmids were diluted and mixed with vaccine plasmids to appropriate concentrations one day prior to injection (DP-RAR: 1μg, 5μg, and 30μg; CYP1B1: 10μg and 30μg; RALDH2: 10μg and 30μg).

Animal vaccination

9 groups of 15, 6-8 week old female C57BL/6J mice (Jackson Labs), were intramuscularly (i.m.) injected in the thigh muscle with a mixture of both vaccine plasmids, either with or without adjuvant plasmid (Table 3.1). Groups received varying dosages of adjuvant either alone or coupled with electroporation, to assess for optimal potency. Groups were vaccinated every 14 days, for a total of 3 doses. Animal work for groups 1-5 were performed separate from groups 6-9 to reduce workload.

Lymphocyte isolation

5 mice per group were euthanized via CO_2 asphyxiation and cervical dislocation 14 days post final vaccination (day 42). Peyer's patches (PPs), spleen, and inguinal lymph nodes (ILNs) were harvested and mechanically dissociated into single cell

suspensions using 70μm nylon mesh strainers (Fisherbrand) prior to antibody staining. Spleens were additionally lysed of red blood cells (RBCs) using Ammonium-Chloride-Potassium (ACK) lysing buffer (ThermoFisher) prior to staining. Small intestine (SI) was digested using collagenase IV (Sigma), and lamina propria lymphocytes (LPL) were isolated using Percoll (Cytiva) density gradient centrifugation.

Antibody staining and flow cytometry

Analysis for vaccine-specific effector CD8+T cell presence in the various tissues was performed using flow cytometry. Cells were stained with a 1:100 dilution of the following fluorochrome-conjugated anti-mouse monoclonal antibodies: CD8α (clone: 53-6.7); CD44 (clone: IM7); and tetramer containing the HIV-1 subtype B gag peptide epitope SQVTNSATI. CD8α and CD44 antibodies were purchased from ThermoFisher, while custom-made tetramer was purchased from ProImmune. Flow cytometry was performed using a BD LSR Fortessa flow cytometer (BD Biosciences) and analyzed using FlowJo 8.8.7 software. Graphs were created using Prism 8 software (GraphPad).

Viral challenge and plaque assay

The remaining 10 mice per group received 3mg subcutaneous (s.c.) medroxyprogesterone injections for menstrual synchronization post final vaccination and were challenged 5 days later at the vaginal mucosal with 3×10^7 PFU recombinant Western Reserve vaccinia virus expressing the HIV-1 subtype B gag protein (VACV$_{gag}$). 6 days following challenge, uterine and ovarian tissue were harvested from euthanized mice, mechanically homogenized (Omni TH), and exposed to 3 rounds of freeze/thaw at -80C/+37C for viral release. Homogenates were then centrifuged at 4C, and supernatants were retained for plaque assay using VERO cells for viral titer quantification.

Results:

DP-RAR and CYP1B1 do not significantly enhance vaccine-specific effector CD8+ T cell migration to mucosal or systemic tissues when delivered with electroporation

DP-RAR has been indicated to be more potent than RALDH2 in stimulating ATRA production, therefore it may possess enhanced mucosal homing adjuvant activity at lower dosages compared to RALDH2. Additionally, the ability of CYP1B1 to mediate ATRA metabolism independent of RALDH2 indicates it may function as a mucosal homing adjuvant. To test these hypotheses, groups of mice were injected i.m. with both vaccine plasmids (10µg each) mixed with either 1µg or 5µg DP-RAR adjuvant plasmid, or 10µg CYP1B1 adjuvant plasmid, every 14 days for a total of 3 doses. Double-negative controls (DNC) received 1X PBS only, negative controls received vaccine only, and positive control received 10µg RALDH2 adjuvant plasmid. Sites were electroporated following injection to facilitate optimal myocyte uptake of plasmid. Mucosal (PPs and SI) and systemic (spleen and ILNs) tissues from 5 mice per group were harvested, processed, and stained for positive expression of CD8α, CD44, and tetramer at day 42. Use of CYP1B1 plasmid resulted in slightly increased numbers of vaccine-specific CD8+ effector cells in the PP, however these data were not determined to be significant when compared to negative or positive control (Figure 3.1A). Mice treated with DP-RAR and CYP1B1 also showed higher percentages of vaccine-specific effector CD8+ T cells in the LP, compared to negative and positive controls, however these findings were not determined to be significant (Figure 3.1B). Both DP-RAR and CYP1B1 treatment resulted in slightly higher numbers of vaccine-specific effector CD8+ T cells in systemic tissues compared to DNC, however these data were not determined to be significant, nor

did they outperform mice treated with negative control (Figure 3.1C) or RALDH2 (Figure 3.1D).

High doses of DP-RAR and CYP1B1 do not significantly enhance vaccine-specific effector CD8+ T cell migration to mucosal or systemic tissues

In vivo electroporation increases the risk of inadvertent muscle damage, therefore we sought to determine if a higher dose of adjuvant plasmid without electroporation resulted in sufficient uptake and increased the presence of vaccine-specific effector CD8+ T cells in mucosal regions. To measure this, groups of mice were injected i.m. with 30μg of both vaccine plasmids mixed with either 30μg DP-RAR or CYP1B1 adjuvant plasmid, every 14 days for a total of 3 doses. DNC received 1X PBS only, negative controls received vaccine only, and positive controls received 30μg RALDH2 adjuvant plasmid. Mucosal and systemic tissues from 5 mice per group were similarly harvested and stained for vaccine-specific effector CD8+ T cells as previously described. While increased doses of DP-RAR and CYP1B1 did result in enhanced T cell migration to mucosal sites compared to DNC, they did not outperform the vaccine only negative control group (Figure 3.2A, 3.2B). Surprisingly, higher numbers of vaccine-specific effector CD8+ T cells were seen in the DNC group compared to all others when examining systemic tissues (Figure 3.2C, 3.2D).

