DEVELOPING LOW-DENSITY LIPOPROTEIN RECEPTOR–RELATED PROTEIN-1 AGONISTS AS A THERAPEUTIC STRATEGY IN ALLERGIC INFLAMMATORY DISEASES

by

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DEDICATION

This work is dedicated to my husband who has supported me through it all. To my children, nothing worth anything is easy. Keep striving, keep persisting through the challenge and always believe you can.

ACKNOWLEDGEMENTS

I would first like to thank God for giving me the grace to get through this process. To my husband, your constant encouragement means the world to me. I am grateful for my friends and family, my dad, who supported me, and my mom who consistently prayed for me. Thank you, Nanny, for helping with the kids, I couldn't have done this without you. To Dr. Gelber, thank you for providing me with an opportunity to continue my education. Dr. Kehn-Hall, I appreciate your advice and guidance. Dr. Luchini, thank you for taking me in under your advisorship and for all the help through the process. To all my committee members, I'm grateful for your time and advice. Caitlin, you've helped me in more ways than you know. Last but not least, Kelly, you have been an integral part of this process and for that, I am truly grateful.

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LIST OF ABBREVIATIONS AND SYMBOLS

Airway Hyperreactivity ..... AHR
Allergic Rhinitis ..... AR
Alpha-1 Antitrypsin ..... AAT
Atopic Dermatitis ..... AD
Bronchoalveolar Lavage Fluid ..... BALF
Dendritic Cell ..... DC
Dulbecco's Modified Eagle Medium ..... DMEM
Eosinophilic Esophagitis ..... EoE
Fetal Bovine Serum ..... FBS
Food and Drug Administration ..... FDA
Half maximum effective concentration EC50
Interleukin IL
Intraperitoneal ..... IP
Intravenous IV
Janus Kinase ..... JAK
Lipopolysaccharide ..... LPS
Liquid Chromatography with tandem mass spectrometry ..... LC/MS/MS
Low-Density Lipoprotein Receptor Related Protein-1 ..... LRP-1
Mechanism of Action ..... MOA
Micro ..... µ
Milligram ..... mg
Milliliter ..... ml
Minimum Toxic Concentration ..... MTC
Nuclear Factor kappa B ..... NFκB
Oral Gavage ..... OG
Ovalbumin ..... OVA
Peripheral blood mononuclear cell ..... PBMC
Pharmacokinetic ..... PK
Poly(deoxyinosinic-deoxycytidylic acid) ..... Poly I:C
Receptor Associated Protein ..... RAP
Serine Protease Inhibitor ..... SERPIN
Serpin Enzyme Complex ..... SEC
Serpin peptide 16 ..... SP16
Signal transducer and activator of transcription ..... STAT
Subcutaneous ..... SC
T helper 2 ..... TH2
Thymic Stromal Lymphopoietin ..... TSLP
Toll-Like Receptor ..... TLR
Tumor Necrosis Factor ..... TNF

ABSTRACT

DEVELOPING LOW-DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN-1 AGONISTS AS A THERAPEUTIC STRATEGY IN ALLERGIC INFLAMMATORY DISEASES

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George Mason University, 2022

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Allergic inflammatory diseases such as atopic dermatitis, eosinophilic esophagitis (EoE) and asthma are a consequence of persistent exposure to allergens leading to impaired innate immune mechanisms and dysregulated immune responses. A large unmet need exists for an effective treatment that is devoid of potentially serious side effects and without immunosuppressive effects. We have previously developed a family of first-in-class drugs derived from naturally occurring Serine Protease Inhibitors (SERPINS) that target Low-Density Lipoprotein Receptor-Related Protein-1 (LRP-1), a homeostatic receptor that regulates a multitude of critical functions of the immune response. The lead peptide, SP16, is a short (17mer) modified peptide derivative of Alpha-1 Antitrypsin (AAT). Recent work has shown that both AAT and LRP-1 play a role in alleviating allergen-driven inflammation. In this study, we have designed a series of SP16-derivative

analogs modeled on the putative LRP-1 binding site, with the goal of developing these peptide therapeutics for inflammatory diseases. We have methodically screened these analogs for their ability to inhibit inflammation, refining the analog design based on structure-activity relationships. In three tissue specific cell lines, analogs showed increased potency, with dramatically improved effective concentrations (EC50s) compared to SP16, and no toxicity, thus having the potential for an increased therapeutic window. In-vitro, ELISA based LRP-1 binding assays showed target engagement. Lead analogs, screened in a rapid in-vivo mouse model of acute inflammation, exhibited significant anti-inflammatory function comparable to SP16. The analogs demonstrated improved pharmacokinetic properties and potential for a wider range of administration routes, including oral. Finally, a few select analogs, alongside SP16, were tested for their ability to inhibit TH2 mediated responses in mouse models of atopic dermatitis and asthma. Both SP16 and analogs showed significant amelioration of disease phenotypes, including thymic stromal lymphopoietin (TSLP) inhibition. Overall, the developmental work has defined two new lead SERPIN-derived LRP-1 agonists for inflammatory diseases and provided a new avenue of SP16 development in allergic inflammation.

CHAPTER ONE: STATEMENT OF UNMET NEED

Inflammation occurs as the immune system mounts a necessary response to injury or infection in order to heal the infected or damaged site. However, when these immune responses become dysregulated, a harmful inflammatory cycle is activated causing uncontrolled tissue responses that can lead to a variety of diseases. In the case of severely acute inflammation, for instance following infection, the initiating insults are highly amplified, resulting in overstimulation of the immune cells and aberrant cytokine release (known as the cytokine storm), resulting in tissue damage that can lead to organ failure and death. In the case of chronic inflammation, on the other hand, long-term exposure to immune stimulating substances (i.e., microbes, toxins such as cigarette smoke, or naturally occurring allergens), causes dysregulated immune responses that can lead to a variety of chronic diseases such as cardiovascular disease, diabetes, peripheral nerve injury, asthma and autoimmune diseases.

Allergic diseases are reaching epidemic proportions in developed countries with an estimated 17.8 million Americans suffering from Atopic Dermatitis (AD) alone (*1*). According to the CDC, more than 25 million Americans suffer from Asthma, a chronic respiratory condition. Allergic inflammatory diseases are a consequence of chronic inflammation due to persistent exposure to allergens. Eosinophilic Esophagitis (EoE) is an orphan indication with no FDA-approved drugs and limited treatment options. This

food-allergen-driven disease is characterized by eosinophil-dominated inflammation that leads to esophageal damage. In the US, an estimated 150,000 patients (largely children) are currently suffering from this disorder that causes esophageal pain, difficulty swallowing, food impaction, nausea, vomiting, and failure to thrive. Atopic march (or the progressive development of multiple allergic diseases in children over time), suggests that there is a common underlying mechanism driving these conditions.

The overwhelming rise in the prevalence of inflammatory disease has prompted an unprecedented interest in developing anti-inflammatory drugs to address the need. While anti-inflammatory drugs such as biologics or corticosteroids do exist, they are not without potentially harmful side effects (immune suppression, stomach bleeding, or liver damage). Therefore, there is an urgent need to discover novel therapeutic targets in order to develop safe and effective anti-inflammatory drugs.

CHAPTER TWO: SPECIFIC AIMS

There is an overwhelming rise in the prevalence of inflammatory disease, prompting an unprecedented interest in developing anti-inflammatory drugs to address the need. Indeed, inflammatory diseases such as ischemic heart failure, respiratory disease, diabetes, cancer, chronic kidney disease, neurodegenerative disease, and autoimmune disease accounts for over half of all deaths and are considered one of the greatest threats to human health. Allergic inflammatory diseases are a consequence of chronic inflammation due to persistent exposure to allergens, and are reaching epidemic proportion in developed countries such as the United States. While anti-inflammatory biologics or corticosteroids that reduce the inflammation in the body do exist, they are not without potentially harmful side effects. Therefore, there is an urgent unmet need to discover novel therapeutic targets in order to advance the development of alternative anti-inflammatory therapeutics devoid of potentially serious adverse effects common to currently available options.

Low-density lipoprotein receptor related protein-1 (LRP-1) is an endocytic and cell signaling receptor found on a variety of tissues such as heart, lung and brain, as well as immune cells. This multifunctional receptor plays an important role in the regulation of diverse cellular processes such as lipid metabolism, protease/inhibitor homeostasis, cell survival and differentiation and has been described as a master regulator of inflammation. We have validated LRP-1 as a viable therapeutic target in cardiac inflammation with the

use of a SERPIN (Serine protease inhibitor) derived peptide drug (SP16) and *hypothesize that LRP-1 can serve as a therapeutic target in a variety of inflammatory diseases, including allergic inflammatory disease, to rebalance the inflammatory response and protect tissue against damage.* **This proposal seeks to further characterize and develop potent SERPIN-derived LRP-1 agonist drugs for the treatment of allergic inflammatory diseases.** The overall objective of the research is to determine a few lead candidates with improved efficacy and/or pharmacokinetic properties (vs. the current lead drug, SP16) allowing a wider range of administrative routes more conducive to the treatment of chronic inflammatory diseases. In order to complete this project, the following aims are proposed:

Aim 1: Screen serpin analogs for anti-inflammatory response and target receptor engagement. A) The analogs will be tested *in-vitro* in tissue specific cell lines to examine the immune modulatory properties of the peptides. Utilization of NF-κB reporter cell lines (human THP1-XBlue™-MD2-CD14 and human THP1-Blue™ NF-κB cells) and IMG mouse microglial cells with an array of stimulants (LPS, Poly I:C, cytokines) will serve to assess the anti-inflammatory potency of the analogs. To examine the effects of the analogs in a relevant model for allergic inflammation, we will assess apoptosis and thymic stromal lymphopoietin (TSLP) release in human keratinocytes. Cell cytotoxicity assays in the THP1-XBlue™-MD2-CD14, THP1-Blue™ NF-κB cells, and mouse IMG microglial cells will also be performed to eliminate any toxic compounds. **B)** *In-vitro*

LRP-1 binding assays using labeled analogs will serve to ensure receptor engagement and measure LRP-1 affinity.

Aim 2: Perform Pharmacokinetic studies with selected serpin analogs. Selected analogs with desirable anti-inflammatory and LRP-1 targeting properties will be administered by either subcutaneous, intravenous, or intraperitoneal route to BALB/c mice. Blood will be collected at specific times following drug administration (up to 24hrs). The plasma levels of the analogs will be measured using LC/MS/MS to determine PK properties (bioavailability, half-life etc.). Other routes of administration (i.e., oral or topical) may be explored.

Aim 3: Characterize the immune modulatory properties of the analogs *in-vivo* in inflammatory and allergic disease models. We hypothesize, based on the mechanism of targeting LRP-1, that treatment with the analogs following induction of acute inflammation with LPS will result in altered cytokine responses through modulation of innate host responses in the acute LPS challenge model in BALB/c mice. **A)** We will utilize an LPS induced acute inflammatory model, as a rapid *in-vivo* screen to demonstrate the systemic anti-inflammatory effects of the analogs. **B)** Recently, LRP-1 has been implicated in mediating immune responses important in allergic inflammatory disease. Therefore, we will utilize two distinct models of allergic inflammation, the ovalbumin-induced mouse model of asthma and the MC903/calcipotriol induced mouse model of atopic dermatitis (both in BALB/c mice), to assess the efficacy of the Serpin

analogs in controlling allergic immune responses. Rescue from tissue damage, edema or other disease specific pathologies (ex. Ear swelling in MC903 induced atopic dermatitis model) will be determined.

The goal of these studies is to discover safe and effective anti-inflammatory serpin analogs for further preclinical development to treat inflammatory disease.

CHAPTER THREE: BACKGROUND

Introduction to Allergic Inflammation

The development of allergic inflammation occurs as a dysregulated adaptive immune response directed towards a typically innocuous environmental substance (allergen). Allergens can encompass a wide variety of substances including certain foods, pollens, pet dander, or house dust-mites. The repeated exposure to the allergens sensitizes the individual by inducing IgE production, with subsequent re-exposures to the allergen leading to an allergic inflammatory response. Chronic inflammation drives allergic disorders such as asthma, allergic rhinitis (AR) (hay fever), atopic dermatitis (AD) (eczema), and rare food allergen-driven diseases such as eosinophilic esophagitis (EoE) (*2*). The diseases are highly heterogenous with many different phenotypes involving complex pathophysiology. At the associated site (i.e., lungs, nasal passage, skin, esophagus), there is local recruitment of white blood cells (leukocytes) such as eosinophils, lymphocytes (T-and B-cells), and basophils. Resident cells such as mast cells and macrophages release mediators such as cytokines, chemokines and proteases that contribute to immune cell infiltration and activation (*3, 4*). Damage to the epithelial barrier and subsequent mediators that are involved in the homeostasis of barrier function have recently been recognized as important mediators in persistent allergic immune responses (*5, 6*). Clinical manifestations, depending on the disease and tissue site, can include edema (swelling), erythema (redness), pruritis (itch), mucous hyper production or airway hyperresponsiveness (AHR). The persistent and repetitive exposure to the

triggering allergen and subsequent chronic eosinophilic inflammation can lead to long-term pathological consequences such as tissue remodeling and fibrosis (*7*). Recent insight into the common pathological mechanisms and underlying dysregulated inflammatory responses that contribute to allergic diseases has widened the potential therapeutic strategies currently under development. Even so, corticosteroids, either inhaled (for asthma), intranasal (AR), topical (AD) or oral (EoE), have been used for decades and continue to be a mainstay treatment option. The immunosuppressive effects and adverse consequences associated with long-term use of steroids supports exploration of additional therapeutics for allergic inflammatory diseases.

Molecular Mechanisms and Therapeutic Targets in Allergic Inflammation

The allergic inflammatory response is associated with activation of a specific type of T-helper cell, TH2 cells. During the sensitization phase, the dendritic cells (DCs), surveying the local environment at the affected site, comes in contact with the allergen (and other surface triggers that induce activation and maturation). The DCs migrate to the lymph nodes and presents the processed antigenic peptides to the naïve T-cells, stimulating differentiation into TH2 cells (as well as other subsets of TH cells, not discussed here) and infiltration into the affected tissues. The TH2 cells produce a specific inflammatory cytokines profile dominated by IL-4, IL-5 and IL-13 that have demonstrated pathogenic roles in allergic inflammation (*8*). These cytokines initiate eosinophilic inflammation, B-cell activation and subsequent IgE mediated responses. IL-5 is particularly crucial in mediating eosinophilic maturation and survival, while

eosinophil specific chemokines such as eotaxin-1 (CCL-11), eotaxin-2 (CCL-24) and eotaxin-3 (CCL-26) are critical for recruitment and accumulation of eosinophils (recognizing the CCR3 receptor of eosinophils) to the affected site (*9*). An over-accumulation of activated eosinophils at the disease site can contribute to cell damage and begin a self-perpetuating inflammatory cycle that worsens disease (*10*).

Given the role of IL-5 in the pathology of eosinophilic driven allergic inflammation, therapies have emerged including monoclonal antibodies targeting IL-5/IL-5 receptor (Mepolizumab, Benralizumab and Reslizumab). These therapies, available for severe and steroid refractory asthma patients, seem effective in reducing exacerbations, however, redundant cytokines (IL-3 and GM-CSF) may explain the lack of complete depletion of eosinophils (which may in turn be a positive outcome to reduce risk of infections) (*11*). Because of the heterogeneity of the allergic inflammatory diseases such as asthma, it would be necessary to select appropriate patient populations that may benefit from this therapy.

Cytokines IL-4 and IL-13 have also been investigated and clinically validated as a therapeutic target for allergic inflammatory diseases. Both IL-4 and IL-13 are produced by a variety of cells including TH2 cells, eosinophils, mast cells and epithelial cells. They have been shown to induce TH2 skewing, production of IgE by B-cells, macrophage activation, dendritic cell function, eosinophil recruitment and activation, as well as fibroblast proliferation and tissue remodeling (*12-14*). These cytokines partly share a receptor (IL-4RII) and can activate signal transduction pathways, notably the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling

axis, in particular STAT6 (*15, 16*). Activation of STAT6 can activate Phosphoinositide 3-kinase – Akt kinase (PI3K/Akt) and nuclear factor kappa B (NF-κB) signaling pathways, which can contribute to proliferative and inflammatory effects (*17*). Additionally, IL-13/IL-4 mediated STAT6 activation can downregulate genes important in proteolytic regulation, such as the protease inhibitor SPINK7, which could contribute to impaired barrier function (*6, 16*).

Given that IL-4 and IL-13 are involved in many aspects of allergic inflammatory pathophysiology, several antibodies targeting IL-13 (Anrukinzumab, Lebrikizumab, Tralokinumab) were evaluated in clinical trials, however, a disconnect in translation resulted in no substantial clinical efficacy. Likewise, monoclonal antibodies targeting IL-4 (Pascolizumab, Pitrakinra) resulted in no significant clinical efficacy (*14*). However, the monoclonal antibody, Dupilumab (Dupixent®), blocking the shared receptor (the α-subunit of the IL-4 receptor complex), capable of inhibiting both IL-4 and IL-13 has shown significant improvements in patients with asthma (lung function, exacerbations) and atopic dermatitis (skin improvements and reduction in pruritis) and has been FDA approved for these indications (*18, 19*). Recent phase III clinical trials (NCT04394351) in eosinophilic esophagitis patients shows improvements in dysphagia (difficulty swallowing) and other symptoms, and will likely result in FDA approval.

The importance of innate cytokines sometimes called “alarmins” mainly released by the epithelial cells, including thymic stromal lymphopoietin (TSLP), and interleukins-(IL)-33, and IL-25 have recently emerged as important therapeutic targets in allergic inflammation (*20*). These cytokines have exhibited a prominent role in allergic

inflammatory pathobiology (*21*). TSLP has been found to be highly overexpressed in serum as well as in bronchial epithelium of asthma patients and in lesions of patients with AD (*22*).

The cytokines are released upon epithelial damage by environmental triggers such as allergens, viruses, bacteria or physical injury. These epithelial cytokines are upstream activators of both innate and adaptive type 2 immune responses. As a result of their release, dendritic cells direct TH2 cell differentiation, thereby resulting in IL-4 and IL-13 mediated B-cell activation and IgE release. Innate responses are directly activated by these alarmins through ILC2 cells (innate lymphoid cells acting similarly to TH2 cells, but lacking T-cell receptors), eosinophils, basophils and mast cells, thereby releasing IL-4, IL-5 and IL-13 (*23*).

Of the epithelial cytokines, TSLP has been most widely studied. Upon interaction to its receptor TSLPR, a ternary complex with the α-subunit of the IL-7 receptor (IL-7Rα) is formed. This interaction results in the activation of the JAK/STAT signaling pathway as well as the PI3K and NF-κB pathways (*24*, *25*). TSLP has been clinically validated as a therapeutic target as the monoclonal antibody Tezepelumab (Tezspire®) was recently approved by the FDA as an add on therapy for severe asthma. Anti-IL-33/IL-33R(ST2) antibodies (Etokimab, Astegolimab, Itepekimab) are also currently being investigated in the clinic. Thus far, clinical trials in asthma, AD and peanut allergy are promising (*26*). Given the apparent redundancy of the epithelial cytokines IL-33, TSLP and IL-25, it may prove that a combination therapy of all three mediators may be needed

for optimal results, and this approach has been shown in a house dust mite (HDM)-induced asthma model to be effective (*27*).

It should be noted that some of the biologics previously discussed, each target a specific event in type 2 immune responses (i.e., IL-4, IL-5 etc.), thereby omitting patients with a non-type 2 response, such as neutrophilic or non-eosinophilic driven asthma, or non-TH2 driven AD (*28*). For these patients, a broader immunomodulatory therapy targeting multiple immune pathways may be beneficial. Likewise, since these allergic inflammatory diseases are heterogenous (even in patients with TH2 dominated responses), a broad-spectrum immunomodulatory strategy may be beneficial. Furthermore, the safety or adverse effects of these biologics may be of concern since the risk of infection (particularly parasitic infections) is increased, as an important arm of the immune response is stifled.

Serine Protease Inhibitors (SERPINS)

Serine Protease inhibitors, or SERPINS, are a highly conserved superfamily of proteins that function to inhibit the proteolytic activity of plasma serine proteases such as elastase, thrombin, plasmin or trypsin. Serine proteases are involved in a diverse range of physiological processes such as coagulation, fibrinolysis, extracellular matrix remodeling, cell growth, immune responses and inflammation (*29*-*32*). The prototypical SERPIN is Alpha-1 Antitrypsin (AAT), an acute phase protein which consists of 394 amino acids, and is the most abundant SERPIN in the human plasma (*33*). AAT predominately inhibits elastase, proteinase 3, trypsin, and select kallikreins (*34*).

AAT is currently used to treat emphysema and COPD in individuals with AAT deficiency. In replacement therapy, AAT purified from donor serum (Zemaira, Prolastin, Glassia/Aralast) functions as a serine protease inhibitor and reduces proteolytic injury to lung tissues. AAT replacement therapy has been FDA approved for over 20 years and has an excellent safety record.

More than a decade ago, data began emerging, which showed AAT could prevent or ameliorate disease in different preclinical mouse models of human disease. Importantly, inflammation and auto-immunity were common threads between these different diseases, suggesting that AAT has anti-inflammatory and immune-modulating properties (*35*). Early work documented the anti-inflammatory properties of AAT in mouse models of Sepsis, where treatment improved survival during lethal endotoxemia, by reducing serum cytokine levels (*36, 37*). Similarly, a series of studies demonstrated that AAT treatment reduces inflammation and NFκB activation in lipopolysaccharide (LPS) induced macrophages and regulated CD14 and TLR4 expression (*38*). Studies in cystic fibrosis patients have shown similar anti-inflammatory effects using inhaled AAT (*39, 40*). The immunomodulatory effects of AAT are seen in modulation of neutrophil migration through antiprotease activity, as well as in eosinophils, which may explain frequent occurrences of asthma in patients with AAT deficiency (*32*). In macrophages and human PBMCs (peripheral blood mononuclear cell), AAT inhibits inflammatory cytokines such as TNF-α, IL-6, IL-8 and IL-1β (*41*). AAT also has effects on balancing the TH1/TH2 profile while enhancing T-regulatory cells (*42*). Therefore, due to its

safety, anti-inflammatory, and immune-modulatory effects, AAT is being explored for therapeutic use in a wide variety of diseases.

In terms of allergic inflammation, recent work has shown that loss of expression of a critical serine protease inhibitor, SPINK7 (Serine peptidase inhibitor, kazal type) is associated with uncontrolled proteolytic activity that causes damage to the epithelial barrier, excessive cytokine release and pro-inflammatory effects (*43*). Furthermore, loss of SPINK7 is associated with increased urokinase plasminogen-type activator (uPA) activity and uPA receptor mediated activation of eosinophils. Treatment with AAT, results in the reversal of these responses, assumingly by balancing the overwhelming proteolytic activity on the epithelium (*6*). In an animal model of Ovalbumin-induced eosinophilic esophagitis, AAT treatment was found to alleviate the pathological effects of EoE (decreased esophageal eosinophilia and inhibited the eosinophilic chemotactic cytokine, CCL24) (*44*).

Together, these studies highlight the significant therapeutic potential of AAT, aside from its current use as a replacement therapy in AAT-deficient patients. As outlined in the next section, we hypothesized the anti-inflammatory and immune-modulating effects of AAT, arise from a small segment of the protein, in which we've developed as an anti-inflammatory peptide drug. These peptides overcome many developmental challenges associated with AAT, such as sourcing, potency, route of administration and cost of manufacturing, while retaining the parent molecule's impeccable safety profile.

Development of Serpin-derived Peptide Therapeutics

As described, AAT has demonstrated significant anti-inflammatory and immune-modulatory effects (*41, 45-51*). Critically important, it was shown that oxidized and denatured AAT, which has no serine protease inhibitory properties, retains the anti-inflammatory effects associated with the protein (*48, 50*). This is relevant because it documents that AAT has anti-inflammatory effects that are separate from the protease inhibitory function of the folded, native protein. Furthermore, it suggests the anti-inflammatory effects arise from primary amino acid sequence elements, and not from conformational properties of the protein.

We have developed a synthetic anti-inflammatory peptide ("SP16") that contains the properties of the SERPIN core motif responsible for the anti-inflammatory and cyto-protective signaling, without inhibiting the plasma serine proteases (Patent application #8,975,224). SP16 is 17 amino acids in length, derived by excision of a 36-39 amino acid long portion of AAT to yield an active, short peptide fragment containing the unique motif. A single amino acid substitution in SP16 peptide was made to enhance anti-inflammatory signaling and plasma stability of the peptide.

SP16 has demonstrated significant proof of concept in several pre-clinical models of acute and chronic inflammatory diseases. For instance, SP16 increased survival in lethal endotoxemia models, as well as lethal *Klebsiella Pneumonia* models and has shown a reduction in pro-inflammatory cytokines in these models. In models of ischemia/reperfusion injury, SP16 treatment was associated with a significant reduction in infarct size and preservation of heart function (*52*). Proof of principle phase II trials in

STEMI (ST-elevation myocardial infarction) patients show reduced C-reactive protein (CRP), an inflammatory marker, and improvement in heart function after a year. In peripheral nerve injury and pain models, SP16 was shown to have both anti-inflammatory and analgesic effects, as well as pro-survival effects (*53*). Thus far, Phase I safety studies have shown no adverse effects of the drug.

Mechanism of Action: Targeting Low-Density Lipoprotein-Receptor Related Protein-1

SERPIN tertiary structure consists of 7-9 α-helices and 3 β-sheets, with a highly conserved core structure and flexible reactive center loop (RCL) containing an enzyme cleavage site (P1-P1') located at the c-terminal end of the protein (*54*). The RCL contains the protease recognition site that acts as bait for the target protease in a highly conserved mechanism. As the target protease binds to the conserved sequence on the RCL, the protease cleaves the P1-P1' bond, locking in the protease to the SEPRIN, but unhinging the SERPIN that undergoes a dramatic conformational change (*30*). As the SERPIN-enzyme complex (SEC) undergoes this structural rearrangement, a unique short peptide motif (5-11 amino acids) is exposed on the SERPIN that targets a cell receptor (previously known as the SEC receptor) responsible for the clearance and lysosomal degradation of the complex (*55-57*). This process is conserved across the entire spectrum of SERPINs, such as AAT (*58-60*). The SEC receptor, now identified as Low-Density Lipoprotein Receptor-Related Protein-1 (LRP-1) was found to mediate, not only the

endocytosis and clearance of the SEC's, but also physiological functions of the SERPINS such as the neutrophil chemotaxis effects of AAT (*61*).

LRP-1 functions both as an endocytic and cell signal transduction receptor and has several ligands that induce specific cell signaling cascades that can contribute to cell survival and anti-inflammatory mechanisms (*55, 58, 60, 62*). LRP-1 is ubiquitously expressed on many different organs (abundantly in brain, lung, heart and immune cells). Because of these unique capabilities and wide expression on both tissues and immune cells, it plays a critical role in regulating inflammation, cellular metabolism, and maintaining homeostasis. For instance, LRP1 regulates inflammatory signaling pathways such as the NFκB pathways that contributes to TH2 mediated responses (*63*, *64*). Targeting LRP-1 has also been show to regulate immune cell responses through the JAK-STAT pathway (*63, 65*). LRP-1 was shown as an important mediator in the conversion of pro-inflammatory (M1) macrophages to the anti-inflammatory (M2) macrophage phenotype, regulating the cytokine output, and contributing to effective migration and phagocytosis (*58, 65, 66*). In neutrophils, LRP-1-dependent mechanisms lead to enhanced cell adhesion, chemotaxis, and antibacterial effects of these cells, thereby resisting immunosuppression (*60*). During acute infection or injury, LRP-1 also promotes inflammatory resolution through scavenging PAMPS (pathogen associated molecular patterns) and DAMPS (damage associated molecular patterns) from dying or injured tissue to prevent the tissue injury cycle (*60*).

In terms of cell survival and regeneration, it has been shown that LRP-1 agonists, including SP16, are capable of promoting axonal growth and repair in neurons after

injury (*53, 67, 68*). Treatment of neurons with SP16 and other LRP1 agonists induces Akt and ERK activation and in Schwann cells, LRP1 activates PI3K/Akt mediated signaling to induce survival following peripheral nerve injury (*53, 68, 69*). The effects of LRP1 agonists on cell survival and preservation of repair mechanisms following cell injury provides a unique and important mechanism in initiation of inflammatory resolution and tissue healing versus other anti-inflammatory drugs.

Therefore, SP16 acts as an LRP-1 agonist to exert its anti-inflammatory and cyto-protective effects, resulting in a rebalancing of immune responses, including cytokine output, and inhibition of key inflammatory pathways such as NFκB. Through LRP-1 dependent mechanisms, SP16 protects cells from inflammatory injury and aids in tissue repair. An overview of the mechanism of action of SP16 is outlined in Figure 1.

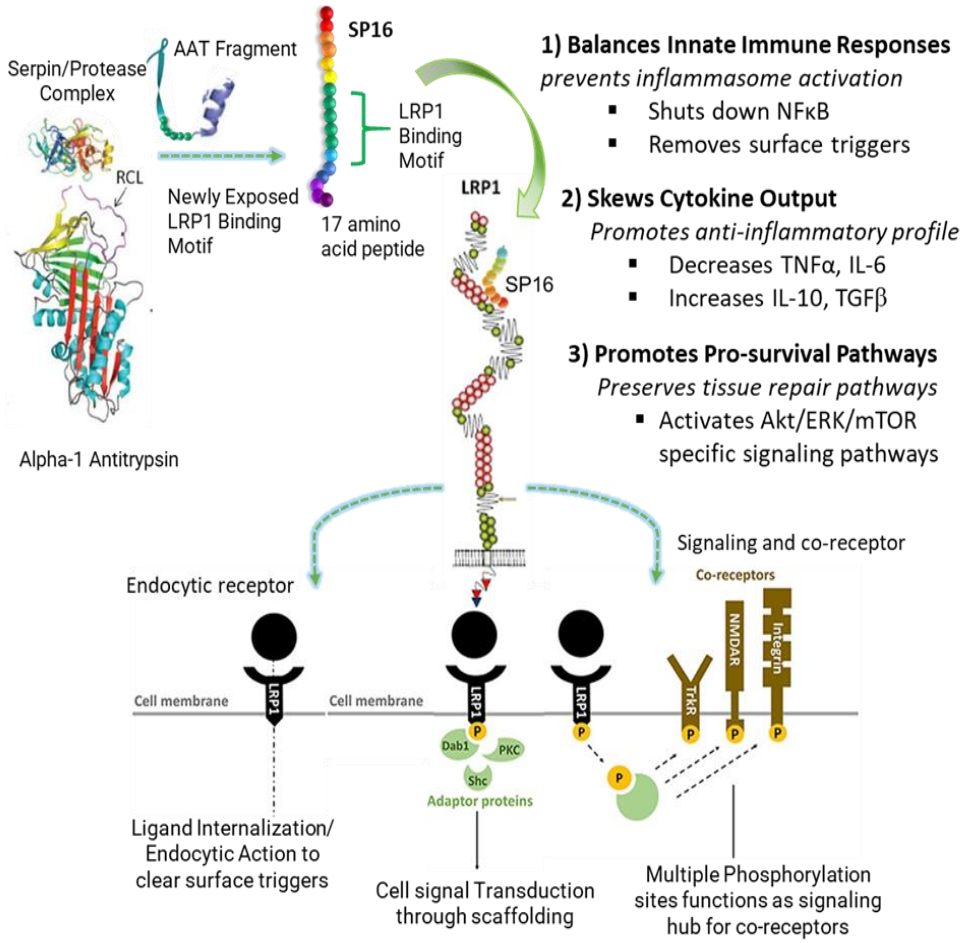


Figure 1. Mechanism of Action of SP16
SP16 is a derivative of the Serpin, Alpha-1 Antitrypsin (AAT). When AAT interacts with its target protease, a conformational change occurs that exposes a short motif that targets LRP1. From this fragment, we excised and modified a short (17AA) peptide (SP16) that retains the anti-inflammatory properties of AAT without the protease inhibitor function. SP16 binds to LRP-1 and activates specific LRP-1 mediated functions and cell signaling cascades that contribute to rebalancing immune responses in order to regain homeostasis and protect cells from injury.

Recently, a novel role for LRP-1 in modulating allergic inflammatory responses was identified. Allergic inflammation involves extensive communication between the epithelium, antigen presenting cells (APCs) and adaptive immune responses. In an animal model of house dust mite (HDM) induced allergic airway disease, deletion of LRP-1 on the dendritic cells causes increased antigen uptake and presentation that leads to

enhanced allergic sensitization, TH2 immune responses and eosinophilic airway inflammation (*70*). Therefore, LRP-1 is a negative regulator of adaptive immune responses mediated by dendritic cells in allergic airway inflammatory disease. Epithelial barrier function is an important innate immune response that is compromised in allergic inflammatory diseases. Proteases, such as the very potent allergen, Der p 1 from HDMs (a cysteine protease), and serine proteases such as kallikrein 5 can lead to direct damage to the epithelial barrier and excessive pro-inflammatory responses (*43*, *44*, *71*). An imbalance of proteases and protease-inhibitor homeostasis is thought to contribute to this barrier breakdown. LRP-1 is known to regulate numerous proteases/protease inhibitors through direct binding or through interaction of secondary mediators (*72*). While it is currently unknown, LRP-1 may play a direct role in protecting epithelial barrier function through mediating protease homeostasis.

We have now designed a series of several SP16-derivative analogs modeled off of the putative LRP-1 binding site, and hypothesize that LRP-1 can serve as a therapeutic target in a variety of inflammatory diseases, including allergic inflammatory disease, to rebalance the inflammatory response and protect tissues against damage. This work seeks to further characterize these LRP-1 agonist drugs and validate LRP-1 as a viable therapeutic target in allergic inflammatory diseases.

CHAPTER FOUR: MATERIALS AND METHODS

Animals. Female BALB/c and (6-8 weeks old) were purchased from Envigo. All animal experiments were approved by the Institutional Animal Care and Use Committee at George Mason University, Fairfax, VA. All mice were housed with a 12hrs:12hrs light: dark cycle with ad libitum access to food and water.

Reagents. SP16 peptide, biotinylated SP16, SP34 (scrambled peptide) and biotinylated SP34 were synthesized by CPC Scientific Inc. (Sunnyvale, CA) with purity >90% as verified by high-performance liquid chromatography and mass spectrometry. Analog 7G was provided by George Mason University (Manassas, VA). Serpin-derived analogs (A1-1 through A3-16 and biotinylated analogs) were synthesized by Serpin Pharma (Manassas, VA). Dexamethasone was purchased from Sigma Aldrich (St. Louis, MO). *Escherichia coli* 0111: B4 lipopolysaccharide (LPS) (Ultrapure) and poly(deoxyinosinic-deoxycytidylic acid) (poly I:C) were purchased from InvivoGen, (San Diego, CA). Recombinant human LRP-1 cluster II or cluster IV Fc chimera was purchased from R&D (Minneapolis, MN).

Cell Culture. THP1-XBlue™-MD2-CD14 Cells (InvivoGen, San Diego, CA) were maintained in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% heat inactivated FBS, 1% penicillin-streptomycin, 100 μg/ml Normocin^{TM}, 200 μg/ml, ZeocinTM, and 250 μg/ml of G418. THP1-Blue™ NF-κB cells

(Invivogen) were maintained in RPMI 1640, 2 mM L-glutamine, 25 mM HEPES, 10% heat-inactivated fetal bovine serum, 100 µg/ml Normocin™, Pen-Strep (100 U/ml-100 µg/ml) and 10 µg/ml of blasticidin, added to every other passage. The Immortalized Microglial (IMG) cell line (Kerafast Inc., Boston, MA) were isolated from the brains of adult mice and were grown in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin. The immortalized human keratinocyte cell line, HaCaT (AddexBio, San Diego, CA), were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin. Cell lines were maintained in 5% CO2 at 37°C and in accordance with the distributor's guidelines.

NFκB Reporter Screening Assays. THP1-XBlue™-MD2-CD14 stably express an NF-kB- and AP-1-inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene and CD14 and MD2. THP1-Blue™ NF-κB cells are a result of stable integration of an NFκB inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene driven by an IFN-β minimal promoter fused to five copies of the NF-κB consensus transcriptional response element and three copies of the c-Rel binding site. The cells were seeded at a density of 1 x 10^5 cells/well in a 96-well cell culture plate. The cells were treated with peptides (or respective vehicle controls) at indicated concentrations for 30 minutes prior to the addition of LPS (5 ng/ml). After an 18–24-hour incubation at 37°C, 20 µl of supernatant was transferred to 180 µl of QUANTI-Blue™ SEAP detection medium.

NFκB inducible SEAP levels were detected by measuring the absorbance using a microplate reader (The CLARIOstar® Plus, BMG LABTECH). In experiments blocking LRP1, cells were pre-incubated with the competitive antagonist of LRP1, GST-RAP (250 nM – 500 nM) (Enzo Life Sciences, Farmingdale, NY) for 15 min prior to the addition of the peptides.

Cytokine Inhibition Screening Assay. In assays determining the effective inhibitory concentrations of the analog peptides, Immortalized Microglial (IMG) cells were seeded at a density of 1 x 10^5 cells/well in a 96-well cell culture plate. The cells were treated with indicated concentration of peptide for 30 minutes prior to the addition of LPS (5 ng/ml) (InvivoGen). After an 18-24-hour incubation at 37°C, the concentration of cytokines TNF-alpha and IL-6 in the supernatant were analyzed via ELISA (Thermo Fisher Scientific). ELISAs were run per manufacturer's protocol.

Human Thymic Stromal Lymphopoietin (TSLP) Assay. Human keratinocytes (HaCaT cells) were seeded (50,000 cells per well) onto a 96-well cell culture treated plate (Corning™) and grown to confluency. Peptides (at concentrations indicated) were incubated with the cells for 30 minutes before the addition of poly I:C at a concentration of 50µg/ml. Cell supernatant was collected after a 24-hour incubation. TSLP (Thermo Fisher Scientific) in the supernatant was measured via ELISA per manufacturer's instructions.

Cell Viability. To assess drug toxicity in the cells or to determine viability following poly I:C treatment in HaCat cells, the CellTiter-Glo luminescent cell viability assay was used according to the manufacturer's instructions (Promega, Madison, WI). Briefly, cells were plated in triplicate onto an opaque 96-well plates. After the given treatment period, cells were then incubated with CellTiter-Glo reagent, and luminescence was measured using a microplate reader (The CLARIOstar® Plus, BMG LABTECH).

LRP1 Binding Assays: Human rhLRP-cluster II/Fc or Cluster IV/Fc Chimera was immobilized at 1µg/ml (100µL) or 100 ng/well in a coating buffer (0.2M BupH, pH 9.4, BupH Carbonate-Bicarbonate Buffer) overnight at 4°C in an ELISA plate (Nunc™ MaxiSorp™ flat-bottom 96 well plates). After thorough washing and blocking, the bound LRP-1 was exposed to increasing concentrations of biotinylated peptides (SP16, scrambled control, or analogs). Binding was visualized using a streptavidin HRP (Jackson Immuno Research, West Grove, PA) and substrate. Absorbance at 450nm was measured using a microplate reader.

Pharmacokinetic Studies. BALB/c mice (6-12 weeks) were administered peptide drugs at doses of either 50µg/mouse (2.5 mg/kg), 100 µg/mouse (5 mg/kg) or 200 µg/mouse (10 mg/kg) by bolus intravenous injection (IV), intraperitoneal injection (IP), or subcutaneous injection (SC). At baseline (before drug) and 15, 30-, 60-, 120- and 240-minutes post dosing, blood was sampled from the mice (n= 2 mice per time-point). For the studies assessing PK parameters following oral gavage (OG) relative to IV or SC

administration, blood was obtained at baseline (before drug), 30 minutes and 75 minutes post drug. Whole blood samples were collected in microtainer tubes (K_2EDTA), and plasma was obtained following centrifugation. Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific) was added to each plasma sample and plasma was stored at -80ºC until analysis. To obtain the plasma concentrations of drug, the plasma samples were diluted with an equal volume of 0.2 M Zinc Sulfate and acetonitrile (containing the internal standard) was added to the sample. The samples were centrifuged for 5 minutes at 13,000 rpm and supernatant collected for analysis by LCMSMS using a Sciex API 3200 mass spectrometer with an Agilent 1100 LC front end. The plasma concentrations of the peptide drugs were determined by comparing the area under curve for the sample versus a standard curve, using Sciex Multiquant software. For determination of bioavailability, the area under the curve was calculated as F = AUC for X route of administration ÷ AUC for IV administration

LPS Challenge Model. BALB/c mice (female 6-8 weeks) were administered the peptide drugs (SP16, 7G, A1-15, A2-5 or A3-10), vehicle control (sterile water, or 5% dextrose in water), or dexamethasone (1-10 mg/kg) as a positive control by intraperitoneal or subcutaneous injection. Thirty-minutes to two-hours later, LPS (*E. coli* 0111: B4) (Inviogen, San Diego, CA) at a dose of 1 mg/kg was administered by intraperitoneal injection. At 90 minutes following LPS injection, mice were euthanized and blood was collected via cardiac puncture. Blood was processed for plasma and protease inhibitor cocktail was added to the samples. TNF-alpha was measured in the plasma by ELISA

(Thermo Fisher Scientific) per manufacturer's instruction. An initial study assessing the cytokine profile following LPS challenge used the LEGENDplex™ Mouse Inflammation panel, which uses fluorescence-encoded beads and included IL-1α, IL1β, IL-6, IL-10, IL-12p70, IL-17A, IL-23, IL-27, CCL2(MCP-1), IFN-β, IFN-γ, TNF-α, and GM-CSF. The LEGENDplex™ kit was used per manufacturer's instruction and samples were run on the Attune NxT flow cytometer (Thermo Fisher Scientific).