Use of DP-RAR and CYP1B1 adjuvant plasmids do not significantly reduce viral titers in mucosal regions following viral challenge

To determine the ability of vaccine-primed memory CD8+ T cells to protect against mucosal viral infection, remaining mice per group were challenged post final vaccination at the vaginal mucosa with 3×10^7 PFU VACV$_{gag}$. Uterine and ovarian tissues

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were harvested and processed 5 days post challenge, and subjected to plaque assay for viral titer quantification. Mice that received 10μg DP-RAR or CYP1B1 in the presence of electroporation showed slightly lower viral titers in uterine tissue compared to DNC, however changes were less than 1 fold and not determined to be significant (Figure 3A). Additionally, treatment with these alternative adjuvant plasmids did not outperform treatment with RALDH2, which showed the largest reduction in uterine viral titers (Figure 3.3A). Treatment with high dose DP-RAR reduced viral titers seen in ovarian tissue compared to DNC, vaccine and electroporation, and RALDH2 groups, however was not significantly different when compared to the high dose vaccine only negative control group (Figure 3.3B).

Discussion:

Current mucosal vaccine development has predominantly been hindered by the inability to amend opposing safety and efficacy profiles (158,159). Identifying adjuvant compounds that enhance T cell immunity at mucosal regions would boost the effectiveness of low-risk vaccines with poor immunogenicity, overcoming this obstacle and improving remedies towards mucosal-transmitted pathogens. Here we tested the ability of candidate proteins involved with ATRA synthesis to mediate effector CD8+ T cell migration to mucosal and systemic tissues during DNA vaccination, and ameliorate immune protection following viral challenge. Our results show that delivery of DP-RAR or CYP1B1 plasmids with electroporation during vaccination can stimulate vaccine-specific effector CD8+ T cell migration to these regions, however increases were either not significant or did not outperform controls (Figure 3.1A-D). Similarly, high dose treatment with DP-RAR or CYP1B1 plasmids did not significantly increase migration or

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consistently outperform controls (Figure 3.2A-D). Mice vaccinated in the presence of high dose DP-RAR showed lowered viral titers in ovarian tissue following intravaginal challenge (Figure 3.3B), however these findings were not significant when compared to vaccine-only controls. Ultimately, our data suggests that DP-RAR and CYP1B1 have weak mucosal adjuvanticity, in this context.

The inability of DP-RAR to significantly enhance T cell migration and subsequent protection *in vivo* was not altogether surprising, when considering the immunologic role of the RAR and mode of vaccine delivery. While Profectus has shown that *in vitro* cellular transfection with DP-RAR enhances ATRA production, *in vivo* immune cell activation of the RAR is more associated with cellular upregulation of homing receptors that induce migration to mucosal regions (59). As delivery of vaccine and adjuvant plasmids via i.m. injection results in uptake by antigen-presenting dendritic cells (DCs) present in the muscle, it is plausible that these DCs are being stimulated to migrate to mucosal regions, not the targeted CD8+ T cells. The reduced numbers of vaccine-specific effector CD8+ T cells seen in systemic tissues supports this hypothesis; DCs being directed to mucosal sites would be inefficient at activating appropriate CD8+ T cells in the muscle draining lymph nodes, therefore fewer would be seen circulating in the body. A high number of vaccine-specific effector CD8+ T cells in systemic tissues would have suggested an issue with T cell migration to mucosal regions, however this was not seen. The hypothesis is worth investigating by antibody staining for DC markers in mucosal regions following vaccination and DP-RAR treatment.

Selection of CYP1B1 as a potential mucosal homing adjuvant was based on literature indicating its ability to generate ATRA by catalyzing the oxidation of retinol

precursors to retinaldehyde, and retinaldehyde to RA (161-163). Similar to DP-RAR, delivery of CYP1B1-containing plasmids via i.m. injection would prevent uptake by target CD8+ T cells. Interestingly, the involvement of CYP1B1 in ATRA synthesis seems to be more prominent *in utero* compared to live birth, which could also explain the minimal effect seen in our young adult mice (163,164). While CYP1B1 does show some ability to promote vaccine-specific CD8+ T cell migration, it is currently not worth pursuing as a lead mucosal adjuvant over RALDH2 or DP-RAR.

Global morbidity and mortality associated with mucosal-transmitted pathogens such as HIV-1 remains exceptionally high, emphasizing the need for effective mucosal vaccines (156,157). Present-day mucosal vaccines that have received licensure do show efficacy in reducing disease and pathogen transmission, however they are either attenuated or inactivated versions, associated with much higher risk (156,157,165). The low risk associated with recombinant DNA vaccines makes them an attractive option, and adjuvant use could boost vaccine immunogenicity to ensure formation of protective T cell responses (166). Adjuvant plasmids containing proteins that influence T cell migration to mucosal regions would increase vaccine immunogenicity, and may provide a higher degree of safety than toxin-based mucosal adjuvants such as cholera toxin (CT) (167-170). While not conclusively shown here, both RALDH2 and DP-RAR do show promise to act as mucosal vaccine adjuvants. Further work should confirm adjuvant uptake by DCs, and focus on optimizing adjuvant delivery to the necessary CD8+ T cells.

Group	Vaccine plasmids	Vaccine dose	Adjuvants plasmids	Adjuvant dose	Delivery
1	HIV_{gag} + HIV_{bal}	10 μg + 10ug	None	N/A	i.m. Electroporation (x3)
2	HIV_{gag} + HIV_{bal}	10 μg + 10ug	CYP1B1	10 μg	i.m. Electroporation (x3)
3	HIV_{gag} + HIV_{bal}	10 μg + 10ug	RALDH2	10 μg	i.m. Electroporation (x3)
4	HIV_{gag} + HIV_{bal}	10 μg + 10ug	DP-RAR	5 μg	i.m. Electroporation (x3)
5	HIV_{gag} + HIV_{bal}	10 μg + 10ug	DP-RAR	1 μg	i.m. Electroporation (x3)
6	HIV_{gag} + HIV_{bal}	30 μg + 30ug	None	N/A	i.m. (x3)
7	HIV_{gag} + HIV_{bal}	30 μg + 30ug	DP-RAR	30 μg	i.m. (x3)
8	HIV_{gag} + HIV_{bal}	30 μg + 30ug	RALDH2	30 μg	i.m. (x3)
9	HIV_{gag} + HIV_{bal}	30 μg + 30ug	CYP1B1	30 μg	i.m. (x3)

Table 3.1 Study Design Showing Vaccination Doses, Adjuvant Doses, and Delivery Method. Groups of 6-8 week old female C57BL/6J mice (n=15) were vaccinated with or without adjuvant as indicated. Mice received treatment every 14 days for a total of 3 treatments (x3), either in the presence or absence of injection site electroporation.
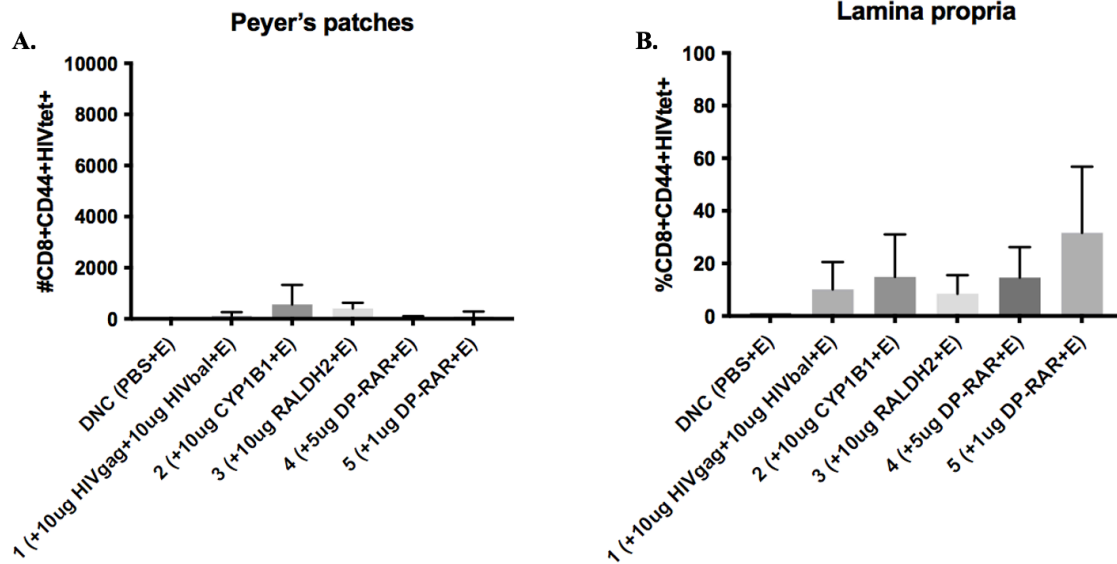
Figure 3.1 Alternative Adjuvant Plasmids Do Not Significantly Increase Vaccine-specific Effector CD8+ T Cell Migration to Mucosal or Systemic Tissues Following Electroporation. Groups of mice (n=15) were vaccinated using 10mg each HIVgag and HIVbal every 14 days for a total of 3 doses, either with or without CYP1B1 or DP-RAR adjuvant plasmids and with electroporation. Mucosal and systemic tissues were harvested at Day 42 and analyzed for vaccine-specific effector CD8+ T cell presence using flow cytometry. Cell counts based on flow cytometry samples only (5×10^4 events collected). **(A)** Mice vaccinated in the presence of 10mg CYP1B1 adjuvant plasmid had higher numbers of