Ovalbumin (OVA-) Induced Allergic Asthma Model. To induce allergic responses in mice, BALB/c mice were sensitized to 100 µg of ovalbumin (Sigma-Aldrich, St. Louis, MO) with 1 mg Alum (Alhydrogel adjuvant® 2%, Invivogen) by a series of two intraperitoneal injections one to two weeks apart. Seven to fourteen days later, during the challenge phase, while lightly anesthetized with isoflurane, the mice were intranasally instilled with 100 µg OVA for a total of at least 4 doses daily or spaced 2 to 3 days apart. The peptide drugs or dexamethasone was given either subcutaneously or intraperitoneally either daily or on the days of the challenge, depending on the study. Peptide drugs were administered at doses between 50µg and 100µg. The mice were euthanized the day following the last challenge and serum, bronchoalveolar lavage fluid (BALF), and lungs were collected for analysis.

Detection of Cytokines in Serum, BALF or Lung Tissue. For analyses of levels of cytokines in BALF, the lung was perfused through the trachea with 1 ml of PBS containing 5 mM of EDTA, repeated three times for 3 total ml of fluid. Lavage fluids

were centrifuged, and the supernatants were collected and concentrated using Amicon 2ml 3K filters (Millipore Sigma, Burlington, MA). Serum was collected one day prior to the end of the study by mandibular bleed and processed as previously described for the LPS challenge. The left and right lung lobes were snap-frozen in dry ice and stored in -80ºC before being weighed and processed. Fifty milligrams of tissue from the lung lobes were homogenized using T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific) and 1.4 mm ceramic beads with the Omni Bead Ruptor system (Omni International, Kennesaw GA). Samples were centrifuged and supernatants were collected. Protein levels in the lung were quantified using the Bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific). In some experiments, the levels of IL-4, IL-5, and IL-13 and TSLP (Thermo Fisher Scientific) in the serum, lung and BALF and serum levels of OVA-specific IgE were measured via ELISA kits (Caymen Chemical, Ann Arbor, Michigan) according to the manufacturer's instructions. In other experiments, the levels of cytokines in the serum, BALF or lungs were measured via the LEGENDplex™ Mouse TH1/TH2 (or TH2 alone) kit, which uses fluorescence-encoded beads to detect IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-17A, IL-17F, IL-22, IFN-γ and TNF-α.

Flow Cytometry Analysis of the Immune Cells in BALF. Flow cytometry analysis of the BALF was performed as reported before (*73*) with slight modifications. Cell infiltrate in the BALF was collected and total WBC count was determined by hemacytometer and trypan blue. For surface staining, cells were incubated with fluorescein-labeled monoclonal antibodies for 30 min at 4°C after blockade of the Fc receptor, then washed

twice and analyzed via the Attune NxT flow cytometer and FlowJo software (Tree Star, Ashland, OR). Antibodies, Alexa Fluor® 488 Rat Anti-Mouse Siglec-F was purchased from BD Biosciences (Franklin Lakes, New Jersey). Antibodies, 7-AAD Viability Staining Solution, MHCII-APC/Cy7, BV421™ anti-mouse CD11c, Alexa Fluor® 647 anti-mouse Ly-6G, Brilliant Violet 711™ anti-mouse/human CD11b were purchased from BioLegend (San Diego, CA, USA). CountBright Absolute Counting Beads (Thermo Fisher Scientific) were used to determine absolute cell numbers of each population. The gating strategy utilized to determine the immune cell populations can be found in the appendix (Figure A1). The equation used to determine the absolute cell population is as follows:

Equation 1. Absolute Cell Count

$$Absolute\ count\ \left(\frac{Cells}{\mu L}\right) =$$

$$\frac{(cell\ \ count\ \ x\ counting\ \ \ beads\ \ volu\,me)}{(Counting\ \ \ beads\ \ count\ \ x\ Cell\ \ volume} \ x\ counting\ beads\ concentration\ (\frac{beads}{\mu L})$$

MC903 Induced Atopic Dermatitis Model. To induce AD like inflammation, 1nmol MC903 (calcipotriol) (Tocris, Minneapolis, MN) in 100% ethanol was applied topically to each mouse ear (10µl per dorsal and ventral side of the ear for a total volume of 20 µl per ear; 1nmol MC903 in 20µl) under light anesthesia (isoflurane 1-3%). The MC903 treatment regimen was as follows: 5 days treatment, 2 days interruption, 5 days treatment, 2 days interruption, and then 1 final day of treatment. Control only animals were treated in parallel with ethanol only (no MC903). The peptide drug treatments or corresponding vehicle control started after 3 days of MC903 treatments and were applied topically to the

ears (25µl per ventral and dorsal side; 50µl per ear) in 70% ethanol (vehicle), at least one hour following MC903 treatments, following the same regimen as MC903 for the remainder of the study. The doses of the peptide drugs ranged from 250µg (for analogs) to 500µg (for SP16) per ear. Vehicle control animals received the same volume of 70% ethanol per ear. Plasma was collected from the animals on day 9 and TSLP was measured via ELISA. Weights were monitored throughout the study. Pictures of the ears were obtained on day 15 of the study. Following the last day of treatments, the animals were euthanized, blood was collected and both ears were snap-frozen in dry ice and stored in -80ºC before being processed. The level of IgE was measured in the serum via IgE ELISA kit (Thermo Fisher Scientific).

Ear Thickness and Clinical Skin Severity Score. Ear thickness was measured (in millimeters) in the same region of the ears by digital calipers and averaged for both ears. Dermatitis severity was scored based on a scoring system of 0-4 for 4 parameters (not to exceed a score of 16) as follows: erythema/hemorrhage (0–4), scale/dryness (0–4), edema (0–4), and excoriation/erosion (0–4). The scores were classified as 0 (none), 1 (mild), 2 (moderate), 3 (severe), or 4 (very severe). Ear thickness and dermatitis scores were assessed on study days 0, 3, 7, 9, 11 and 14. Pictures of the ears were obtained on day 15 of the study.

Detection of Cytokines in Ears. The ears were homogenized using T-PER™ Tissue Protein Extraction Reagent (Thermo Fisher Scientific) and 1.4 mm ceramic beads with

the Omni Bead Ruptor system (Omni International, Kennesaw GA). Protein levels in the ear were quantified using the BCA kit (Thermo Fisher Scientific). Cytokines in the ear lysate were analyzed using the LEGENDplex^{TM} Mouse TH2 panel (Biolegend) that allows for simultaneous quantification of IL-5, IL-13, IL-6, IL-10, TNF-α, and IL-4, typically released by TH2 cells.

Statistical Analysis. All acquired data were statistically analyzed with the help of GraphPad Prism software (version 9.3.1, GraphPad Software Inc., La Jolla, CA). Statistical analysis was calculated using an unpaired, two-tailed student t-test, for single comparisons, or one-way analysis of variance (ANOVA) for multiple comparisons between groups with post-hoc analysis (Dunnett's test). A comparison was considered significant, if probability values were lower than 0.05. In certain data sets, after estimation of Gaussian distribution with Shapiro-Wilk normality test, the non-parametric Kruskal-Wallis test was utilized. For all analysis, the level of significance was indicated as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and **** $p < 0.0001$. Data are presented as mean ± standard error of the mean (SEM), or in cases in which there is potential deviation from Gaussian distribution, shown as median and interquartile range [IQR].

CHAPTER FIVE: RESULTS

Shortened SERPIN-derived peptides retain anti-inflammatory activity.

We have previously shown that the SERPIN-derived peptide, SP16, contains 17 amino acids from the C-terminal end of AAT that interacts with LRP1 to exert anti-inflammatory activity in ischemia-reperfusion injury as well as peripheral nerve injury (*52, 53*). However, based on previous reports only a small domain of a series of 5 amino acids is predominately responsible for the binding to LRP-1 (*55, 56*). To test whether the longer SERPIN-derived peptides could be shortened, while retaining its anti-inflammatory activity, we used three different SERPINS (AAT and two closely related SERPIN proteins) with shortened sequences around the core LRP-1 binding domain (the full sequences to remain undisclosed) (Table 1).

Table 1. Sequence Alignment of Serpin Derived Peptides

Peptide	Sequence
AAT C-36 Peptide	**PP**K***N*P***L*IE**S*LF**KV**PT*K
AAT Derived SP16	*K***N*P***L*IE**TK
AAT Derived, Short Core	*K***N*P***L*
AAT Derived, Short Core, Poly Rs	RRR*K***N*P***L*RRR
Serpin 2 Derived Short	*R***N*P**V*IP**TQ
Serpin 2 Derived Short, Poly R's	RRR*R***N*P**V*IRRR
Serpin 3 Derived, Short Core	*R***N*P****I
Serpin 3 Derived, Short,Poly R's	RRR*R***N*P****IRRR
LRP1 Binding site altered	*R***N*P****
LRP1 Binding site altered, Poly R's	RRR*R***N*P***RRR
SP16 Modified Core	Ac-*K***N*P****IE**TK-NH2
Scrambled Control Peptide	FPK**P*FNT**KIF*E*NIK

To measure anti-inflammatory activity, we used NF-κB reporter cells (THP1-XBlue-MD2-CD14 cells) treated with each peptide analog as well as SP16 (50 μg/ml) before being insulted with LPS (5 ng/ml) and incubated overnight. The NF-κB inducible Secreted Embryonic Alkaline Phosphatase (SEAP) was measured in the supernatant and read for absorbance.

We show in Figure 2, that the shortened peptides (short core peptides) lose activity, presumably due to the high degree of hydrophobicity of the core sequence causing instability of the peptides. When however, the shortened core peptides are stabilized with positively charged amino acids (ex. arginine) flanked on either side of varying lengths of the core motif, the peptides increase the ability to reduce NF-κB activation compared to SP16. If, however, amino acids in the core LRP-1 binding site are truncated, the peptide loses activity and the activity cannot be rescued by flanking these peptides with poly-arginine amino acids. This indicates that, while SERPINs contain a highly conserved domain not known to be involved in LRP-1 binding, the moderately conserved LRP-1 binding domain is what is important for anti-inflammatory function.

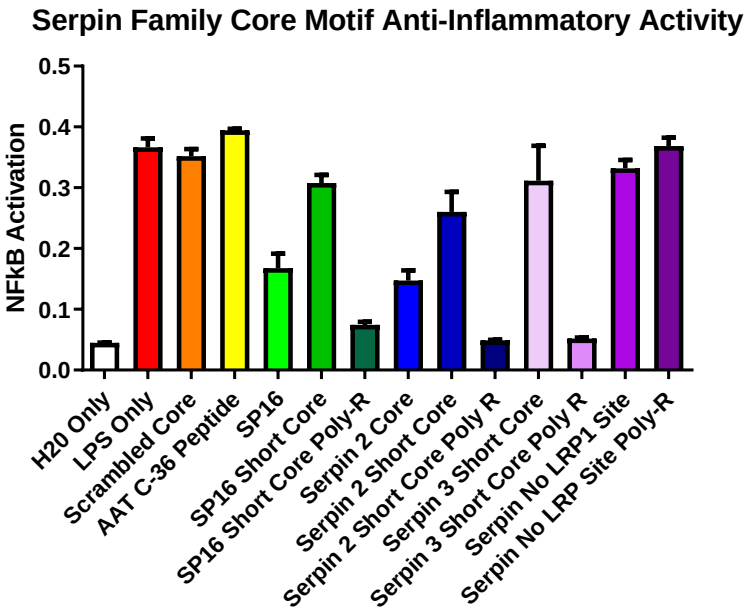


Figure 2. Serpin-derived peptides share a highly conserved anti-inflammatory sequence.
A) Using a systematic approach, a short anti-inflammatory sequence domain was identified in several human serpins (partial sequences shown). The shorter peptides retain the anti-inflammatory effect, but require short poly-arginine ends due to the hydrophobicity of the central domain. The shortest fragment truncates part of the sequence responsible for binding to LRP1 and this peptide loses activity that addition of poly-arginine ends cannot restore. SP16 remains the shortest AAT derived peptide able to bind LRP1 and retain anti-inflammatory activity in its natural state was chosen for further development. SP16 motif was further modified through replacement of an amino acid residue in the core binding site and end modifications to increase stability. This peptide (referred to as SP16) demonstrates increased potency.

Structure activity relationship of short-core SERPIN-derived peptides.

We continued exploring the structure activity relationship of the peptides by designing additional shortened peptides (proof of concept analogs) that contain the core LRP-1 binding motif and comparing the activity in the NF-κB reporter cells, using SP16 as a positive control. As shown in Figure 3, the SERPIN peptide analogs that retain the shortest LRP-1 binding sequence and a triple arginine as a flanker to improve solubility (such as 3C, 6F, and 7G) demonstrated improved activity in NF-κB inhibition when compared to SP16. However, the SERPIN peptide analogs flanked with a positively

charged tripeptide or with negatively charged tripeptides (such as 1A, 2B, 4D, 5E) demonstrated minimal NF-κB inhibition activity.

This data shows that a shortened peptide (e.g., 3C) can be made and that the shortened peptide is more effective in reducing NF-κB activation than the SERPIN peptides SP16 and SP22 (longer core sequence flanked on either side with triple arginine residues). Of these SERPIN-derived peptides, only 7G was cyclized (side chain cyclization with cysteine-cysteine disulfide (s-s) bond).

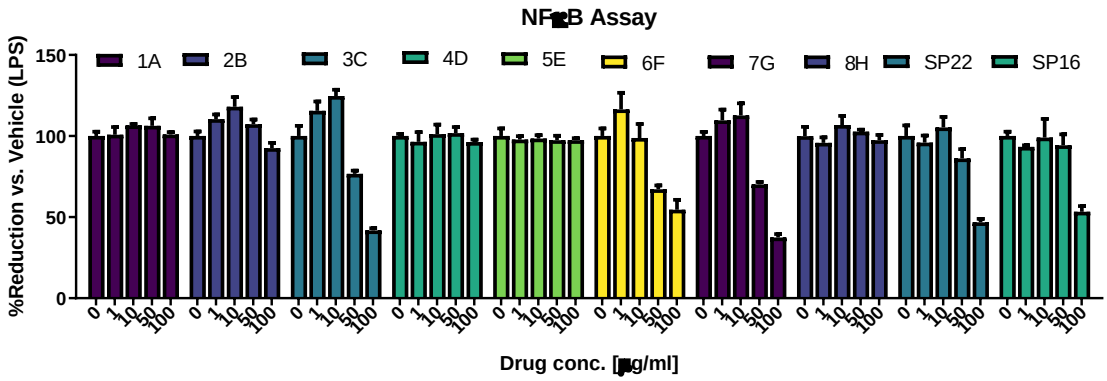


Figure 3. Structure-Activity Relationship of SERPIN-Derived peptides 1A-8H.
The activities in reducing NFκB activation for peptide analogs 1A-8H at various concentrations, 0, 1, 10, 50, and 100 μg/mL. Compared to the SERPIN peptide SP16, truncating the peptide while retaining the LRP1 binding site and adding arginine residues conferred improved activity in the NFκB reporter assay.

Anti-inflammatory activity of LRP-1 agonist peptide analogs (Series 1).

Structure activity relationships of the Serpin-derived peptide analogs revealed that shortened core peptides can be stabilized and that cyclization of the analogs maintains activity. We then designed several series of novel analogs modeled off of the binding of the interaction between SERPIN-enzyme complexes (SECs) and the LRP-1 receptor to replicate the LRP-1 agonist activity we have seen with SP16. Since the active core

sequence is made up of mainly hydrophobic amino acids, we tested variable sequences of positively charged amino acids to flank the core motif. We also tested whether any of the amino acids in the hydrophobic core could be replaced with a hydrophilic amino acid in order to improve solubility of the drugs. Analogs A1-A15 were screened *in vitro* for their ability to reduce TNFα (Figure 4A) and NFκB secretion (Figure 4B). The NFκB reporter cells or the IMG microglial cells were treated with each peptide analog at various concentrations, up to 100 μg/ml before being insulted with LPS (5 ng/ml) and incubated overnight. The percentage of reduction in NFκB activation or level of TNFα in the supernatant (pg/ml) vs. vehicle (LPS stimulated) cells is shown in the figures.

It was determined, based on the improved activity seen in A8 and A10 that modifications to the positively charged amino acids flanking the core motif could be made, and that certain hydrophobic amino acids in the core motif could be replaced with hydrophilic amino acids. This series of analogs also explored appropriate cyclization strategies based off of the SEC-LRP-1 interaction. The improved activity seen with A15 helped to identify an optimal ring structure.

The analogs were tested for any cell toxicity, NFκB reporter cells or IMG microglial cells were treated with either SP16 or peptide analog at concentrations of 100 μg/mL for 24 hours and then cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay. Typically, we do not observe any cytotoxic effects with SP16. Initial cell viability of the first set of analogs was comparable to SP16 (Figure 4C).