vaccine-specific effector CD8+ T cells in Peyer's patches, however increases were not significant. **(B)** Treatment with either DP-RAR and CYP1B1 resulted in higher percentages of vaccine-specific effector CD8+ T cells in LP, however these data were not significant compared to vaccine only controls.
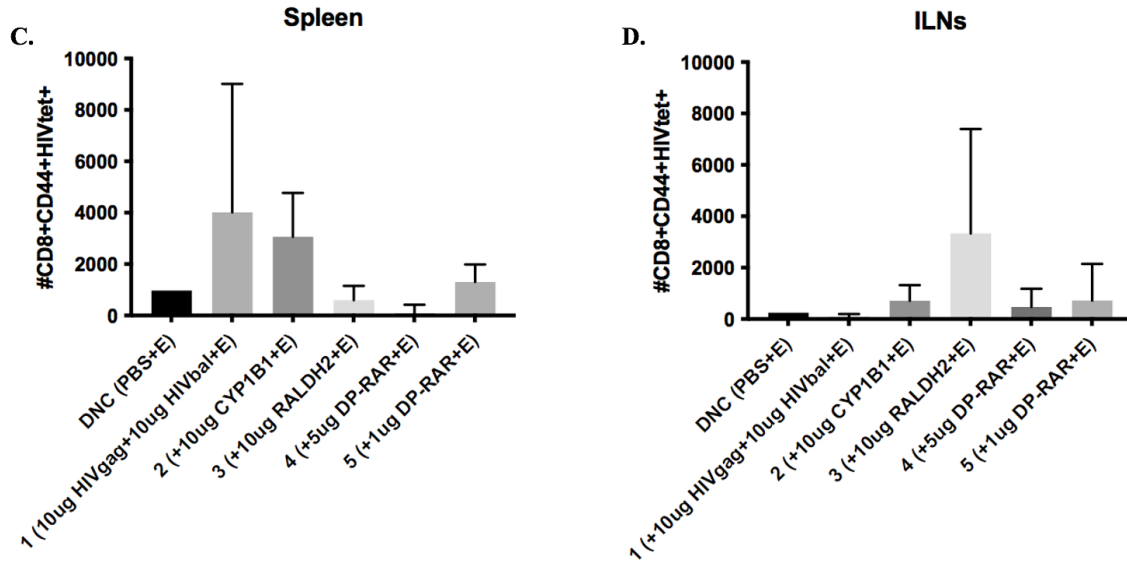
Figure 3.1 cont. (C) Vaccination in the presence of either 10mg CYP1B1 or 1mg DP-RAR enhances vaccine-specific effector CD8+ T cell numbers in the spleen compared to DNC, however these data are not significant compared to either negative or positive control. **(D)** Vaccination in the presence of RALDH2 increases vaccine-specific CD8+ T cell numbers in the ILNs, however increases seen with DP-RAR and CYP1B1 are minimal.
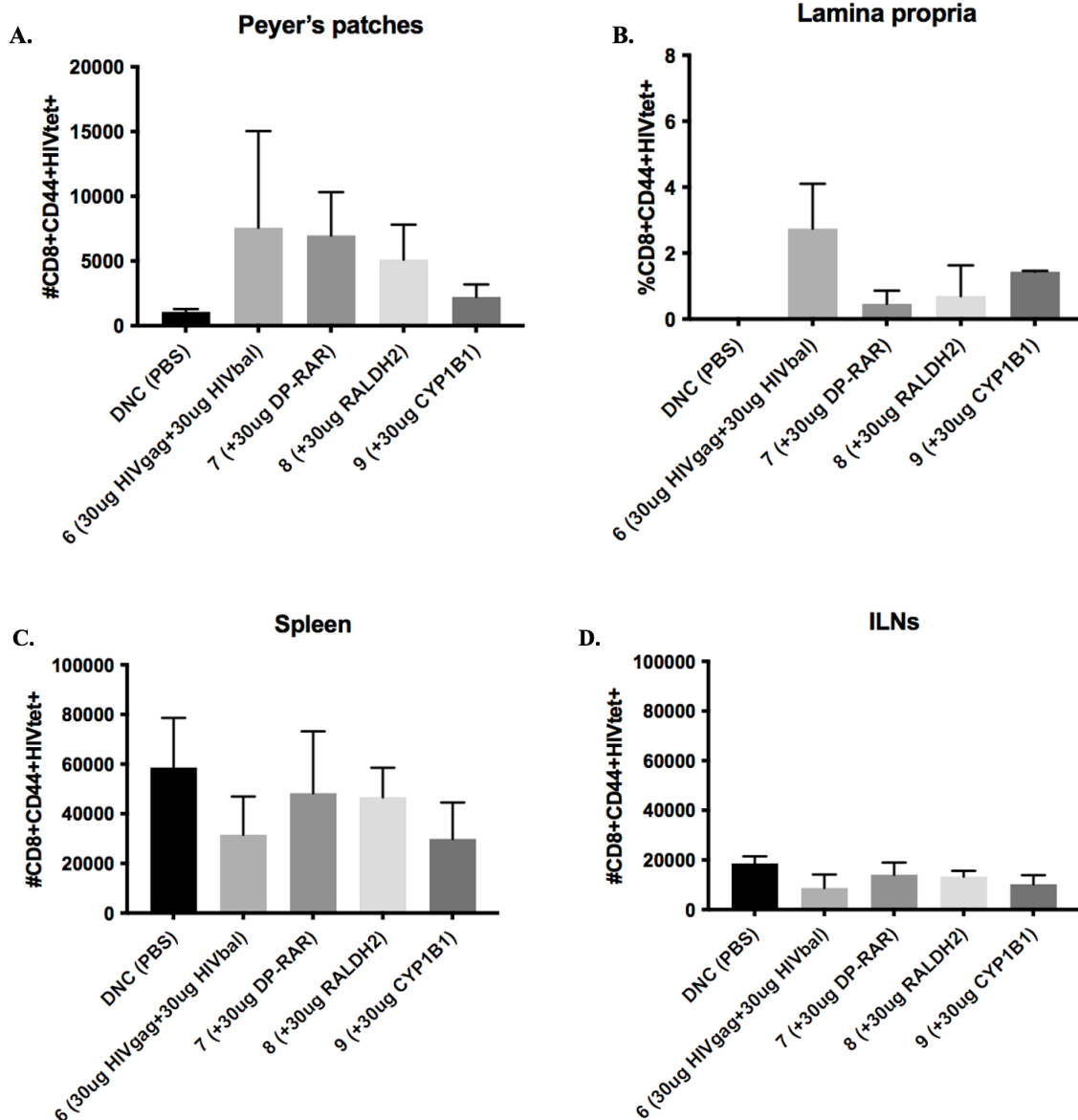
Figure 3.2 High Dose Alternative Adjuvant Plasmid Delivery Does Not Significantly Increase Vaccine-specific Effector CD8+ T Cell Migration to Mucosal or Systemic Tissues. Groups of mice (n=15) were vaccinated using 30mg each HIVgag and HIVbal every 14 days for a total of 3 doses, either with or without 30mg CYP1B1 or DP-RAR adjuvant plasmids. Mucosal and systemic tissues were harvested and analyzed for vaccine-specific effector CD8+ T cell presence at Day 42 using flow cytometry. Cell counts based on total cells per organ. **(A)** High dose DP-RAR and CYP1B1 treatment enhance vaccine-specific effector CD8+ T cell migration to Peyer's patches compared to DNC, however do not outperform vaccine-only mice. Similar results were observed in the lamina propria **(B)**. Mice that received high dose DP-RAR or CYP1B1 showed vaccine-specific effector CD8+ T cell migration to spleen **(C)** and ILNs **(D)**, however did not outperform DNC mice.

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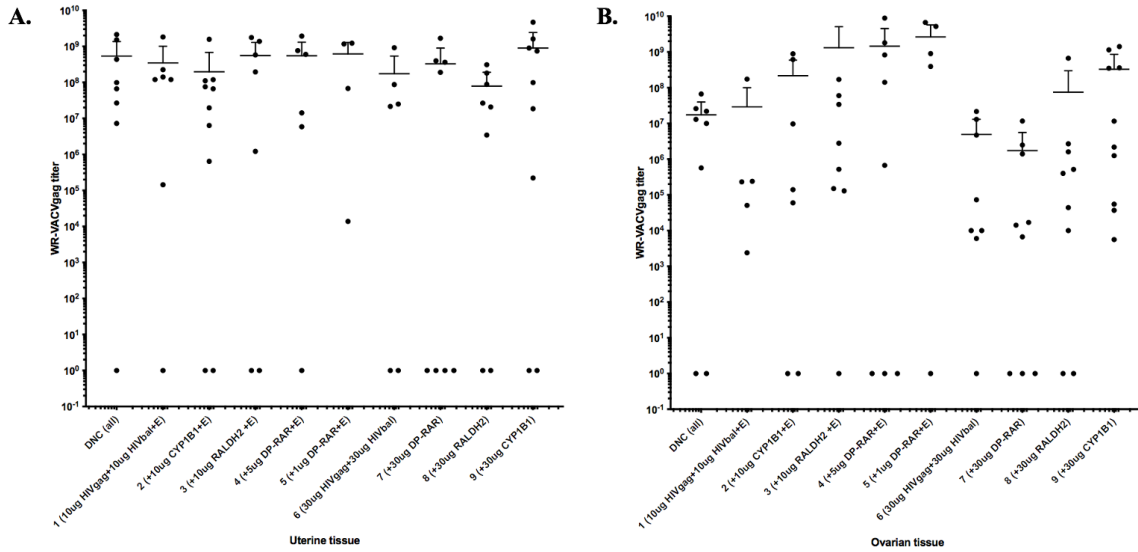
Figure 3.3. Treatment with Alternative Adjuvant Plasmids Do Not Significantly Reduce Viral Titers in Mucosal Regions Following Viral Challenge. Uterine and ovarian tissues from mice (n =5-10) treated with alternative adjuvant plasmids (DP-RAR or CYP1B1) were obtained and subjected to plaque assay following vaccinations and subsequent VACVgag challenge at the vaginal mucosa. **(A)** Groups vaccinated in the presence of 10mg DP-RAR or 10mg CYP1B1 both showed lower VACVgag titers in uterine tissues, however reduction was not determined to be significant. **(B)** Mice vaccinated in the presence of 30mg DP-RAR showed the lowest viral titers in ovarian tissue compared to other groups, however these findings were not significant when compared to negative control.