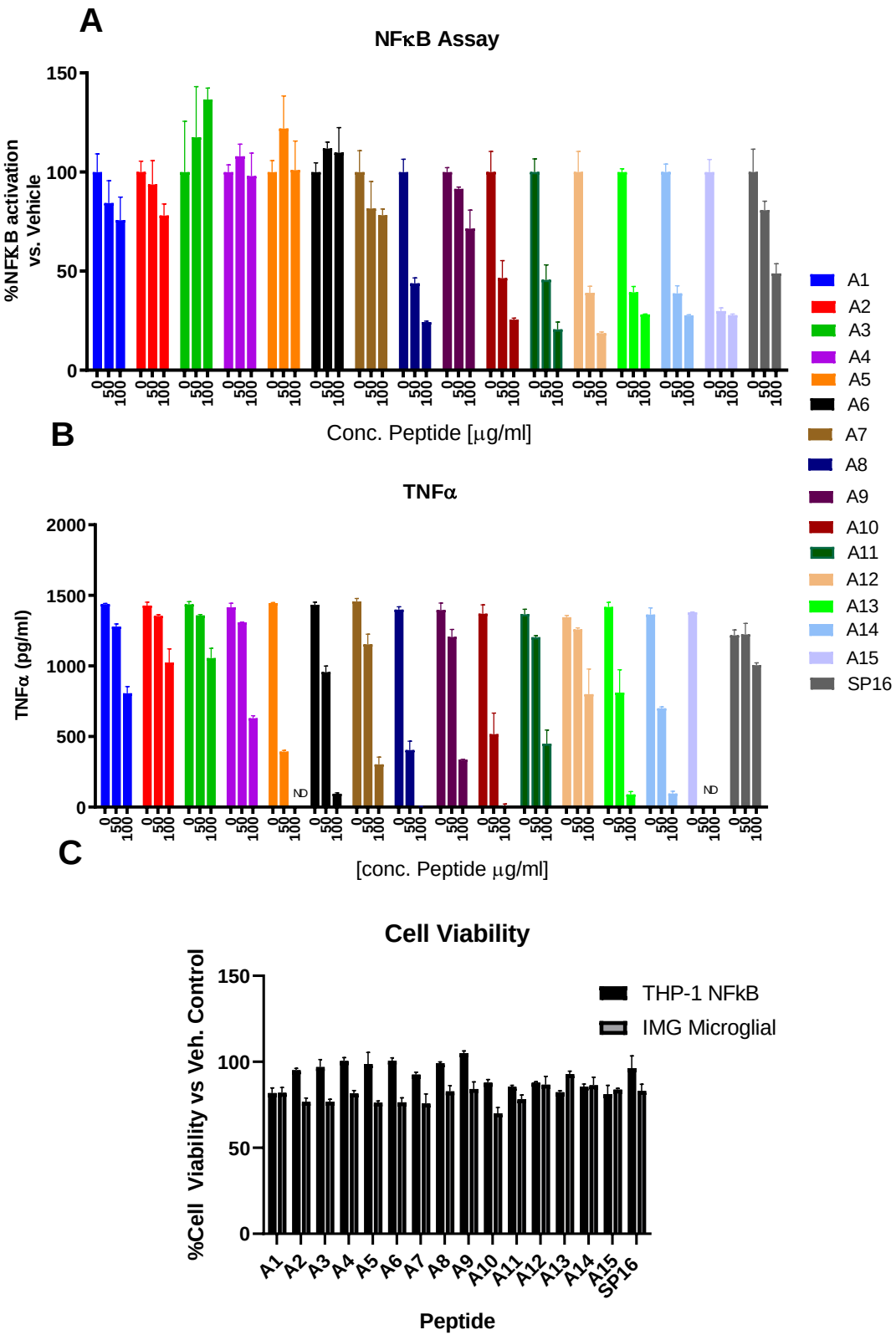
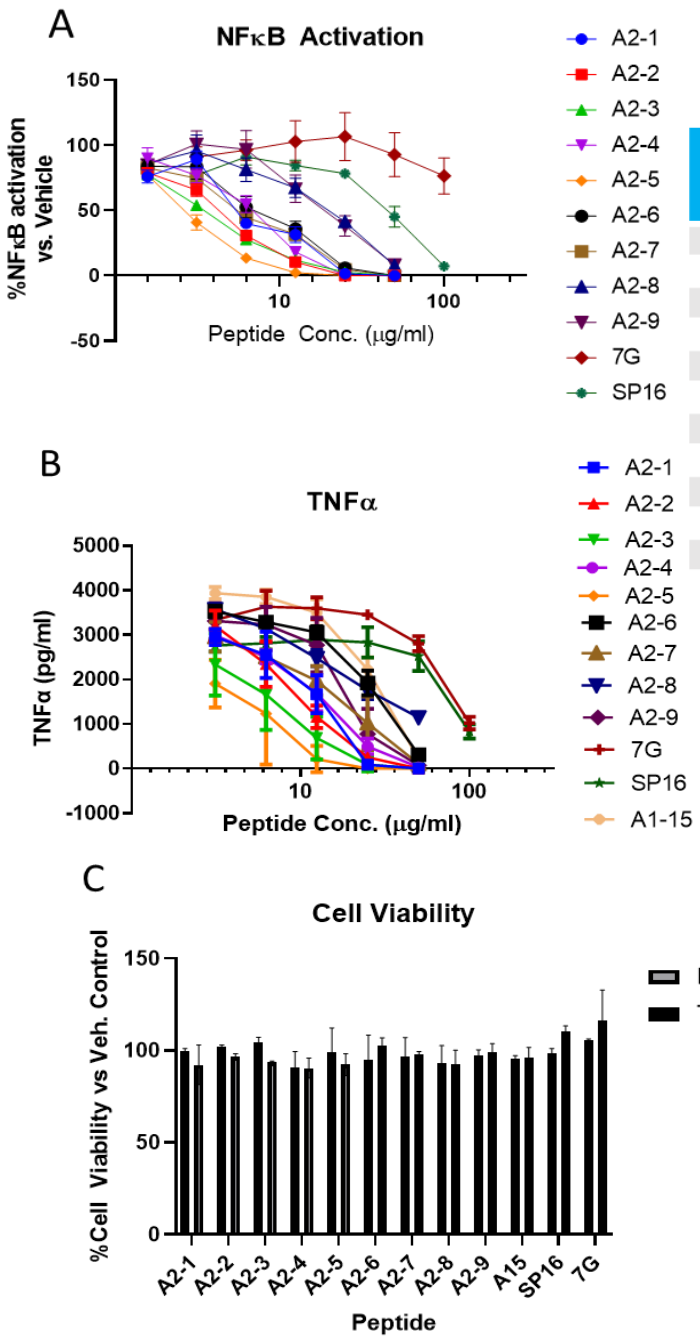

A
NFκB Assay
%NFKB activation vs. Vehicle
150
100
50
0
Conc. Peptide [μg/ml]
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
A11
A12
A13
A14
A15
SP16
B
TNFα
TNFα (pg/ml)
2000
1500
1000
500
0
ND
[conc. Peptide μg/ml]
C
Cell Viability
%Cell Viability vs Veh. Control
THP-1 NFkB
IMG Microglial
Peptide

Figure 4. Anti-inflammatory function and toxicity of A1-A15 SERPIN Analogs.
The peptide derivatives (50 or 100 μg/ml) were tested in the A) NFκB reporter assay in response to LPS (5 ng/ml) and screened for B) TNFα secretion in IMG microglial cells in response to LPS (*E.coli* 0111:B4) stimulation (100 ng/ml, 24 hours). In the NFκB reporter cells, the NFκB inducible Secreted Embryonic Alkaline Phosphatase (SEAP) was measured in the supernatant. TNFα secretion in the supernatant was measured via ELISA. C) THP-1 NFκB reporter cells and IMG microglial cells were treated with peptide analogs at 100μg/ml for 24 hours. Percent cell viability vs. the respective vehicle control is shown (mean ± SD of triplicates)

Screening for anti-inflammatory activity and safety of series 2 analogs reveals several potential lead drugs.

The next series of analogs (Series 2, A2-1 through A2-9) utilized the three ideal strategies identified in the first series screen for amino acid substitutions, ring structures and stabilizing flanking amino acids to explore whether the sequence could be shortened further. Additionally, we explored whether substituting some of the amino acids with D-configuration would improve LRP-1 binding, given that not all the amino acids in the sequence are facing in the same direction. These peptide analogs were screened for anti-inflammatory activity in the NFκB reporter assay (Figure 5A), as well as for TNFα release (Figure 5B) in the supernatant following LPS stimulation (5 ng/ml) for 24 hours. Dose response curves starting at 50μg/ml for the analogs was used to establish an IC50 for both NFκB activation inhibition and TNFα inhibition (shown for each assay). Compared to SP16, all of the series 2 analogs had increased inhibitory function in both NFκB THP-1 (monocytic) cells as well as the ability to inhibit TNFα in microglial cells, both following LPS stimulation. A2-5 displays the lowest IC50, with concentrations of 2.54 μg/ml and 7.12 μg/ml in the NFκB assay and TNFα assay, respectively. Comparatively speaking, SP16 has an IC50 for the NFκB activation assay in the range of 50 μg/ml, indicating that A2-5 has an increased potency of nearly 20 times.

In order to eliminate any potentially cytotoxic analogs, cell viability was assessed in the NFκB and IMG microglial cells at the highest concentration tested in each assay (50µg/ml for the analogs, and 100µg/ml for SP16 and 7G, as controls for non-toxic drugs). Figure 5C shows that none of the analogs display any cell toxicity. The effective concentration (EC) is less than 10µg/ml in both assays for the more potent analogs such as A2-3, A2-4 and A2-5, and no toxicity is seen at doses five times higher than effective concentrations, therefore, a large therapeutic window exists for the second series analogs, and the minimum toxic concentration (MTC) of the drug remains to be achieved. This series of analogs reveals amino acid substitutions and alternate configurations can increase the potency of the drugs, while maintaining non-toxic profiles.



Analog	NFκB IC50 (μg/ml)	TNFα IC50 (μg/ml)
A2-1	7.85	13.60
A2-2	5.17	8.85
A2-3	3.45	8.23
A2-4	7.41	13.90
A2-5	2.54	7.12
A2-6	9.54	32.79
A2-7	7.55	30.00
A2-8	30.57	16.23
A2-9	20.18	18.71
7G	NA	65.90
SP16	52.11	57.37
A1-15	NA	33.26

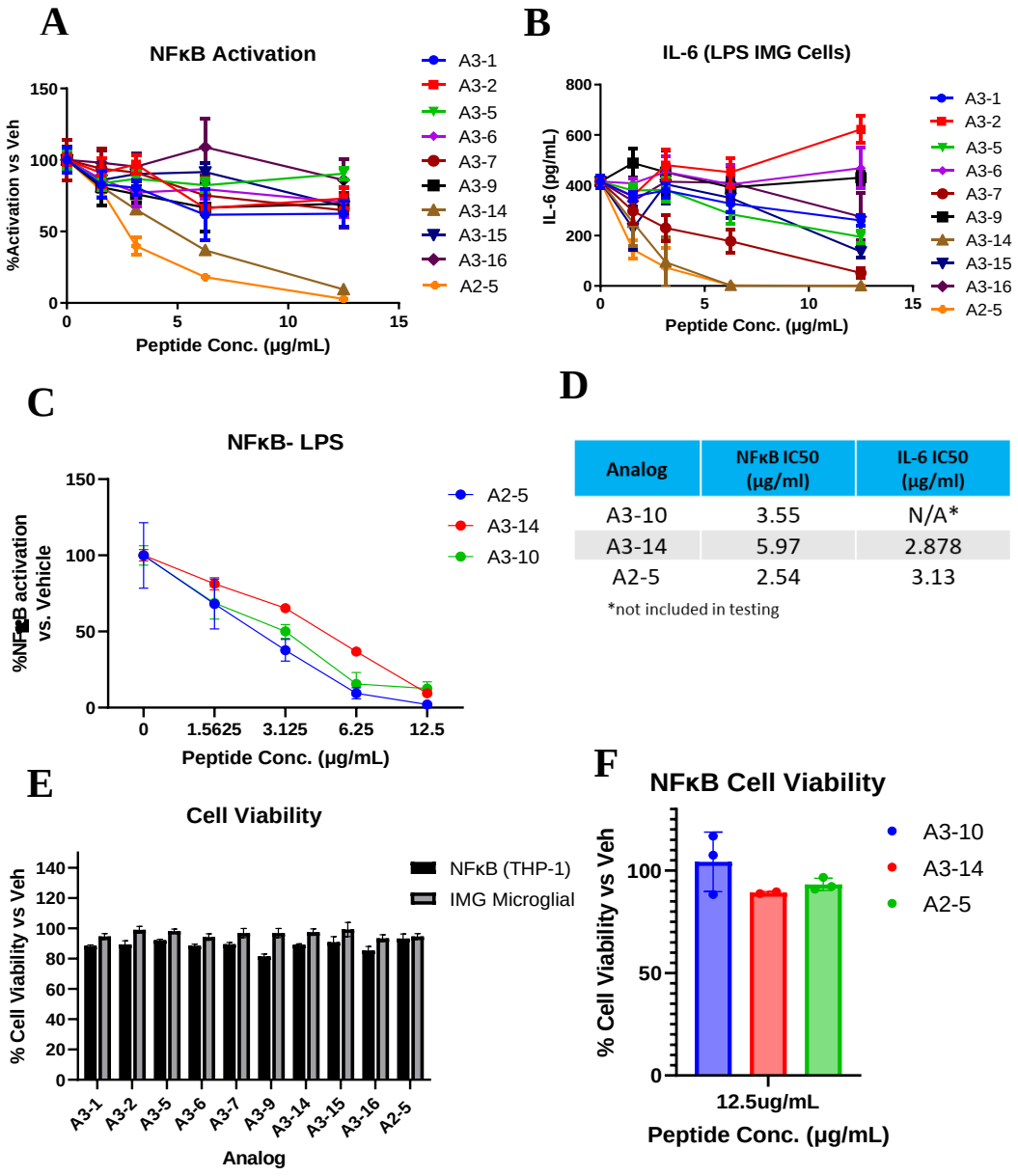
Figure 5. Inhibitory Concentrations and Cell Viability of Series 2 Analogs.
Analogs A2-1 through A2-9, as well as A1-15 from the initial screens were run at dose ranges up to 50 μg/ml in the A) NF-kB reporter cells and B) IMG cells following LPS activation (5ng/ml) for 24 hours. NFkB inducible SEAP and TNFα were measured in the supernatant. As controls, SP16 and 7G were tested starting at a higher range of 100μg/ml. The concentration of analog that results in 50% inhibition for each assay is shown (IC50 concentrations). C) cell

viability of the IMG cells and THP-1 NFkB reporter cells following 24 hours of treatment with the highest concentrations tested in the assay systems. Mean ± SD is shown for triplicate values.

Anti-inflammatory screen of additional LRP-1 agonist analogs reveals extensive modification the LRP-1 binding domain reduces activity (Series 3 analogs).

Based on the previous findings from the first two sets of analogs, additional modifications to the ring structure, as well as amino acid optimization, resulted in a third series of analogs (A3-1, A3-2, A3-5, A3-6, A3-7, A3-9, A3-10 A3-14, A3-15 and A3-16). Figure 6A and 6B shows the effects of peptide analogs A3-1, A3-2, A3-5, A3-6, A3-7, A3-9, A3-14, A3-15, and A3-16 on NFκB activation (in NFκB reporter cells) and IL-6 reduction (in IMG microglial cells) in response to LPS stimulation (5 ng/ml, 24 hours). Analog A3-10 was not included in the first set of experiments due to a synthesis issue. The peptide analogs were tested at concentrations between 1.56 µg/ml to 12.5 µg/ml (given the low IC50's found in the previous set of analogs). The analog with the greatest inhibitory effects from the second series (A2-5) was included for comparison. These sets of analogs were not as effective as the previous series of analogs with the exception of A3-14, which shows inhibitory effects in both the NFκB and IL-6 assay similar to the A2-5 analog. Because A3-10 was not included in the first set of screens, we tested it in comparison with the leading analogs, A2-5 and A3-14 in the NFκB reporter assay. Figure 6C and 6D shows that the A3-10 analog has similar activity and IC50 concentration when compared to the leading analogs and A3-14 has a slight advantage in IL-6 inhibition compared to A2-5. We also tested the cell cytotoxicity as we had done in the previous screens with the series 2 analogs, this time testing concentrations up to

12.5µg/ml. No significant cell cytotoxicity was observed for the analogs (Figure 6E, F). These data show that extensively modifying the LRP1 binding amino acid sequence results in reduced activity. The leading compounds that emerged from the 3 sets of analogs were A1-15, A2-5, A3-10 and A3-14.



Analog	NFκB IC50 (µg/ml)	IL-6 IC50 (µg/ml)
A3-10	3.55	N/A*
A3-14	5.97	2.878
A2-5	2.54	3.13

*not included in testing

Figure 6. Anti-inflammatory Activity and Toxicity of Series 3 Analogs.
Analogs A3-1, A3-2, A3-5, A3-6, A3-7, A3-9, A3-10 A3-14, A3-15 and A3-16 were tested at concentrations of 1.56 µg/ml to 12.5 µg/ml before LPS (5 ng/ml) stimulation of A) NFκB reporter cells (THP-1) or B) IMG microglial cells. SEAP inducible NFκB or IL-6 was measured in the supernatant and reported as either %activation vs. LPS treated vehicle control or pg/ml, respectively. C) Analogs A2-5, A3-14 or A3-10 were tested in the NFκB reporter cells as described in A. D) IC50 concentrations for NFκB and IL-6 inhibition were calculated for analogs A3-10, A3-14 and A2-5. E) Cell viability following analog treatment up to 12.5µg/ml, for 24hrs was tested in THP-1 NFκB reporter cells and IMG microglial cells. F) THP-1 NFκB reporter cell viability following treatment of analog A3-10, A3-14 and A2-5 at 12.5µg/ml were compared.

***In-vitro* LRP-1 binding assay to determine target engagement of the analogs.**

In order to test the analogs' ability to bind to LRP-1, we synthesized versions of the peptides with a biotin label and performed a direct ELISA based assay by immobilizing a fixed amount of recombinant LRP-1 to an amiable surface. We have previously used this assay as a tool to measure the affinity of SP16 to both cluster II and cluster IV of LRP-1. Recombinant LRP1 (Cluster II) bound to ELISA plate was exposed to increasing concentrations of biotinylated SP16 or scrambled control (SP34) before visualization using streptavidin HRP and substrate. SP16 was capable of binding to LRP-1 Cluster II and Cluster IV with approximately equal affinities (1.3μM and 1.0μM, respectively), while SP34 containing the amino acids found in SP16 in scrambled orientation did not bind to LRP1 Cluster II or Cluster IV (Figure 7A).

We also tested whether the placement of the biotin label had an impact on the affinity to LRP-1. Figure 7B shows that when the biotin label is placed on the C-terminal end of SP16, the ability of SP16 to engage LRP-1 is negatively impacted, with significantly less binding when compared to the biotin labeled SP16 on the N-terminus.

Next, we performed the assay using the biotin-labeled Analogs (A2-5, A3-10, A3-14, with 7G and SP16 as controls). As a control for background, in the event that the biotin-labeled analogs non-specifically bound to the surface, we included a non-LRP-1 coated control. As an additional control for a non-LRP-1 targeting peptide, biotin labeled SP34 was included. We show in Figure 7C that biotin-SP16 (BSP16) bound to LRP-1, with increased binding in a dose dependent manner. At the higher concentrations of B-SP16, there was a small amount of non-specific binding (as shown with the B-SP16

treated, non-LRP1 coated samples (B-SP16-Unc.). Likewise, the biotin-A2-5 (BA2-5) bound to LRP1 in a dose dependent fashion, increasing intensely at the highest concentration of 10 μg/ml, ultimately hitting the saturation threshold of the assay. As we saw with the biotin labeled SP16, there was also some non-specific binding of the BA2-5 analog at the highest concentration (BA2-5 unc.). The earlier generation analog biotin-7G also showed strong targeting of LRP-1, with similar background. Of the two third-generation analogs, Biotin A3-10 and Biotin A3-14 surprisingly showed little to no binding of LRP-1, with the biotin A3-14 analog showing strong non-specific background. The non-LRP-1 targeting negative control, biotin-SP34, showed neither LRP-1 binding nor any non-specific targeting (B-SP34 unc.), as seen in previous assays.

We showed that the placement of the biotin label can have a significant impact on binding to the target protein, an issue that has been widely recognized when studying the interactions of molecules (*74*). In order to test whether the biotin label may have a direct impact on the function of the peptides, we assessed the biotin-labeled analogs as well as the N- and C- terminal biotin SP16 peptides for their inhibitory function in the LPS-induced NFκB reporter assay. Placement of the biotin label on the N-terminal end of SP16 allowed LRP-1 binding. When the cells are treated with N-terminal biotin SP16, the NFκB inhibition concentration is similar to that seen with the unlabeled SP16 peptide (Figure 7D). On the contrary, when the cells are treated with the C-terminal biotin SP16 peptide that showed reduced LRP-1 targeting, NFκB inhibitory function is negated.

With similar logic, we next tested the biotin-labeled A2-5, A3-10 and A3-14 as well as the non-labeled versions of the analogs in the LPS-induced NFκB reporter cell

assay (Figure 7E). The analogs were tested at concentrations of 10µg/ml and 5µg/ml, which typically shows significant inhibitory effects in this assay. Biotin A2-5, which showed binding to LRP-1, displays activity in reducing NFκB induction, albeit to a lesser extent than its non-labeled A2-5 version, indicating that LRP-1 targeting is likely partially inhibited by the biotin label. As expected, biotin labeled A3-10, which showed no LRP1 targeting, also shows no activity in inhibiting LPS-induced NFκB activation, while the non-labeled A3-10 shows normal function. This suggests that the biotin label placement on the A3-10 analog interfered with the LRP-1 interaction and therefore any activity. Surprisingly though, the biotin labeled A3-14, which displays only minimal LRP-1 binding and a high degree of non-specific binding, had increased inhibitory activity in the functional assay as compared to the non-labeled A3-14. It is not clear whether this is due to non-LRP-1 dependent mechanisms or whether the particular properties of BA3-14 peptide are causing interference with the assay system, since a high degree of non-specific interactions were observed in the LRP-1 binding assay. It should be noted that of the A3 series peptides, A3-14 contained the highest degree of modifications to the LRP-1 binding motif. Due to potential off-target effects, no further development of A3-14 was continued. A2-5 and A3-10 on the other-hand, were still strong potential candidates and were further investigated for PK properties and in-vivo efficacy.