CHAPTER 4

DISCUSSION

Favorably altering T cell migration to specific body regions can improve immune-based therapies towards diseases that affect these sites. It is well-understood that vitamin A can modulate effector T cell migration to mucosal surfaces; the active vitamin A metabolite all-trans-retinoic acid (ATRA) signaling through the retinoic acid receptor (RAR) of the nuclear RAR/retinoid X receptor (RXR) heterodimer promotes mucosal homing receptor expression on responding T cells (58-63,94,95). Unfortunately use of exogenous ATRA as a therapeutic is limited due to its instability and high toxicity potential, therefore our work focused on identifying safer yet equally efficacious alternatives (82-89,100). We showed that several novel rexinoids, low toxicity RXR-selective ligands, possess clinical relevance as ATRA alternatives, as they enhanced T cell expression of mucosal homing receptors and migration *in vitro* either independently or synergistically with subtoxic doses of ATRA (100). We additionally showed that high dose oral rexinoid treatment is well-tolerated in *in vivo* cancer models, further supporting their medicinal use (100). Although our attempts to modulate T cell mucosal migration during vaccination using plasmid adjuvants containing ATRA-synthesizing enzymes did not show significant results, it does not rule out this modality as a prospective treatment option. This Discussion section consider the future use of rexinoids and plasmid-based adjuvants as remedial choices.

Rexinoid Use in Cancer Treatment

Immune checkpoint blockade therapy

Cancer treatment focus has largely shifted from traditional chemotherapy and radiation to now include immunotherapy, which harnesses the power of the immune system to destroy malignant cells (171,172). As effector CD8+ T cells are indispensable fighters in the war on cancer, it should come as no surprise that the goal of many immunotherapies is to maintain or enhance their function. One of the most promising T cell-based immunotherapies is immune checkpoint blockade (ICB) therapy, which uses monoclonal antibodies to block inhibitory interactions and promote T cell activity. Two prominent targets of ICB therapy include the PD-L1/PD-1 pathway and CTLA-4/CD80/86 pathway (173). Blocking tumor-expressed PD-L1 interactions with T cell expressed PD-1 prevents CD8+ T cell apoptosis in the TME, allowing them to maintain their killing function, while blockade of CTLA-4 and CD80/86 interactions enhances the activation of new cancer-specific T cells. Combination anti-PD-L1 and anti-CTLA-4 treatment has been shown to result in a synergistic anti-tumor response, however surprisingly this efficacy is limited to patient subsets and cancer types (55-57,149,152,154,174). We hypothesize that this may be due to inefficient migration of newly activated cancer-specific T cells from their lymph node of activation to the cancer site. This would result in CD8+ T cells within the TME becoming overwhelmed, despite the use of ICB therapy. In the case of mucosal-associated cancers, this could be remedied by coupling ICB therapy with our selected rexinoids that promote mucosal migration. Showing proof of concept in an animal mucosal cancer model would strengthen the prospective use of these rexinoids clinically. Furthermore, it would pave the way into research focusing on T cell migration to additional body regions, expanding the efficacy of ICB therapy in non-mucosal cancers.

Adoptive cell transfer therapy

In addition to ICB therapy, another up-and-coming T cell-based immunotherapy which rexinoids could synergize with is adoptive cell transfer (ACT) therapy. With this treatment, cancer-specific T cells isolated from patient tumors are expanded *ex vivo* and reinfused back into the patient, with the increased number of cytotoxic T cells anticipated to provide a better anti-tumor response (150-153). ACT therapy currently shows considerable success in individuals with metastatic melanoma, with at least 50% of patients showing cancer regression in response to treatment (151). As common sites of melanoma metastases are the lung and gut mucosa, use of our current rexinoids could also improve ACT therapeutic efficacy by modulating the migration of reinfused T cells to these regions (175).

Chimeric antigen receptor T cell therapy

Considered to be a variation of the original ACT therapy, chimeric antigen receptor T cell (CAR-T) therapy involves the genetic engineering of T cell receptors (TCRs) on autologous T cells isolated from blood or tumor (176). With this method, the extracellular TCR receptor is modified to enhance antigen recognition and specificity, while the intracellular signaling domains are manipulated to optimize activation for a heightened cytotoxic response (176,177). Currently approved CAR-T therapies are limited to B cell cancers, however our tested rexinoids could be coupled with CAR-T therapies that target B cell lymphomas and leukemias that arise in or metastasize to mucosal regions (176).

Oncolytic virotherapy

Exploiting the ability of certain viruses to preferentially infect human tumor cells has introduced oncolytic virotherapy (OV) as another promising cancer treatment. With this therapy, non-pathogenic viruses that show selective ability to replicate in and kill malignant cells are administered to patients (178). Originally named for the discovery and use of naturally occurring viruses that infect malignant cells, current oncolytic viruses also include genetically engineered viruses that can directly deliver cytotoxic and immune enhancing molecules to the tumor microenvironment (TME) (178). It has been seen that loading tumor-targeting cells with oncolytic viruses prior to patient delivery increases the success rate of virus reaching the TME, a finding which could be further enhanced with the use of rexinoids (178). Currently approved OV option T-VEC has shown success in shrinking metastatic melanoma tumors; as with ACT and CAR-T therapy, use of our rexinoids could further enhance regression and possible elimination of mucosal metastasized melanoma tumors by directing oncolytic virus-loaded cells to these regions (179).

Independent rexinoid therapy

In addition to acting in combination immunotherapies, our rexinoid candidates may be able to act as standalone treatments to stimulate cancer cell apoptosis. As noted in Chapter 2, ATRA use has been shown to upregulate the expression of pro-apoptotic genes, supporting its use as a chemotherapeutic. As rexinoids A18, A20, and A41 showed strong ability to act like ATRA in our studies, they may also mimic the cytotoxic effect of ATRA, albeit with reduced negative side effects (100). The clinical use of bexarotene (BEX), a rexinoid used for treating cutaneous T cell lymphoma (CTCL), supports this hypothesis. BEX has been shown to upregulate anti-proliferative and pro-apoptotic gene

expression in T cells, hence its use in CTCL, which suggests that our rexinoids may have a yet untapped ability to stimulate apoptosis in other malignant cell types (180).

Rexinoid Use in Vaccination

Infectious diseases persist as a global health concern, particularly those transmitted across mucosal surfaces (155). As highlighted in Chapter 3, vaccines remain the most effective tool in defending against pathogenic microorganisms. As further noted, effective mucosal vaccines must be able to stimulate vaccine-specific effector T cell migration to mucosal regions. While our work in Chapter 3 considered the use of plasmids containing ATRA-synthesizing enzymes to act as mucosal vaccine adjuvants, it may be possible for our rexinoids to fulfill this role. It is worth testing both our ATRA mimicking and ATRA cooperating rexinoids with our HIV-1 DNA vaccine; it could validate their ability to enhance T cell migration to mucosal regions in an *in vivo* model, and also expand their use for treating non-cancer-related mucosal diseases.

Rexinoid Use for Non-mucosal Diseases

While our work focused on using rexinoids in the context of enhancing mucosal migration, it is important to mention that they may play a role in modulating immune cell migration to other body regions. As previously noted in Chapters 1 and 2, the RXR is a highly promiscuous receptor, partnering with a slew of other nuclear receptors to enable their function. One notable receptor is the vitamin D receptor (VDR), which has been shown to upregulate T cell expression of homing receptors that direct them to skin-associated regions, such as chemokine receptor 10 (CCR10) (181). The VDR displays signaling patterns highly similar to the RAR; it partners with the RXR and associates with vitamin D response elements (VDREs) found in promoter regions of vitamin D

responsive genes (181,182). Binding of calcitriol, the biologically active form of vitamin D, to the VDR results in corepressor proteins bound to the VDR/RXR heterodimer dissociating, coactivators binding, and transcription occurring (182). It is possible that our tested rexinoids may also enhance VDR-associated CCR10 expression in responding T cells, and is worth testing in appropriate model systems. Unfortunately the nocturnal nature of mice results in the lack of necessary VDREs in their CCR10 gene (181). Humanized mouse models may be effective alternatives to conventional models, and could be pursued further.

Plasmid-based Adjuvant Use During Vaccination

The relationship between vaccine potency and safety is often negatively correlated (158,159). Use of adjuvants can bridge this gap, increasing the efficacy of benign vaccines that produce subpar results. Plasmid-based compounds offer a high degree of safety and control, making them ideal candidates for vaccine adjuvants. The protein products of plasmid expression have minimal possibility to induce toxicity, due to their biologic and non-replicative nature. Furthermore, a large selection of potentially immunogenic proteins could be incorporated into plasmid vectors, widening the repertoire of prospective adjuvants. Here we tested proteins that can modulate immune cell migration, however pro-inflammatory cytokines could also be included, as well as known antigenic proteins for the pathogen target.

As mentioned in Chapter 3, the efficacy of dominant-positive RAR (DP-RAR) as a mucosal vaccine adjuvant may be enhanced if delivery and uptake were directed to T cells undergoing activation, rather than dendritic cells (DCs) at the site of vaccine injection. The previous success of RALDH2 plasmids to stimulate T cell mucosal

migration is credibly due to DCs upregulating their production of ATRA after plasmid uptake, which would then act on T cells undergoing activation (75). As the RAR is more associated with enhancing mucosal homing receptor expression, attempting to deliver DP-RAR plasmids the same way as RALDH2 would not produce the same results, as seen in our work (59).