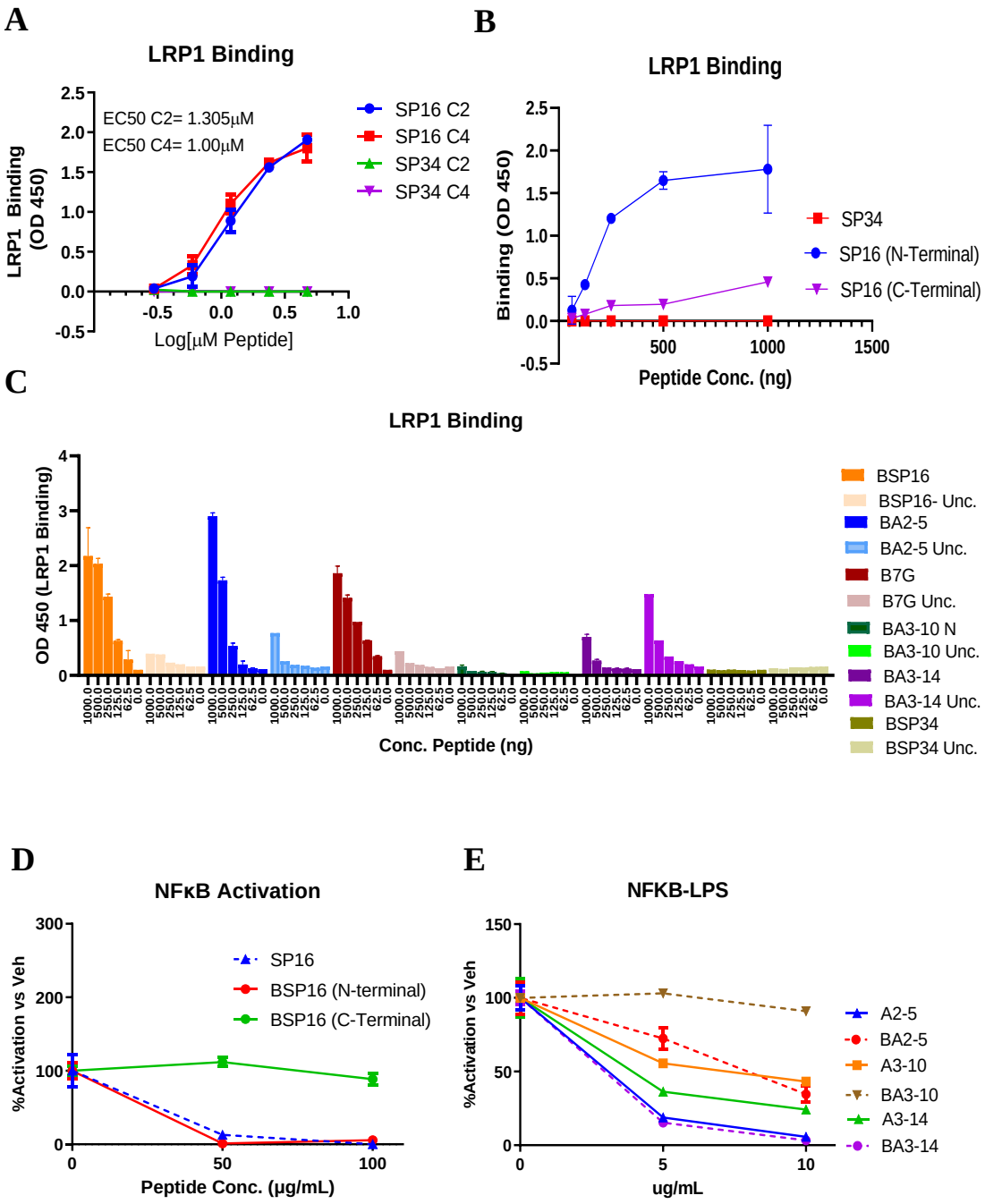


Figure 7. In-vitro assessment of Biotin-Analogs Binding to LRP-1.
A) Recombinant LRP-1 (Cluster II or cluster IV) bound to ELISA plate was exposed to increasing concentrations of biotinylated SP16 or scrambled control (SP34) before visualization using streptavidin HRP and substrate. **B)** SP16 biotinylated either on the N-terminal end or C-terminal end of the peptide was run in the same LRP-1 binding assay (Cluster II) with biotin SP34 as the negative control. **C)** Biotin labeled analogs SP16, A2-5, 7G, A3-10 and A3-14 were added at increasing concentrations to bound LRP-1, or uncoated ELISA plate (unc. for each peptide, as a control for background), Biotin-SP34 was run as a negative control that does not target LRP-1. **D)** The N or C-terminal biotinylated SP16 peptides or

unlabeled SP16 were tested for inhibition of LPS-induced (5ng/ml) NFκB activation as a measure of function. **E)** Analogs A2-5, A3-10 and A3-14 as well as the biotinylated counterparts were tested in the same NFκB assay for activity. The NFκB reporter assays were run as previously described with concentrations of analogs up to 10μg/ml.

LRP-1 specific dependency on in-vitro anti-inflammatory effects of the analogs.

We have previously shown that the cell signaling effects of SP16 in PC12 cells is mediated by LRP-1 (*53*). In these studies, SP16 induced strong p-AKT and p-ERK1/2 signaling that was blocked using Receptor-Associated Protein (RAP), an antagonist to all identified ligands that binds with high affinity (nM range) to LRP-1 (*75*). Binding of ligands occurs to regions of the receptors that are built from clusters of non-identical copies of the CR (complement-like repeat) domain, including CR4 and CR5 (*76*). Using a novel identification method of protein-protein interactions called protein painting, previously described (*77*), We identified binding sites located in the CR4 and CR5 domain of LRP-1 as primary binding sites of SP16 (Figure 8A).

We therefore sought to determine whether the anti-inflammatory effects we see in the NFκB reporter assay could be blocked using the RAP LRP-1 antagonist. RAP (250nM) added to the cells 15 minutes prior to the addition of SP16 or analogs A2-5 and A3-10 at concentrations shown. LPS induced NFκB activation was measured after 24 hours. Figure 8B shows that SP16 and analogs A2-5 and A3-10 decrease LPS induced NFκB in a dose dependent fashion as previously seen. Unexpectedly, however, RAP added into the assay results in significantly higher LPS induced NFκB activation compared to non-RAP treated conditions. Interestingly, SP16 and analogs dose-dependently inhibit this RAP induced response. We show in figure 8C, that using the

more potent stabilized form of RAP D3 domain (*75*) at a higher concentration, results in SP16's inhibitory function in the NFκB reporter cell assay being blocked, as expected.

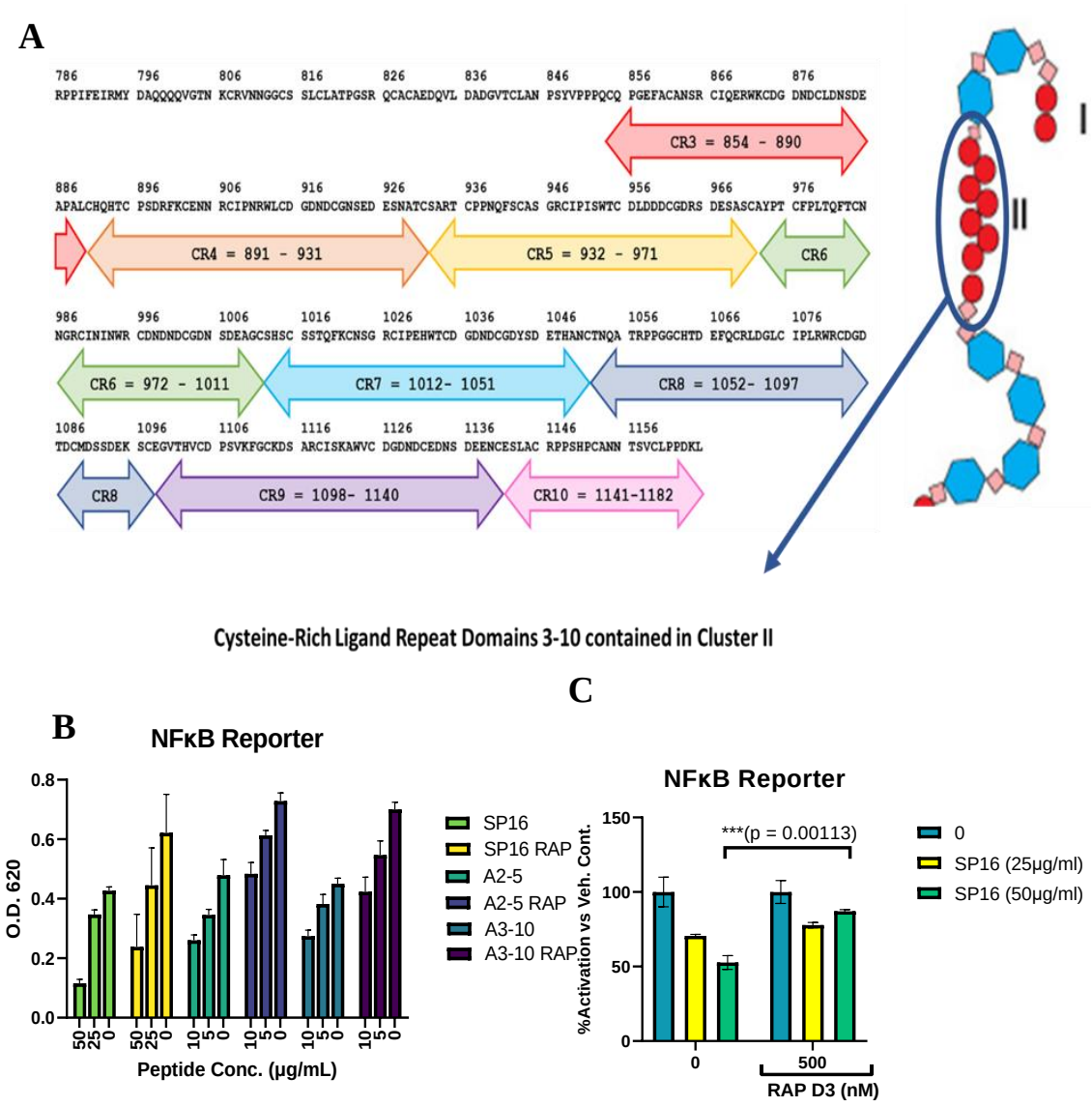


Figure 8. Inhibition of Anti-inflammatory Activity of Analogs using Receptor Antagonist Protein (RAP).
A) Sequence identity of cysteine-rich-ligand repeat domains contained in cluster II of LRP-1 containing binding sites for SP16. B) RAP (250nM) was added 15 minutes before SP16 (0, 25, 50µg/ml) or analogs A2-5 and A3-10 (0, 5, 10 µg/ml) to NFkB reporter cells. Cells were incubated with treatments for 30 minutes before LPS (5ng/ml, 24hrs) (O.D.

at 620nm is shown, mean ± SD shown). C) The same experiment described in B was performed with SP16 (0, 25, 50 µg/ml), with the RAP D3 stabilized protein at 500nM. *** $p \leq 0.0001$.

SP16 and analogs inhibit poly I:C induced TSLP in Keratinocytes

We next wanted to elucidate the effects of SP16 and analogs A2-5 and A3-10 in a model of skin inflammation. TSLP expression has been shown to be increased in AD lesions, playing a significant role in AD progression (*22*). Pathogen associated molecular patterns (PAMPs), including the TLR3 agonist poly I:C, and other surface triggers present at the epithelial barrier are recognized as potent inducers of TSLP. We therefore, utilized a model of poly I:C (50µg/ml) induced TSLP in keratinocytes (HaCat) cells to explore whether the LRP-1 agonist peptides had TSLP inhibitory effects as an *in-vitro* AD-like inflammatory model. Figure 9A shows that SP16, A2-5 and A3-10 significantly inhibit poly I:C induced TSLP at concentrations of 50 and 100µg/ml. Further, it was noted that poly I:C (50µg/ml) significantly induced cell death in the HaCat cells. The cell viability after SP16 treatment at 100µg/ml, was significantly increased compared to non-peptide treated cells (vehicle only) (Figure 9B). The analogs A2-5 and A3-10, however, were not capable of rescuing the viability induced by poly I:C. These results show that the LRP-1 agonists SP16 and analogs significantly inhibit a key cytokine, TSLP, that initiates a cascade of inflammatory processes that contribute to AD and other allergic inflammatory diseases. SP16, likely due to its activity in upregulating pro-survival pathways such as AKT, is also capable of rescuing the keratinocytes from poly I:C induced cell death, while the analogs seem to lack these effects. In figure 9B we also

show that treatment with the peptides (SP16, A2-5 and A3-10) was non-toxic to these cells at a concentration of 100µg/ml (peptide treatment in absence of poly I:C induction).

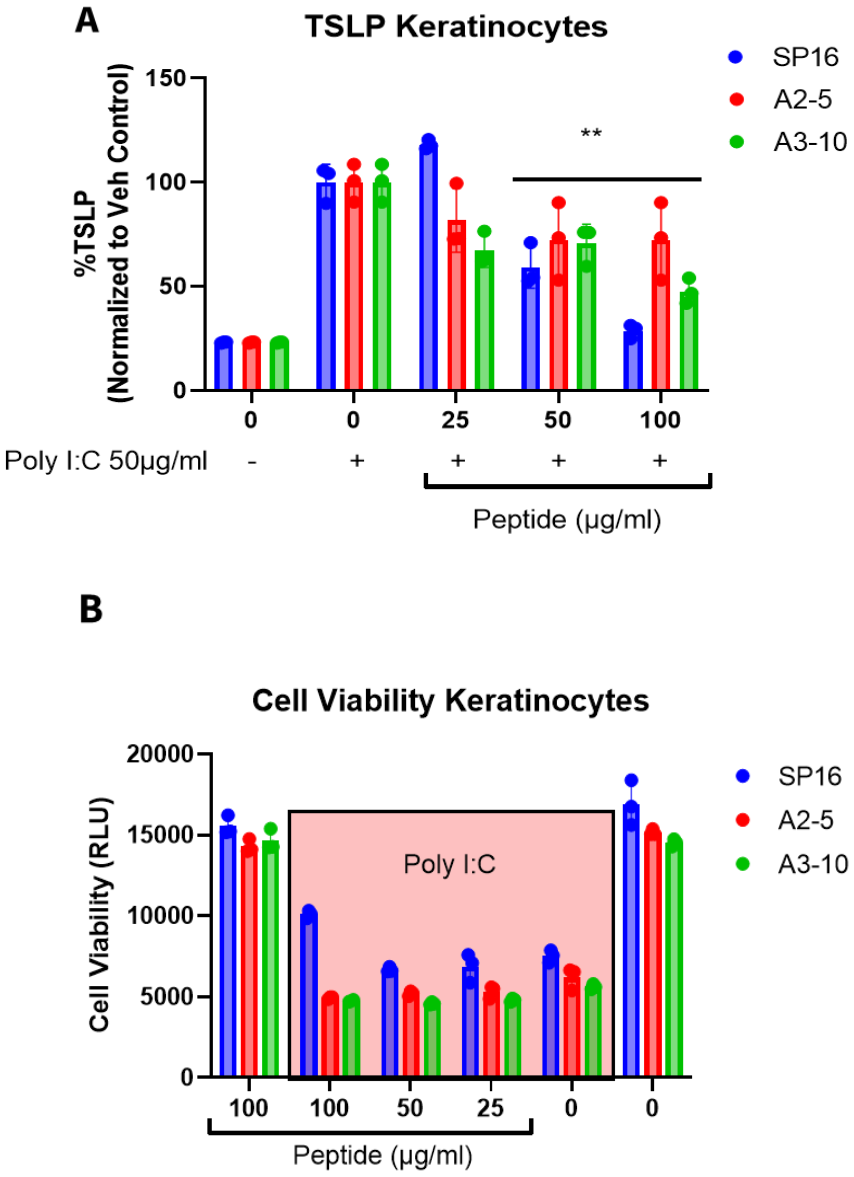


Figure 9. SP16, A2-5 and A3-10 inhibit TSLP in Keratinocytes.
A) HaCat keratinocyte cells were treated with SP16, A2-5 or A3-10 analog at increasing doses (up to 100µg/ml) before addition of poly I:C (50µg/ml) for 24 hours. TSLP in the supernatant was analyzed via ELISA. B) Cell viability was

assessed in the same assay by Cell-TiterGlo®, reading luminescent signal (RLU) after 24hrs. Cells were also treated with each peptide (SP16, A2-5 and A3-10) at 100µg/ml with no poly I:C and show no toxicity of these cells. **p<0.001.

The pharmacokinetic properties of SP16 by subcutaneous injection.

Previous preclinical work with SP16 in rats has shown the half-life of the peptide to be approximately 3.3 hours when given at a dose of 5mg/kg by intravenous injection, with a maximum plasma concentration of 989 ng/ml and AUC_{last} of 266 h*ng/ml (Figure 10A). We compared the plasma concentrations of SP16 when given by different routes of administration to mice, as outlined in Figure 10B, SP16 was given by either intravenous (IV), intraperitoneal (IP) or subcutaneous (SC) administration. Blood samples were taken before SP16 (100µg, 5mg/kg) injection and 30 minutes following drug administration by each route. Figure 10C shows that compared to IV injection (typically considered 100% bioavailability (*78*)), SP16 plasma concentrations given by both IP and SC injection is lower than anticipated (10.94, and 7.58%, respectively). Although we have seen pharmacodynamic effects of SP16 following subcutaneous injection, recently shown in models of nerve injury and pain (*53*) and previously in animal models of ischemia/reperfusion injury (*52*), at relatively low concentrations (0.2 mg/kg), low bioavailability and potential issue with solubility or plasma stability can be restrictive in terms of appropriate dosing due to volume limitations of certain administrative routes (i.e., subcutaneous).

A

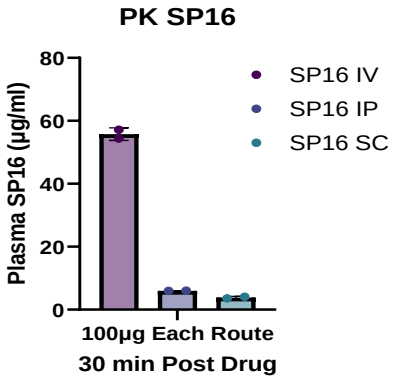
PK parameters	Unit	5mg/kg
Cl_obs	mL/min/kg	310
$T_{1/2}$	h	3.33
C_0 /C_{max}	ng/mL	989
AUC_{last}	h*ng/mL	266
AUC_{Inf}	h*ng/mL	269
$AUC_{_\%Extrap_}$obs	%	1.20
$MRT_{Inf_}$obs	h	0.462
AUC_{last}/D	h*mg/mL	53.2
V_{ss}_obs	L/kg	8.58

B

Analog	Route	Time-Points (min)	N	Dose (µg)	Dose (mg/kg)
SP16	IV	0, 30	2	100	5
SP16	IP	0, 30	2		
SP16	SC	0, 30	2		

SC, subcutaneous. IP, intraperitoneal. IV, intravenous

C



SP16 Bioavailability	
Route	% IV
IP	10.94
SC	7.58
Time= 30 minutes	

Figure 10. Plasma Concentration of SP16 Given by Various Routes of Administration.
A) PK properties of SP16 given by IV injection at 5mg/kg in SD rats in a previous study. B) Outline of experiment comparing the plasma concentration of SP16 given by IV injection, IP injection or SC injection to mice at a dose of 100µg (5 mg/kg). C) Comparison of the plasma concentration of SP16 sampled 30 minutes following various administrative routes, and analyzed via LC-MS/MS. SC, IP vs. IV dosing (% of IP/IV or %SC/IV) at 30 minutes.

Comparing the pharmacokinetic profile of SP16 and analogs.

Given certain advantages of subcutaneous injection compared to intravenous injection, such as ease of administration and higher patient compliance, we sought to determine whether the analogs could improve the bioavailability issues seen with SP16

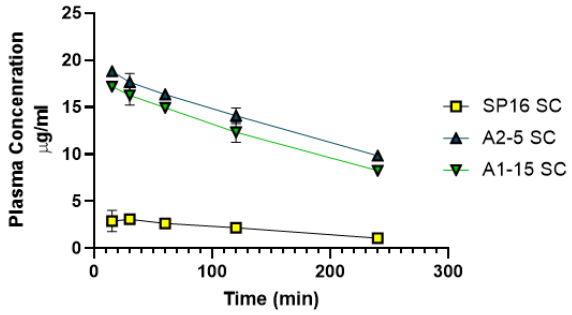
via this route. Analogs A2-5 and A1-15 (leading analog from the first series of analogs) as well as SP16 were administered to mice at 50µg (2.5 mg/kg) as outlined in Figure 11A. Plasma was collected from the mice at six different time-points and analyzed for the concentration of each analog or SP16. Compared to SP16, both analogs A2-5 and A1-15 show significantly higher levels of drug at all time-points, with peak plasma concentrations of 2.9, 18.9 and 17.2 µg/ml, respectively (Figure 11B). After four hours, there was still detectable levels of peptide in the plasma. Calculation of the Area Under the Curve (AUC) shows that compared to SP16, which has an AUC of 474.8 (min*µg/ml), analog A2-5 and A1-15 show AUCs of 3134 and 2774 (min*µg/ml), respectively, a roughly six times higher exposure level (Figure 11 C, D).

The pharmacokinetic profile following subcutaneous injection of the selected analogs are improved, compared with SP16, according to the plasma concentration at each time-point and overall AUC. This may translate to a lower dose needed to achieve pharmacodynamic effects following subcutaneous administration and may help ease any dosing limitations associated with drugs suffering from poor bioavailability issues.

A

Analog	Route	Time-points	N	Dose	Dose (mg/kg)
SP16	SC	0, 15, 30, 60, 120, 240	2	50µg	2.5 mg/kg
A2-5	SC	0, 15, 30, 60, 120, 240	2	50µg	2.5 mg/kg
A1-15	SC	0, 15, 30, 60, 120, 240	2	50µg	2.5 mg/kg

B



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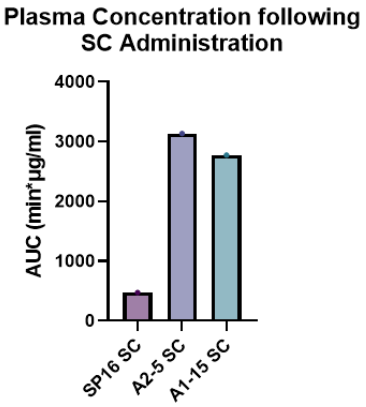


D

Analog Plasma AUC			
Analog	**SP16 SC**	**A2-5 SC**	**A1-15 SC**
Total Area	474.8	3134	2774
Std. Error	32.05	61.5	74.78
95% Confidence Interval	411.9 to 537.6	3013 to 3254	2627 to 2920

Figure 11. Comparison of PK Curves Following SC Administration in Mice.
A) Study design of PK experiment performed in mice B) Mice were administered either SP16, A2-5 or A1-15 by subcutaneous injection, at time-points shown in outline, plasma was collected and the plasma concentrations were analyzed for the peptides by LC-MS/MS. C, D) Area under the curve (AUC) was calculated for each peptide using Graphpad prism and shown as a function of the concentration over total time period (min*µg/ml).

Determination of the oral bioavailability of analogs A2-5 and A3-10.

In terms of drug development, achieving high oral bioavailability is key in design of the drug. Particularly for chronic indications, in which long-term dosing may be required. A drug in the form of a pill remains ideal, particularly if the half-life of the drugs is short, requiring daily dosing. Ultimately, it would be a significant advantage if a

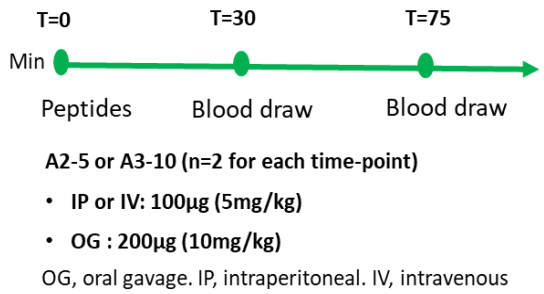
drug were to have oral bioavailability. The analogs were designed as cyclic peptides as cyclization can increase oral bioavailability by removing cleavable N- and C-termini minimizes enzyme degradation and by shielding components from metabolic enzymes (*79*).

In this experiment, we compared the plasma concentration of the analogs A2-5 and A3-10 following IV, IP and oral administration via oral gavage (OG). Figure 12A details the study design, with A2-5 and A3-10 being administered at doses of 100µg, (5mg/kg) for IV and IP injection and 200µg/ml (10mg/kg) for OG. Plasma was taken before drug administration (time = 0), as well as 30 minutes and 75 minutes following drug administration. Figure 12B displays the plasma concentrations of both analog A2-5 and A3-10 when administered IV, IP or OG. When we consider the IV route as the maximum bioavailability attainable (100% bioavailability), the drugs given by IP administration attain 70.2 and 74.9% bioavailability (Figure 12C). Comparing in the same manner the bioavailability of the drugs given by oral gavage, we see oral bioavailability of A2-5 and A3-10 at 17.01 and 19.1%, respectively (Figure 12C). If we then compare the PK properties of the analog A2-5 and A3-10 to that of SP16 at 30 minutes post drug administration (shown in Figure 10), we see that all peptides achieve roughly similar plasma levels when administered by IV. However, when the peptides are administered by IP route, SP16 has significantly reduced levels (about 10%) vs. around 70% seen with the analogs. Although we did not test the oral administration of SP16, we see that A2-5 and A3-10 given by oral gavage obtain higher plasma concentrations than SP16 given by SC route (Figure 12D). Because SP16 has shown efficacy following SC

administration, even with low plasma concentrations (indicating its potency), we can surmise that the analogs may also achieve efficacy when given by the oral route. Efficacy by oral administration, however, was not addressed here.

Although a full PK curve (with 6 time-points) was only achieved for analogs A1-15 and A2-5 as well as SP16, via subcutaneous route, we show that indeed SP16 does suffer some bioavailability issues and the analogs display a more favorable pharmacokinetic profile, which may benefit future development of the drugs. Furthermore, analogs A2-5 and A3-10 show respectable oral bioavailability levels, a huge advantage in dosing for more chronic indications.

A



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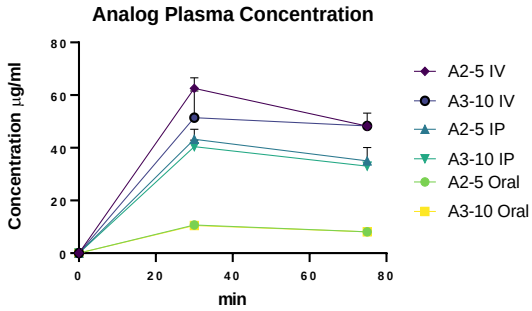


C

Analog Plasma AUC						
	A2-5 Oral	A3-10 Oral	A2-5 IP	A3-10 IP	A2-5 IV	A3-10 IV
Baseline	0	0	0	0	0	0
Bioavailability (%)	**17.01**	**19.10**	**70.20**	**74.90**	**100**	**100**
Total Area (AUC)	583.5	576	2408	2259	3430	3016
Std. Error	16.57	34.48	154.2	67	155.8	273.7
95% Confidence Interval	551.0 to 616.0	508.4 to 643.6	2105 to 2710	2128 to 2391	3125 to 3736	2480 to 3553

*Oral bioavailability is calculated as (AUC oral/AUC IV) and is expressed as a percent.

D

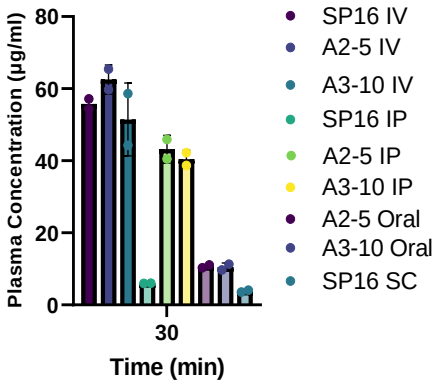


Figure 12. PK assessment of Analogs by IV, IP and Oral Gavage.
A) Outline of the PK study to assess oral bioavailability of analogs. B) A2-5 or A3-10 was administered by the routes outlined in A, plasma concentrations shown at 0-, 30- and 75-minutes post drug as analyzed by LC/MS/MS. C) Area under the curve (AUC) was calculated for each peptide using Graphpad prism. Bioavailability (%) calculated as AUC route/AUC IV. D) Plasma concentrations at 30 minutes post drug for SP16 (IV, IP and SC) and Analogs A2-5 and A3-10 (IV, IP, and oral).

Screening of the LRP-1 agonist analogs for efficacy in an *in-vivo* model of acute inflammation.

The analogs were next screened for efficacy in an *in-vivo* rapid mouse model of acute inflammation. To examine the cytokine profile in the LPS challenge mouse model, we induced cytokines with LPS (IP, 1mg/kg) and sampled the mice (n=3) at 90 minutes post LPS injection. The mice were treated with the immune-suppressive corticosteroid, dexamethasone (IP, 1mg/kg), 2 hours before LPS as a positive control for a cytokine inhibitory drug. Mice left untreated were used as normal controls. The cytokine profile in mice at 90 minutes post LPS is shown in Figure 13A. Of the 13 cytokines examined in the serum, only three were significantly increased vs. the untreated control; TNFα, IL-6, and MCP-1. Dexamethasone worked well to reduce TNFα to levels near the untreated control. For subsequent experiments, we chose to examine only the levels of TNFα in the blood.

We started with examining the anti-inflammatory effects of the analogs when given by IP administration, since we saw good bioavailability with the analogs via this route. The analogs A1-15 and A2-5, as well as SP16, were given at 100μg (5mg/kg) via IP administration, 30 minutes before LPS administration. Dexamethasone was given at 10mg/kg, IP, considered a high dose of the drug. Both the analog A2-5 and SP16 significantly inhibited LPS induced TNFα vs. the vehicle treated LPS induced control animals (even with the small sample sizes) (Figure 13B). Analog A1-15 showed some reduction in TNFα vs. the LPS, vehicle control group, however, significance was not reached.

Next, we examined the efficacy of analogs 7G, A3-10 and A2-5 when given by subcutaneous injection. SP16 was included for comparison. Non-LPS untreated animals were included as normal controls. Peptide treated animals were compared to LPS induced vehicle control animals. The experiment was executed as before, with peptide drugs given 30 minutes before LPS injection (1mg/kg, IP). Peptide drugs were administered at 100μg (5mg/kg), by SC injection. Although the plasma TNFα levels in all of the peptide drug treatment animals resulted in lower means as compared to the LPS induced vehicle control animals, only A3-10 treated animals reached significance (Figure 13C).

These results show that our current lead analogs A2-5 and A3-10 exhibit anti-inflammatory effects in-vivo, showing significantly lower plasma levels of TNFα, even in these preliminary screening experiments with a small sample size.

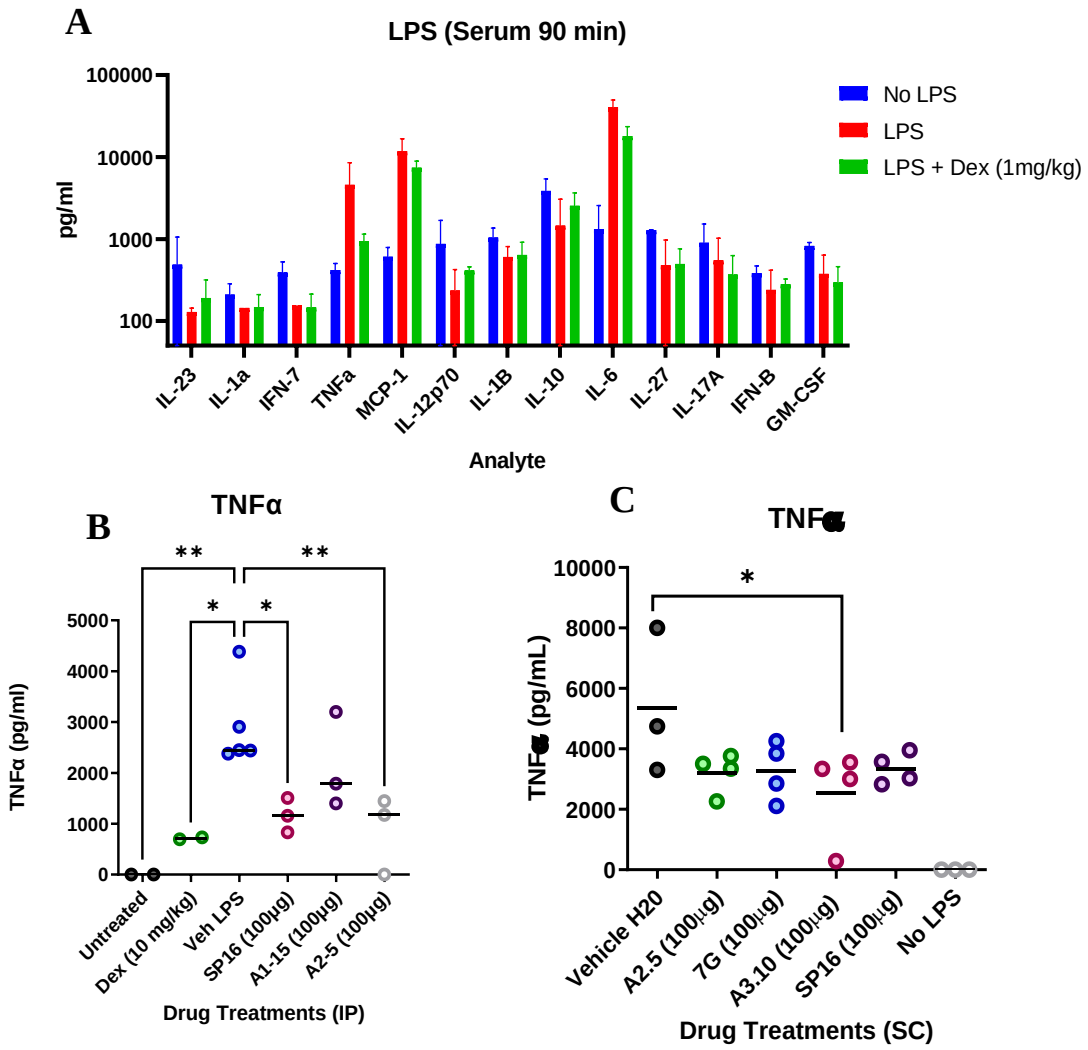


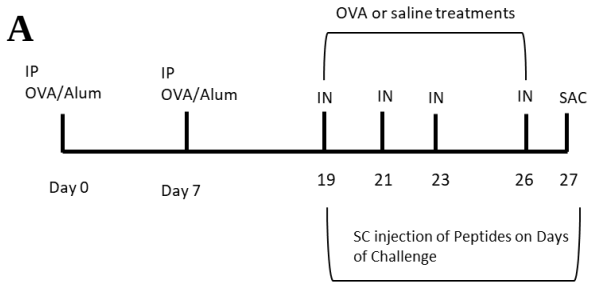
Figure 13. In-vivo Screen of Analogs Using LPS Challenge.
A) Mice were treated with dexamethasone (IP, 1mg/kg) 2 hours before challenge with LPS (1mg/kg) or PBS as a normal control group (n=3). Blood was drawn from the mice 90 minutes post LPS and analyzed for serum multiple cytokines via the Legendplex™ by flow cytometry of fluorescently labeled beads with each analyte. Cytokines are shown in pg/ml. B) Mice (n=3-4) were treated either dexamethasone (IP, 10mg/kg), vehicle control, SP16, A1-15 or A2-5 (IP, 100µg each) 30 minutes before LPS (1mg/kg), plasma was drawn 90 minutes following LPS challenge and analyzed for TNFα via ELISA. C) Mice (n=3-4) were treated with vehicle, A2-5, 7G, A3-10, SP16 (SC, 100µg) or left untreated, 30 minutes later, LPS (IP, 1mg/kg) was given. Plasma was analyzed for TNFα via ELISA. *p ≤ 0.05, ** ≤ 0.01.

Pilot study exploring the use of the LRP-1 agonists, SP16 and analogs, in an allergic asthma model

In order to explore whether LRP-1 agonists such as SP16 and the analogs could be used as a therapeutic treatment in allergic inflammatory diseases, we utilized the mouse model of ovalbumin (OVA)-induced allergic inflammation. This model recapitulates key features of asthma such as airway hyperresponsiveness as well as TH2/eosinophilic mediated airway inflammation (*80*). In this model, mice were sensitized to OVA with two IP injections of OVA with the adjuvant alum seven days apart. To induce allergic inflammatory responses 4 challenges of OVA were given by intranasal administration spaced 2-3 days apart. During the challenge phase, starting on day 19, the peptide drugs (SP16, A2-5, A3-10, and A1-15, each 50μg, SC) were given only on OVA challenge days (an hour after OVA challenge). As controls, mice were treated with dexamethasone (1mg/kg), vehicle treated OVA-induced disease controls and non-treated non-OVA normal controls (see outline, Figure 14A).

Serum collected at the end of the study shows significantly higher IgE levels in the OVA-induced vehicle control groups vs. the untreated control. However, none of the treatment groups, including 1mg/kg of dexamethasone was capable of reducing IgE (Figure 14B). Total protein in the lung lysate was quantified and show significantly lower concentrations of total protein levels per milligram of lung tissue in all groups (except A3-10) vs. the OVA/vehicle group (Figure 14C). TSLP in the lung lysate was measured via ELISA and while the untreated control animals show negligible levels of TSLP, all OVA challenged mice had significantly higher TSLP quantities, with the A2-5 treated mice being the only treatment group showing a significantly lower level ($p=0.0262$) (Figure 14D).

Elevated levels of IgE in the serum of the OVA-challenged mice, as well as an increased TSLP level of in the lung homogenate compared to the non-treated controls indicate that we were successful in inducing an allergic response in the mice. Surprisingly, the positive control dexamethasone, could not suppress either IgE or lung TSLP. Likewise, SP16 or the analogs tested were unable to suppress these responses with a dosing scheme of 4 days at 50μg given SC. The exception to these results was the A2-5 treated mice, in which significantly lower levels of lung homogenate TSLP was measured in these mice. A modified dosing strategy may be needed.



Group (n)	Treatment	Dose
3	Saline/Saline	N/A
5	Vehicle/OVA	N/A
3	Dexamethasone	(1mg/kg)
5	SP16	50µg
5	A2-5	50µg
5	A3-10	50µg
5	A1-15	50µg

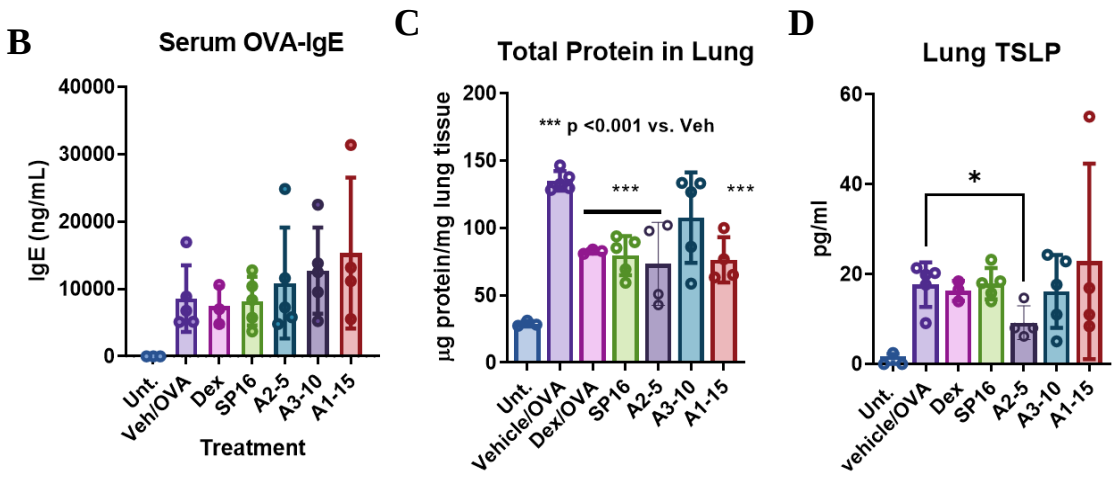


Figure 14. Pilot Study of LRP-1 Agonists in the OVA-induced Allergic Inflammatory Model.
A) Outline of the Ovalbumin-induced Asthma model and treatment regimens. B) OVA-specific IgE in the serum at Day 27, end of study) (pg/ml via ELISA). C) Total protein measured in the lung lysate following OVA-induced allergic inflammation (quantified by BCA). D) Lung TSLP levels were measured in the serum via ELISA (pg/ml). *$p \leq 0.05$, *** $p \leq 0.001$. Mean ± SD shown.

BALF cytokine profile following treatment with LRP-1 agonists SP16 and analogs in the Ovalbumin allergic mouse model.

We also examined the cytokine profile in the experiment (outlined in Figure 15A) following allergic challenges to assess whether we could induce predominately TH2 mediated cytokine expression (IL-4, IL-13, IL-5), and whether any of the peptides could modulate this response. We utilized the Legendplex™ system to simultaneously measure

a panel of 8 cytokines (IFNγ, IL-5, TNFα, IL-2, IL-6, IL-4, IL-10 and IL-13) in the bronchoalveolar lavage fluid.

Following ovalbumin challenge of the mice, Figure 15A shows that compared to the non-OVA induced group, cytokines IL-5, IL4, and IL-13 were upregulated in the OVA/vehicle treated mice, indicating a predominately TH2 mediated immune response was successfully mounted. Cytokines IL-4, IL-5, IL-13 and IL-10 are separated out and shown in Figure 15B, in order to better visualize the treatment effects. Although none of the treatment regimens (including the positive control, dexamethasone) were capable of significantly reducing any of the cytokines tested, we did see lowered overall means in the A1-15, and more so in the A2-5 treatment groups of cytokines IL-5 and IL-4. Treatment with analog A2-5 reduced IL-13 to near baseline levels (however, did not reach significance). Interestingly, as we have seen in other models of inflammation, SP16 increased levels of the anti-inflammatory cytokine IL-10 (in 3 out of the 5 mice), however, none of the analogs show similar trends.