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APPENDIX A

SUPPLEMENTARY MATERIAL FOR REXINOIDS MODULATE EFFECTOR T

CELL EXPRESSION OF MUCOSAL HOMING MARKERS CCR9 AND α4β7

INTEGRIN AND DIRECT THEIR MIGRATION *IN VITRO*
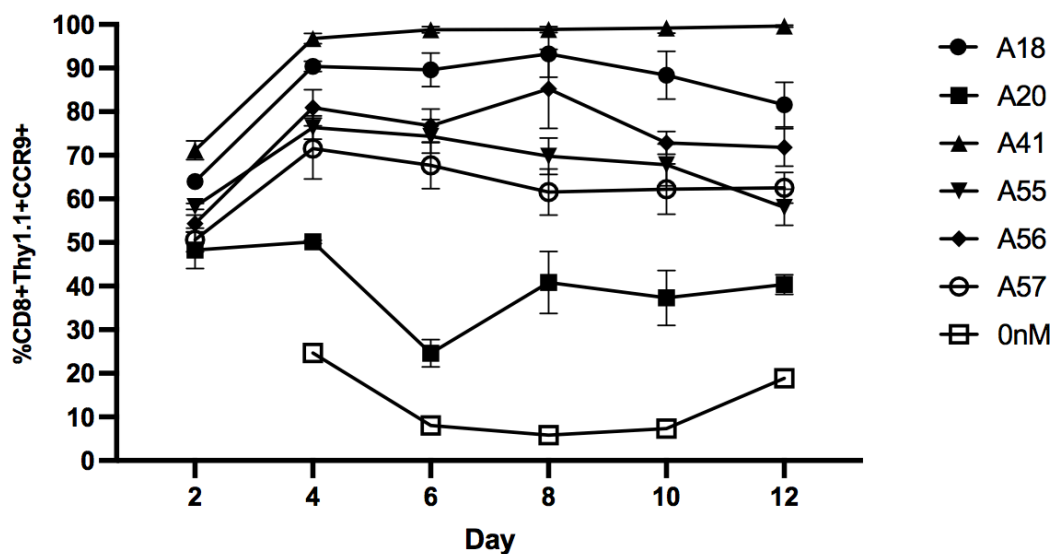
Supplementary Figure S2.1 Time Course Assay Shows Optimal CCR9 Expression at Day 8. Splenocytes obtained from naïve P14 mice were stimulated with GP_{33-41} peptide and cultured with 100nM select rexinoids (key to right). Flow cytometric analysis of CCR9 expression was performed every 2 days. A majority of rexinoids stimulated cells to optimally express CCR9 by day 8, which corresponds with the peak of primary murine immune responses.
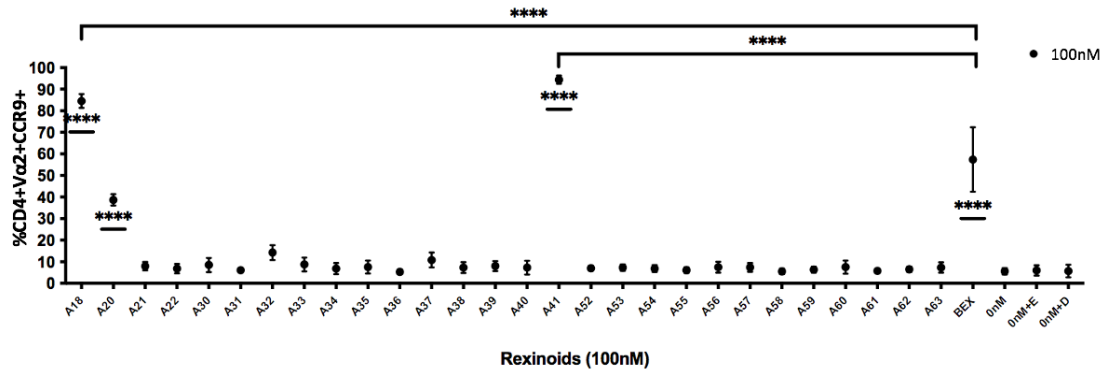
Supplementary Figure S2.2 Rexinoids can Enhance Effector CD4+ T Cell Expression of CCR9 Independently of ATRA. Splenocytes obtained from naïve SMARTA mice were stimulated with GP_{61-80} peptide and cultured with 100nM rexinoids in vitamin A deficient media. After 8 day culture, effector CD4+ T cells were analyzed for expression of CCR9 using flow cytometry. **(A)** Rexinoids A18, A20, A41 and BEX are able to significantly enhance CCR9 expression independent of ATRA presence compared to no treatment, similar to their activity on effector CD8+ T cells. Rexinoids A18 and A41 also significantly enhance CCR9 expression compared to BEX. Experiment performed in triplicate. Connecting letters report used to determine statistical significance, with ordered differences report used to compare p-values between groups (**** = $p<0.0001$).Error bars represent SD from the mean.
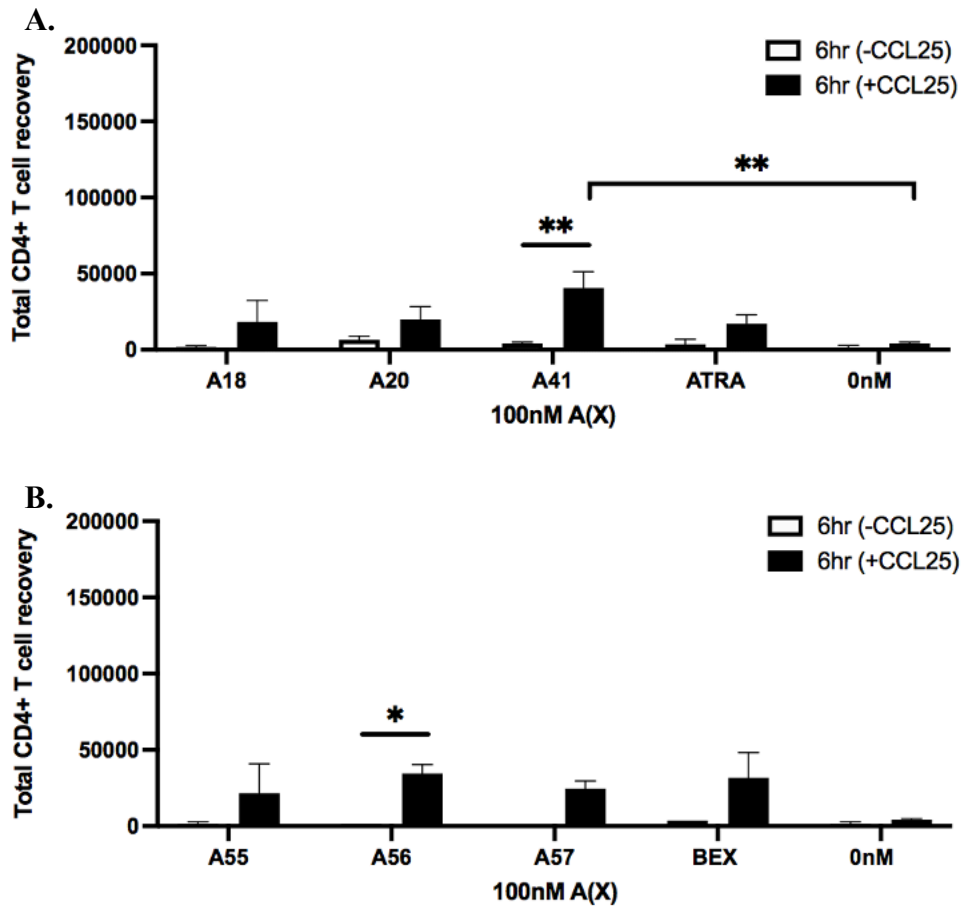
Supplementary Figure S2.3 Treatment with ATRA Independent and ATRA Cooperating Rexinoids can Enhance Effector CD4+ T Cell Migration Towards the Mucosally Expressed Chemokine CCL25 *In Vitro*. Splenocytes obtained from SMARTA mice were stimulated with GP_{61-80} peptide and cultured for 7 days with 100nM selected rexinoids or 10nM ATRA. Cells were then subjected to a Boyden chamber assay, as described in Figure 4 legend. **(A)** Migration following cell culture with ATRA independent rexinoids or ATRA. T cell migration towards CCL25 was improved when cells were cultured with rexinoids A18, A20, A41, and BEX. Migration was significantly improved when cells were cultured with A41 in the presence of CCL25 (adjusted p value = 0.006). Rexinoid treatment also improved migration towards CCL25 compared to no treatment given, with significantly improved migration seen following A41 treatment (adjusted p value = 0.006). Treatments tested in duplicate. **(B)** Migration following cell culture with ATRA cooperating rexinoids or BEX. Migration towards CCL25 was improved when cells were cultured with A55, A56, A57, and BEX, with significantly improved migration seen following A56 treatment (adjusted p value = 0.04). Treatments tested in duplicate. Statistics were calculated using a two-way ANOVA (* = p <0.05, ** = p<0.005). Error bars represent SD from the mean.
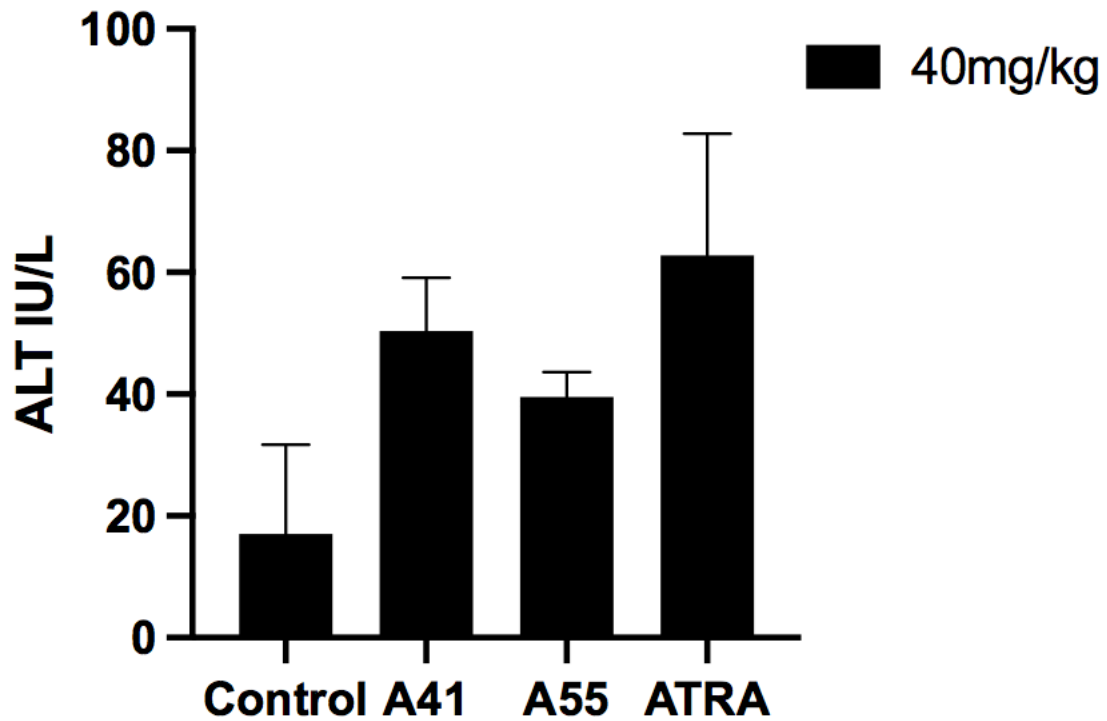
Supplementary Figure S2.4 ATRA-treated Mice Show Higher Levels of ALT.
Balb/cJ mice were treated orally with 40mg/kg vehicle control (n=2), A41 (n=2), A55 (n=2), or ATRA (n=2) for two weeks, with serum obtained at day 14 to measure for levels of the liver enzyme ALT. Mice treated with ATRA showed higher levels of ALT compared to other groups, however these differences were not seen to be significant.