While we were successful in inducing many allergic responses (IgE, TH2 dominated cytokines in the BALF and TSLP in the lung), curbing these responses may require an alternative dosing regimen for both SP16 and the analogs, such as a higher dose or daily dosing may be required. Likewise, the dexamethasone regimen in this model may need to be reevaluated.

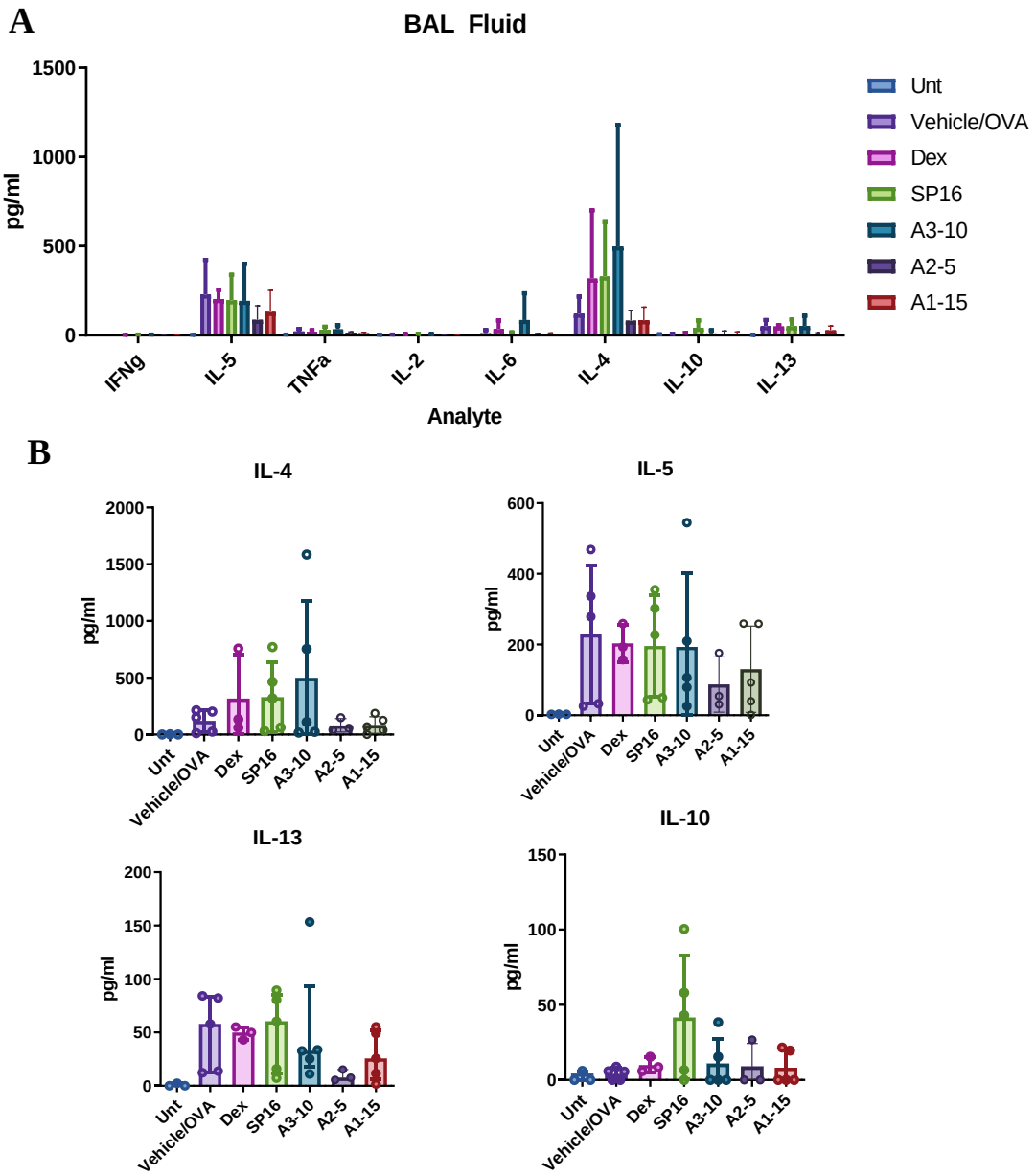


Figure 15. BALF cytokine profile shows TH2 mediated allergic responses.
A) Cytokines in the bronchoalveolar Lavage Fluid (BALF) of the mice from the experiment described in Figure 12A were analyzed via the Legendplex™. IFNγ, IL-5, TNFα, IL-2, IL-6, IL-4, IL-10 and IL-13 shown (pg/ml). B) cytokines IL-5, IL-4, IL-13 and IL-10 displayed to scale.

Additional assessment of the efficacy of LRP-1 agonists in allergic inflammatory asthma model.

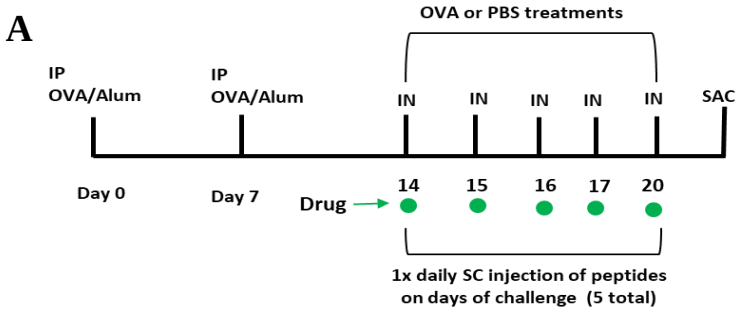
As an additional experiment to test whether SP16 or any of the analogs could modulate allergic responses, we used a shorter regimen of ovalbumin-induced allergic asthma and treated the animals as outlined in Figure 16A. Briefly, the animals were sensitized to ovalbumin with two IP injections of OVA/Alum spaced 7 days apart. One-week later (Day 14) the animals were challenged with OVA by IN instillation daily for four consecutive challenges, and then on day 20, challenged one additional time before the study end on day 21. The animals were treated with dexamethasone (1mg/kg) as a positive control. LRP-1 agonist peptide treated groups included SP16, A2-5 or 7G (100μg, SC). OVA-induced vehicle controls or untreated controls were included.

In this experiment we isolated the cellular infiltrate following lavage of the lungs and measured total WBC count. We collected a single lobe of the lung and determine lung weight, as well as TSLP in the lung homogenate for all the treatment groups.

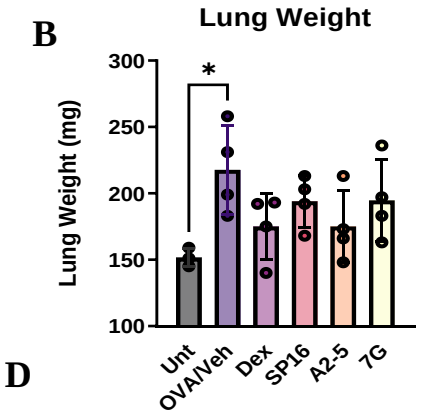
In terms of lung weight, the OVA-induced/vehicle treated group had significantly higher weights compared to non-OVA induced controls. However, none of the lower means achieved by the peptide treated groups reached significance (Figure 16B). The total cell counts isolated from the cellular infiltrate of the BALF also show no significant differences among treatment groups, although the dexamethasone treated group does show a lower mean compared to the other treatments (Figure 16C). We measured TSLP in the lung tissue at day 21, however, at this time-point, no significant change was seen

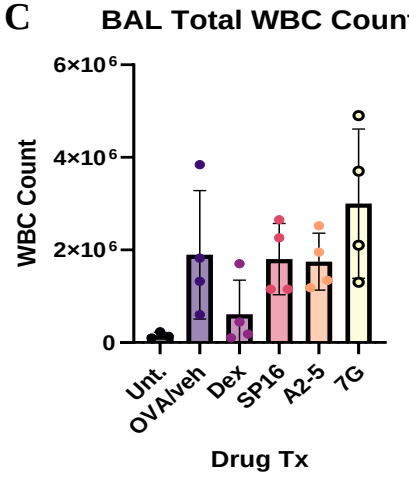
between the untreated control and the OVA/vehicle treated control. Likewise, no significant difference was shown among treatment groups (Figure 16D).

These results show minimal changes between the untreated and OVA/vehicle treated control in the parameters tested, and no significant differences in the treatment groups, even with the positive control dexamethasone. This could indicate a potential issue with the dosing regimen of the drugs and protocol to induce asthma that were used.



Study Outline of OVA induced Asthma Model			
Drug Tx	**Dose**	**Dose volume**	**Route**
PBS Control (No OVA)	N/A	N/A	N/A
OVA (Vehicle Control)	Equal Volume	100µl	SC
Dexamethasone	1mg/kg		
SP16	100µg		
A2-5			
7G			





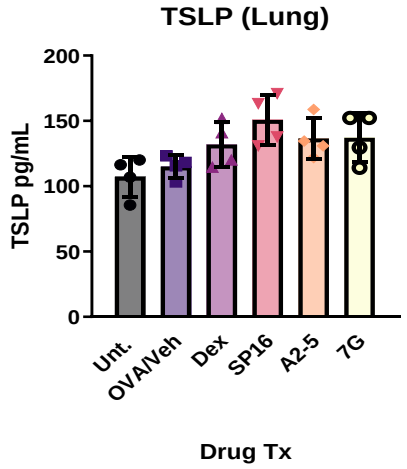


Figure 16. Assessment of SP16 and Analogs in shortened protocol of ovalbumin-induced allergic asthma model. A) Protocol used to induce allergic asthma in mice and treatment regimens explored. B) Lung weight at day 21 of OVA-induced allergic model in treated groups. C) Lungs were lavaged and cellular infiltrate was counted using the countess™ hemacytometer and trypan blue exclusion. D) TSLP in the lung homogenate was analyzed via ELISA

Immune cell profile in the BALF following treatment with SP16 or analogs in the OVA-induced asthma model.

To examine the immune cell populations within the BALF, we utilized a previously described method, with some modifications (*73*). The gating strategy can be found in the appendix (A1). Only the untreated/non-OVA groups, OVA-vehicle treated group, SP16 or A2-5 groups were included in the flow analysis. The results show that of the cell populations examined (macrophages, neutrophils, eosinophils, and dendritic cells), the macrophages dominated the profile (making up about half of the total WBC count), with neutrophils constituting the second highest population, followed by eosinophils and dendritic cells (Figure 17). While there was a significant increase in each population in the OVA-induced vehicle treated mice compared to the non-OVA treated control mice, there were no significant changes among the treatment groups (SP16 or A2-5) compared to the OVA-induced control group, likely due to the small sample sizes (n=3-4). There were some non-significant trends in the means of the groups, with SP16 and A2-5 showing lower means in the eosinophil and neutrophil populations. A2-5 also showed lower mean dendritic cells, however, not reaching significance.

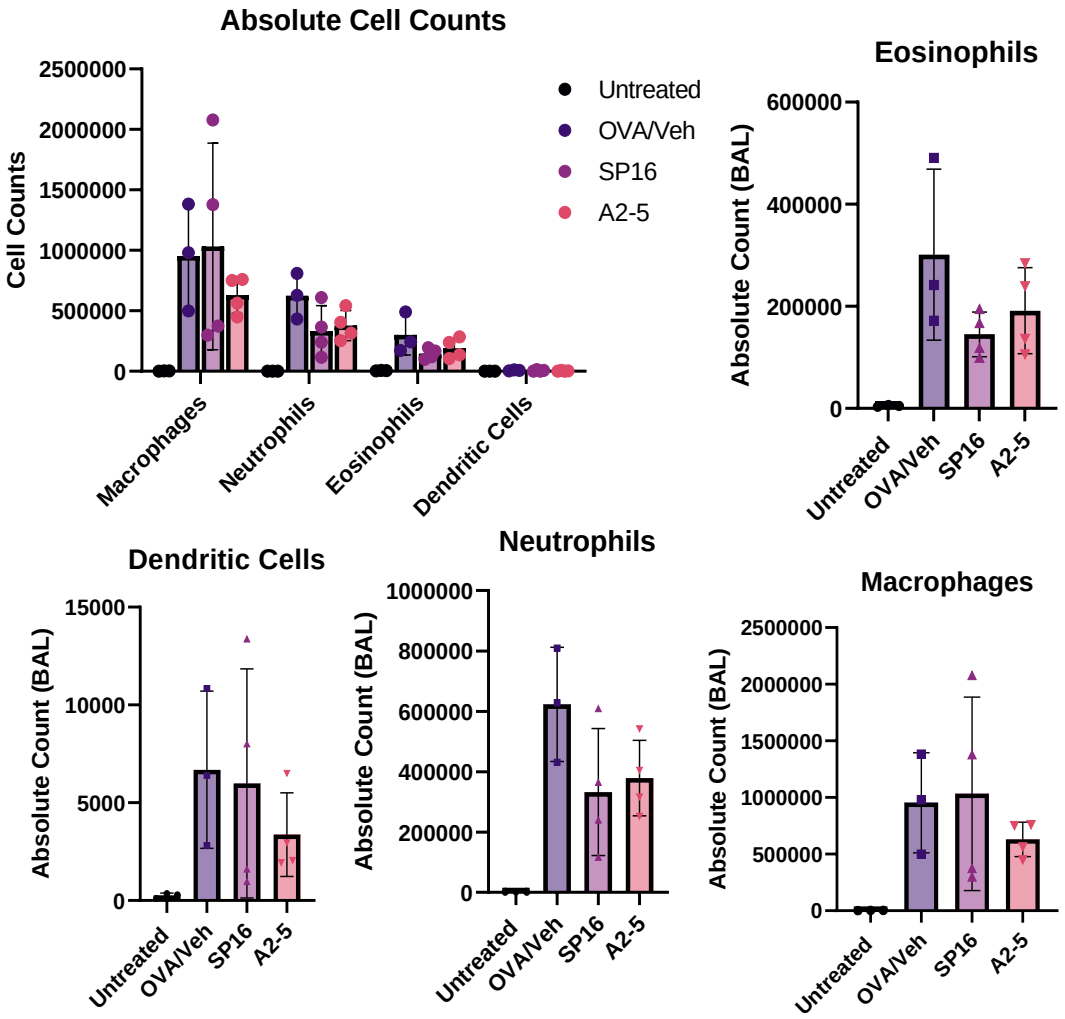


Figure 17. Immune cell infiltrate following treatment with SP16 or A2-5 in the OVA-induced Asthma model. The immune cells in the BALF were stained with fluorescently labeled antibodies for surface markers, CD11b, CD11c, MHCII, Ly6G and Siglec-F as well as 7-AAD for viability. The populations were determined by CD11c low/CD11b+/Ly6G- (Eosinophils), CD11c low/CD11b+/Ly6G+ (Neutrophils), CD11c high/SiglecF+ (Macrophages) and CD11c High /Siglec-/MHCII+ (Dendritic cells). Counting beads were added in order to determine absolute cell numbers.

Assessment of the efficacy of SP16 and analogs in the MC903 induced model of atopic dermatitis.

We next tested SP16 and the analogs in a model of atopic dermatitis. Topical application of MC903 (calcipotriol) induced key features of atopic dermatitis, including inflammation of the skin, with redness and edema, as well as gradual dryness/scaling of the skin. These features were graded and reported as the dermatitis score. Initially, hypervascularization was noted, followed by gradual thickening of the ears, which was measured with a caliper. Although pruritis is a common occurrence in atopic dermatitis, we did not assess it in this study. Weights were taken to ensure no MC903 associated weight loss occurred. The animals were treated topically to both ears with SP16 (500μg), A7-2 (a modified A2-5) (250μg), or A3-10 (250μg), or the vehicle control (equal volume of 70% ethanol) on the days of MC903 treatments. Figure 18A outlines the protocol used to induce AD as well as the treatment regimens and dosing. Topical application of SP16 and the analogs was chosen based on the significant local inflammatory response of the skin that occurs in AD to directly target the local environment.

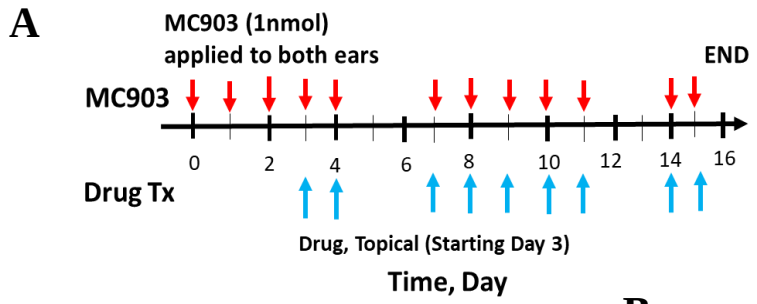
The dose of the MC903 (calcipotriol) was carefully chosen, as we have observed significant weight loss in animals receiving 4nmol applied to the ears. We therefore, applied 1nmol of MC903 to each ear according to the schedule outlined in previously described protocols (*81*). We show in Figure 18B, no significant weight loss occurred with this dose and treatment regimen.

Compared to the MC903-vehicle treated mice, the mice treated with SP16 and analog A3-10 showed significant improvement in dermatitis scores (Figure 18C). At the

end of the study, the MC903-vehicle control group continued to show swelling, redness and dryness of the ears, while SP16 treated mice continually improved with each treatment. Mice in the A3-10 treated group were able to maintain improvements in dermatitis throughout the study. Treatment with A7-2 also showed some efficacy in reducing dermatitis; however, significance was lost on the last day of assessments.

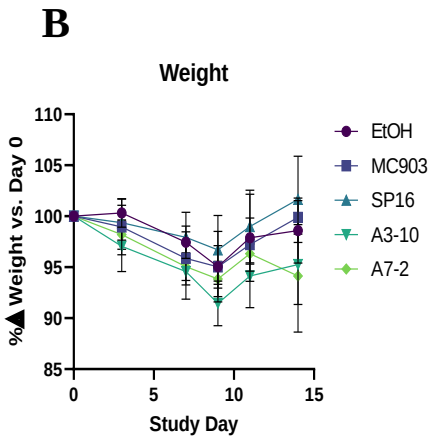
The ear-thickness in the MC903-vehicle treated mice significantly increased during the course of the study compared to the untreated control group. However, mice in all of the peptide treated groups showed significantly less ear-thickening compared to MC903 mice (Figure 18D).

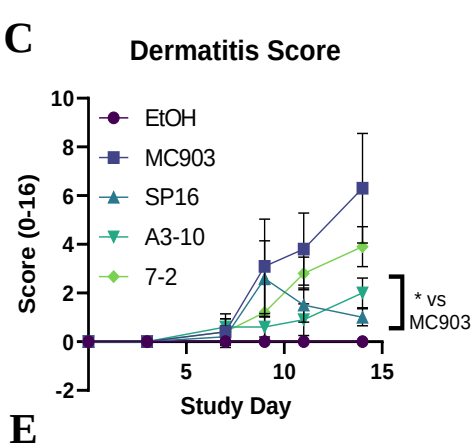
The images taken on the last day of the study show the red, swollen and scaly features of the MC903-vehicle treated mice, while the mice in the peptide treated groups resemble more closely the untreated control, with significantly less clinical features of AD present (Figure 18E).



Study Outline of MC903 Induced Atopic Dermatitis Model			
Drug Tx	**Dose**	**Dose volume**	**Route**
EtOH (No MC903)	N/A	N/A	N/A
MC903 (Vehicle Control)	Equal Volume	50µl	Topical (applied to ears)
SP16	500µg		
A3-10	250µg		
A7-2			

***Vehicle for peptide drugs was 70% Ethanol**





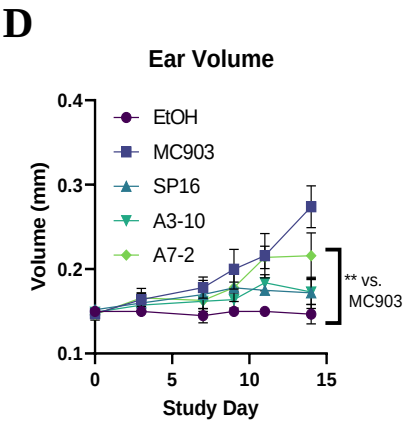


Figure 18. SP16 and Analogs Improve Outcomes in an AD-like Inflammatory Skin Model.
A) Schematic outline of the protocol used to induce atopic dermatitis like inflammation in mice and treatment regimens (n=5). The peptide drugs were applied topically to the ears on the days of the MC903 applications. In-life parameters: B) Weights C) Dermatitis score and D) Ear volume measured on days 0, 3, 7, 9, 11 and 14. E) Pictures were taken on the last day of the study (Day 15) and a randomly chosen representative mouse from each group is shown.

SP16 and analog A3-10 reduce important mediators of allergic skin inflammation.

The MC903 induced model of AD is associated with an upregulation of Type 2 inflammatory cytokines such as IL-4, IL-13 and IL-5, as well as TSLP in the skin and systemic levels of IgE (*82*). We therefore measured the serum IgE levels, collected at the end of the study (Day 15) (as outlined in Figure 18A). TSLP in the serum was measured at day 8 of the study, while TSLP in the ear was measured at the end of the study. The cytokine profile of the skin was assessed on day 15.

We show that the systemic IgE levels were elevated in the MC903/vehicle treated group compared to the untreated group, however, the difference was not statistically significant (Figure 19A). Among treatment groups, there was no change in IgE levels. The serum TSLP on day 8 also did not reach significance among treatment groups due to high variation in the MC903 treated mice at this time-point (Figure 19B). The TSLP levels measured in the ears of the mice at the end of the study (Day 15) did show differences among groups (Figure 19C), with both the SP16 and A3-10 treated animals displaying significantly lower levels of TSLP compared to MC903/vehicle group. The A7-2 treated animals, however, did not show differences in TSLP compared to the MC903 control group.

An examination of the local inflammatory response in the ear tissue, as measured by a panel of TH2 cytokines, shows that at the later time-point, IL-4 was significantly increased in the MC903 control animals compared to the untreated control animals.

While treatment with analog A7-2 did not have significant effects, treatment with either SP16 or the analog A3-10 was successful in inhibiting IL-4 in the tissue (Figure 19D).

These results indicate that SP16 and analog A3-10 have a significant impact on inhibiting cytokines that initiate TH2 responses.

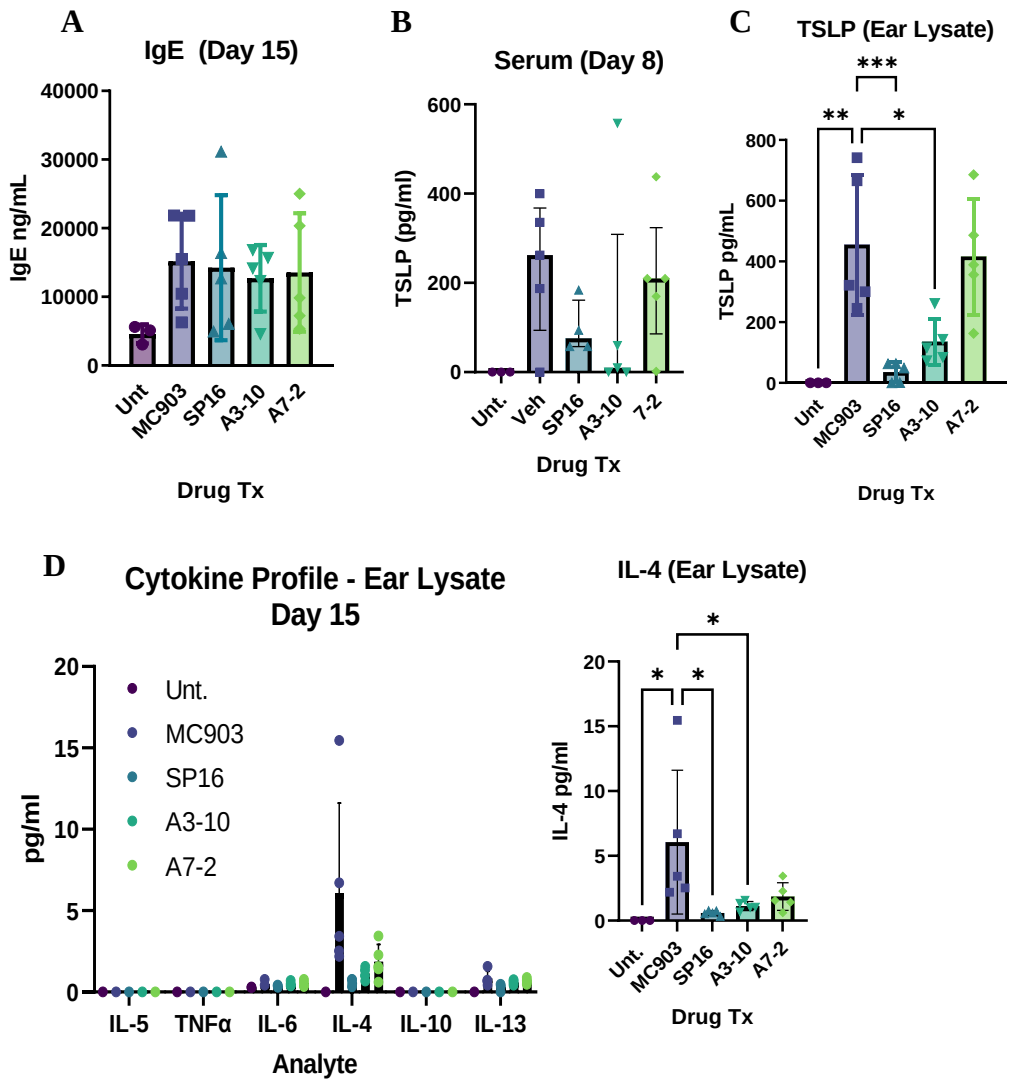


Figure 19. LRP-1 agonists SP16 and A3-10 mediate drivers of allergic responses in AD model.
A) Plasma was collected from mice treated as outlined in figure 18A on day 15 (end of the study) and analyzed for levels of IgE via ELISA. B) TSLP was measured in the blood collected from the mice on Day 8 of the study. C) Ear

homogenate collected on Day 15 of the study was analyzed for TSLP via ELISA. D) Ear homogenate was analyzed via the Legendplex™ for TH1/TH2 specific cytokines (Day 15), the IL-4 is shown individually.

CHAPTER SIX: DISCUSSION

These studies demonstrate the therapeutic potential of using LRP-1 agonists for inflammatory diseases, including allergic inflammatory diseases. SERPIN proteins, including AAT (from which these LRP-1 agonists are derived) have long been investigated for their effects in a variety of biological processes including inflammatory regulation and tissue repair (*83, 84*). We show here that SERPIN proteins contain a small active amino acid sequence that can be exploited for its anti-inflammatory function. We have synthesized several SERPIN-derived peptides (analogs of the lead peptide, SP16) largely modeled off of the LRP-1/AAT interaction, as we have previously determined the mechanism of action of SP16's anti-inflammatory and pro-survival effects to be through LRP-1 interaction (*52, 53*). These analog peptides are designed to target LRP-1 in order to initiate a cascade of cellular and molecular processes that initiate inflammatory resolution. Due to the fact that the LRP-1 binding motif consists of nearly all hydrophobic amino acids, an important consideration throughout the design of the SERPIN-derived analogs was how to exploit the hydrophobic side chain interactions between the peptides and the LRP-1 protein, while maintaining improved stability and solubility. This is a challenge because as we shorten the peptide sequence, positively charged amino acids are potentially truncated resulting in a peptide with potentially poor drug properties due to plasma instability (*85, 86*).

The initial screening process utilizing LPS induced NFκB or TNFα in cell models, revealed several potential analogs with highly improved effective concentrations

compared to SP16. Using structure-activity relationships we were able to optimize three key features of the analogs including the positively charged amino acid chain length required to stabilize the hydrophobic core, the amino acids within the core motif that contributed to activity, as well as the ideal cyclization scaffold. These studies revealed that certain amino acids within the core binding motif could be substituted in favor of more hydrophilic amino acids, which contributes to increased solubility. We also sought to benefit from cyclization of the analog peptides, with the intent of creating analogs with improved PK properties and potentially even oral bioavailability. Therefore, additional modifications to the core binding sequence were made to improve oral properties, including the incorporation of non-natural amino acid residues and d-stereoisomers (*87*). Based on the activity of the third series of analogs, a highly modified core motif loses anti-inflammatory activity, compared to the second series of analogs which contained mostly natural amino acids. Incorporating a d-stereoisomer in an amino acid position that was deemed unnecessary for LRP-1 binding did yield a highly active analog and could contribute to the oral bioavailability of these analogs. Because the d-amino acid faces the opposite direction compared to the L-amino acid, it is possible that this slight change in the peptide structure could enhance LRP-1 affinity.

A key feature of SP16 is that it is a short peptide derived from a natural plasma protein with few modifications and therefore, not expected to be associated with toxicity. Indeed, we have seen no drug associated adverse effects of SP16 in initial clinical trials (*88*). Likewise, the analogs are anticipated to be safe and well tolerated, having been derived from SP16. The initial cell viability assay screens show no concern for toxicity,

even at concentrations well above the effective range. This can translate to a wide therapeutic window for use in pre-clinical models and clinical trials, a substantial benefit. The PK studies utilized several different routes of administration (IV, SC, IP and oral), with therapeutic doses of the analogs A1-15, A2-5 and A3-10, showing no safety concerns in the mice. Future studies testing the safety of lead analog peptides for off-target effects, including *in-vitro* testing for any potential interactions with G-protein coupled receptors, ion channels, transporters, nuclear receptors or other potential targets that could pose a risk for undesirable adverse effects should be explored before proceeding to non-clinical toxicology and safety studies in animals.

Although a clear understanding of the mechanism of action is not required for FDA drug approval (*89*), our intent in the design of these analogs was to create potent LRP-1 agonists with favorable drug properties. Having identified a few lead analog peptides from each series of modifications (7G, A2-5, A3-10 and A3-14) that were designed from the putative LRP-1 binding site of SP16, it would be reasonable to assume that they bind to LRP-1. Given the increased activity of the analogs *in-vitro*, an increased affinity to LRP-1 would also be logical to assume. The binding assessments of the analog peptides show clear LRP-1 binding of one of the analogs, A2-5, however, increased LRP-1 affinity of A2-5 cannot be demonstrated from this study. When the NFκB inhibition of A2-5 was assessed, it was found that the biotin labeled A2-5 was less effective compared to the non-labeled A2-5. One possibility is that the biotin label placement on the analog partially blocked the LRP-1 interaction, thereby, showing both a decreased affinity to LRP-1 compared to SP16, and corresponding blunted anti-inflammatory activity

compared to the non-labeled version. Of consideration, since the biotin label is potentially interfering with the active binding site of the peptides, we could add a spacer to increase the distance between the biotin label and active pharmacophore. As support for this, the 7G analog contains additional amino acids between the LRP-1 binding site and N-terminal end of the peptide (where it was biotin-labeled) and showed affinity to LRP-1 similar to SP16. We could also use an alternative labeling method (*90, 91*), or an alternative method utilizing an unlabeled drug (i.e., surface plasmon resonance (SPR) (*92*)).

We did not pursue A3-14 which showed no binding activity to LRP-1, but did show activity, due to potential off-target effects of the highly altered core motif. However, we did continue with the A3-10 analog which also showed no LRP-1 binding, yet no activity, as expected, and therefore further studies are needed to confirm LRP-1 target engagement. One method we could employ would be a competition assay using a known LRP-1 ligand such as Receptor Associated Protein (RAP) (*93*). The benefit of this method is that we could utilize an unlabeled analog peptide, avoiding any potential interference issues. Although we attempted to block the NFκB inhibitory activity of the analogs with the RAP protein, no conclusive LRP-1 dependence could be demonstrated with either SP16 (positive control) or analogs, potentially because the RAP concentration was not sufficient to block the peptide-LRP-1 interaction. We have shown however, that a stable RAP mutant at a higher concentration is effective in blocking SP16's activity and this should be explored using the analogs (*75*).

We show increased anti-inflammatory effects of the lead analogs *in-vitro*, however, an additional aim was to assess the pharmacokinetic properties of the analogs, as a major issue with SP16 is its poor bioavailability when administered subcutaneously. Although SP16 has shown efficacy *in-vivo* due to its incredible potency and targeted action, despite its potential bioavailability issues, the analogs demonstrate considerably higher bioavailability when administered subcutaneously. The combined increased potency of the analogs with the increased bioavailability could equate to a highly effective anti-inflammatory drug. In addition, the analogs demonstrate significant oral bioavailability for small peptide drugs. The clinical utility of a highly potent and targeted orally available anti-inflammatory drug is substantial.

The lead analogs A2-5 and A3-10 demonstrate efficacy in the acute LPS challenge mouse model, similar to both SP16 and a high dose of dexamethasone. Since this model is extremely acute, inducing very rapid upregulation of inflammatory cytokines, a higher dose of both SP16 and analogs was used. This study did not test the dose dependency of the analogs; therefore, we cannot make conclusive comparisons regarding the effective concentrations between SP16 and analogs. It also was not powered for significance (only 3-4 mice per group), even so, SP16 and analogs did demonstrate significant anti-inflammatory effects.

Although recent findings suggest a role of LRP-1 and AAT in allergic inflammatory diseases, it is unknown whether treatment with an LRP-1 agonist is an effective strategy in allergic diseases. Here, we show that SP16 and the analogs inhibit key mediators that drive allergic inflammatory responses. For instance, *in-vitro*

assessment of TSLP release, shows significant inhibitory effects of SP16 and analogs in an inflammatory skin model. In the AD mouse model, both SP16 and A3-10 analog significantly reduce TSLP in the inflamed ear. In the mouse model of allergic asthma, we also show that analog A2-5 was effective in significantly reducing TSLP in the lung tissue. TSLP released from the epithelium can initiate both innate and adaptive immune responses that contribute significantly to disease and recent clinical trials have shown that inhibition of TSLP has profound effects in disease outcomes in asthma, AD and EoE (*23, 94*).

Although we were capable of inducing some inflammatory immune cell infiltration in the lungs among the OVA-induced groups, it is unclear if the shortened protocol (using the 3-week model with five rapid daily challenges) instead of a longer protocol, resulted in only mild TH2 allergic responses. Subsequent OVA-induced asthma studies should utilize a longer protocol (additional time between sensitization and challenge). However, models utilizing similar protocols do show significant increases in the eosinophilic population in the BALF (*95*), as opposed to a profile dominated by neutrophils and macrophages (indicating perhaps TH1 instead of TH2 responses) that we report here. Further examination of the cell markers and gating strategy should be conducted upon further studies.

Utilizing a slightly longer OVA-induced asthma protocol, we did show an increase in TH2 cytokines in the OVA-induced controls compared to non-OVA controls. We show some inhibitory effects with treatment of the A2-5 analog (although it did not reach significance) in IL-4, IL-5 and IL-13 from the lung lavage fluid compared to

vehicle treated mice. Animals in all the peptide treatment groups received the same dose, and this may need to be reevaluated, as SP16 may require a higher dose to see significant effects. It is also interesting that the dexamethasone did not show significant responses in this model, as it has been shown to alter inflammation in similar OVA-induced asthma models (*96*). Lack of significance among treatment groups is likely due to small sample sizes, and additional studies should be conducted. It would also be interesting to explore whether inhaled or intranasal administration of the peptides as a more direct delivery to the lungs (as opposed to systemic administration) would be more effective.

One of the key features of SP16 is that it is not an anti-inflammatory drug, but immunomodulatory drug, upregulating anti-inflammatory cytokines such as IL-10. The cytokine profile in the BALF shows that SP16 treatment increases IL-10 in some of the mice, while none of the analogs exhibited these effects. We also see pro-survival effects in the keratinocytes that were not replicated by the analogs. Retention of these effects by the analogs is an important attribute of the drug and it should be explored in more depth.

One of the significant findings of this work is that SP16 was extremely effective in mitigating skin inflammation in the AD mouse model upon topical administration. Of the biomarkers examined, clinically validated mediators of TH2 allergic responses, TSLP and IL-4, were inhibited by SP16 treatment. Although the analog A3-10 had similar effects, because SP16 is already a clinical phase drug, it would be an easier developmental path to transition to allergic inflammatory skin diseases. Still, development of the analogs for more chronic inflammatory indications in which oral

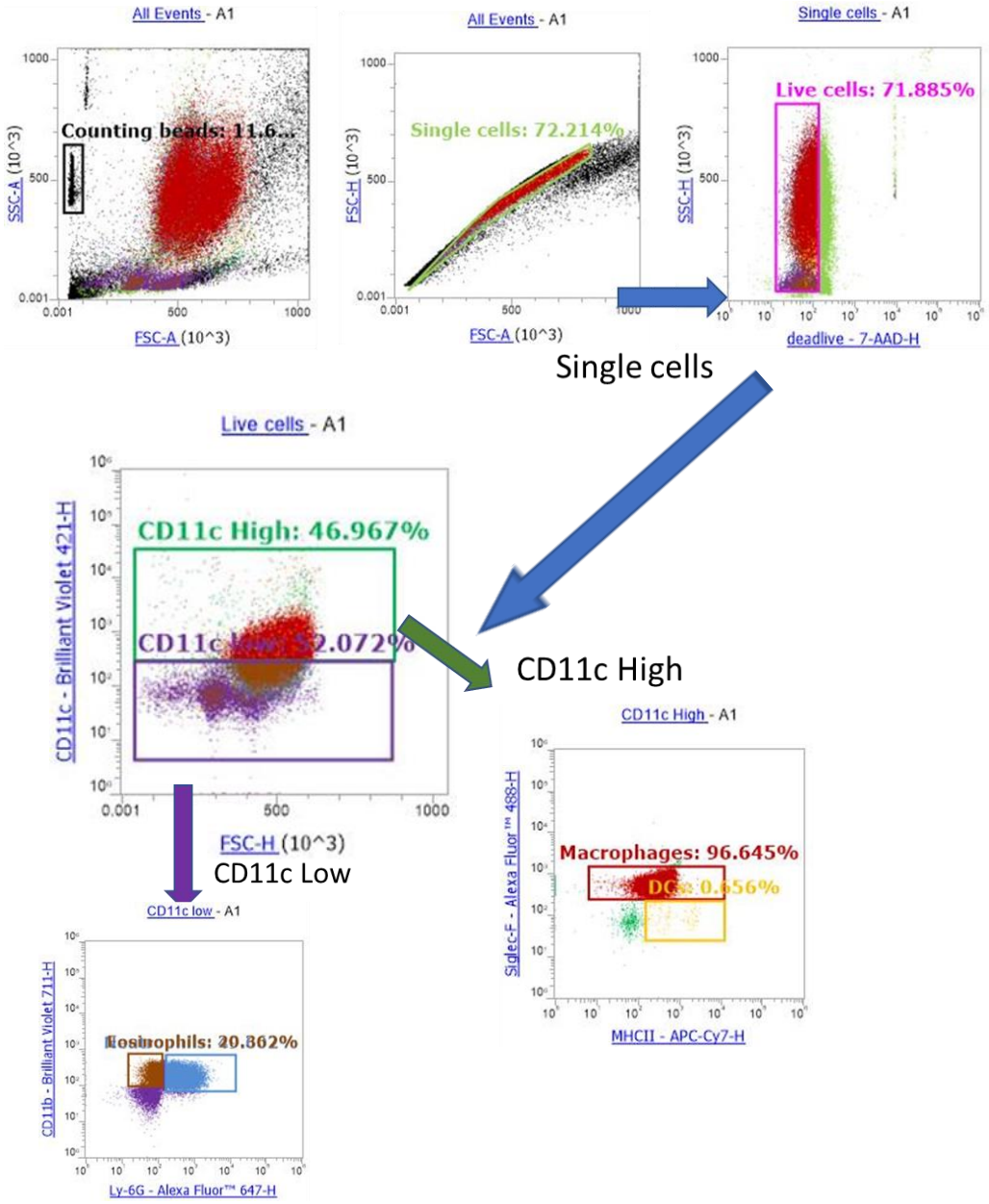
administration would be beneficial is extremely important, given that thus far, they appear safe and have potent anti-inflammatory effects.

Conclusions

This developmental work has defined a series of highly potent anti-inflammatory SERPIN-derived analogs, designed to target a key homeostatic receptor LRP-1. These highly potent analogs, appear to have no cell toxic effects, at least preliminarily, which can significantly impact dosing strategies in the clinic. Therefore, given the low effective concentrations of the analogs with no corresponding toxicity, a wide range of therapeutic doses could be effectively employed in clinical studies. Given the improvement in plasma concentrations following subcutaneous injection and significant plasma levels following oral administration, these drugs may have clinical utility in chronic inflammatory diseases. The long-term safety and mechanism of action through LRP-1 of the analogs should be further investigated.

LRP-1 plays a role as a negative regulator of innate and adaptive immune responses in allergic inflammatory diseases. The lead drug, SP16, off of which these analogs were modeled, is a potent LRP-1 agonist, and is shown here to inhibit key mediators that drive allergic inflammatory responses, resulting in significant improvement in clinical features of the disease in preclinical studies. These findings suggest that targeting LRP-1 in allergic inflammatory diseases is an attractive therapeutic strategy that should be further explored.

APPENDIX



Appendix A. Gating Strategy to Determine Immune Cell Populations.

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BIOGRAPHY

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