THE REGULATION OF ALLERGIC AIRWAY DISEASE BY TYPE V COLLAGEN–INDUCED TOLERANCE

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DEDICATION

This thesis is dedicated to my wonderful parents, Louis and Daisy Lott, for giving me life and supporting me in every endeavor I have untaken during my life.

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ABSTRACT

Jeremy M. Lott

THE REGULATION OF ALLERGIC AIRWAY DISEASE BY TYPE V COLLAGEN–INDUCED TOLERANCE

Rationale: Tissue remodeling and complement activation are asthma hallmarks. Type V collagen [col(V)], a cryptic antigen, becomes exposed during lung remodeling. IL-17 is key to anti-col(V) immunity, and regulates complement activation. We have reported that col(V)-induced tolerance down regulates IL-17 and prevents immune-mediated lung diseases.

Objectives: Determine a role for anti-col(V) immunity in asthma.

Methods: Serum anti-col(V) antibodies were measured in asthma patients, and immunohistochemistry utilized to detect interstitial col(V) in fatal asthma. Balb/c mice were tolerized with col(V) prior to sensitization with ovalbumin (OVA), and subsequent OVA intranasal challenge. Airway hyper-responsiveness (AHR) to methacholine was measured; and RT-PCR utilized to determine local *Il17* transcripts. Bronchoalveolar lavage levels of C3a, C5a and OVA-specific IgE were measured; and immunohistochemistry utilized to detect expression of complement regulatory proteins, expression, CD46/Crry and CD55, in lung tissue.

Results: Compared to normal subjects, anti-col(V) antibodies were increased in asthmatics; and interstitial col(V) was over expressed in fatal asthma. OVA-induced AHR up regulated anti-col(V) antibodies systemically, and increased OVA-specific IgE and C3a in BAL, and parenchymal *Il17* transcripts. Col(V)-induced tolerance abrogated

AHR, down regulated OVA-induced T cell proliferation, as well as total and OVA-specific IgE, C3a, IL-17 expression and tracheal smooth muscle contraction. Crry/CD46 and CD55, key to preventing complement activation, were down regulated on goblet cells in murine allergic airway disease.

Conclusions: Anti-col(V) immunity correlates with asthma pathogenesis, and col(V)-induced tolerance may be a novel therapeutic for asthma. Decreased expression of Crry/CD46 and CD55 on goblet cells may in part account for complement activation in asthma.

David S. Wilkes, M.D. Chair

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AAD	Allergic airway disease
AHR	Airway hyper-responsiveness
Alum	Aluminum hydroxide
APC	Antigen presenting cell
BAL	Bronchoalveolar lavage fluid
CBA	Cytokine bead assay
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD25	Cluster of differentiation 25
CD28	Cluster of differentiation 28
$CD45RC^{high}$	Cluster of differentiation 45 isoform C high cells
CD46	Cluster of differentiation 46
CD55	Cluster of differentiation 55
CFA	Complete Freund's adjuvant
Col(II)	Type II collagen
Col(V)	Type V collagen
COPD	Chronic obstructive pulmonary disorder
CRP	Complement regulatory protein
Crry	Complement receptor-1 related protein y
cTEC	Cortical thymic epithelial cell
DAF	Decay-accelerating factor

DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
FEV1	Forced expiratory volume
GPI	Glycosylphosphatidylinositol
IgE	Immunoglobulin E
IFA	Incomplete Freund's adjuvant
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-9	Interleukin 9
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-17A	Interleukin 17A
IL-21	Interleukin 21
IL-22	Interleukin 22
IL-23	Interleukin 23
IPF	Idiopathic pulmonary fibrosis
i.v.	Intravenous
MAC	Membrane-attack complex
MASP	Mannose-binding lectin-associated proteases
MBL	Mannose-binding lectin
MBP	Major binding protein
MCP	Membrane cofactor protein
MHCII	Major histocompatibility complex II

mTEC	Medullary thymic epithelial cell
NK	Natural killer cell
OB	Obliterative bronchiolitis
OVA	Ovalbumin
PAMP	Pathogen-associated molecular protein
PBS	Phosphate buffered saline
RORγt	RAR-related orphan receptor gamma t
RT-PCR	Quantitative reverse transcription polymerase chain reaction
STAT	Signal transducer and activator of transcription
TGF-β	Transforming Growth Factor beta
Th	T helper cell
Th1	T helper 1 cell
Th2	T helper 2 cell
Th17	T helper 17 cell
TNF-α	Tumor necrosis factor alpha
Treg	T regulatory cell

INTRODUCTION

The Immune System

Many organisms have developed defense systems to protect themselves from threats. In many cases this means thick armor, claws or other such structures. However, these very physical features provide no such protection against the greatest threat that vertebrates must deal with, the threat of microorganisms and viruses (1).

The human body constantly exists in an environment filled with many types of organisms. Some of these organisms can be harmful while others can be beneficial to humans. Remarkably, many of these organisms that co-exist with humans can cause severe bodily harm when the health of the individual is compromised. An interior line of defense, after pathogens have penetrated the exterior defenses is the immune system. It is through this system that humans can survive in this harsh environment and thrive even if the pathogens enter the body.

The immune system is divided into separate categories that perform distinct tasks in the fight against the body's foreign invaders. Thus, the immune system employs many levels of protection for the body (1). The first line of defense involves the body's largest immune organ, the skin and mucous membranes. These tissues function like a shield system, providing a physical barrier that microscopic organisms must overcome to enter the body. Optimally, the skin will prevent invaders from entering and the mucous membranes will trap any particles; those ideal conditions do not always exist and pathogens can penetrate these protective barriers.

If the invading organisms penetrate the first defenses the body possesses, they must then activate the next line of defense, specific and nonspecific immune defenses. The nonspecific cells function as the first wave of a large group mobilized by the body. The cells circulate within the body as roaming patrols and respond to any penetration of the body's outer defenses, without determining the identity of the invader. Examples of nonspecific defenses include: macrophages, which are cells that kill microbes by ingesting them; and neutrophils, which are cells very similar to macrophages which also ingest foreign microbes. Eosinophils also make up a part of the nonspecific defenses by each of these cells produce chemicals that kill internalized bacteria although neutrophils have a higher capacity for producing these toxic substances. Natural killer cells (NK cells) are another type of white blood cell and a member of the initial salvo activated by the body's defenses. NK cells kill infected cells. Other cells that help with nonspecific immune defenses include: monocytes, mast cells, basophils, eosinophils and granulocytes.

In the family of cells that make up the immune system, there are two types of cells that do not ingest cells, but are extremely critical to the survival of the organism and the species; T and B lymphocytes. T and B lymphocytes originate in the bone marrow. B cells remain in the bone marrow and mature, while T cells migrate to the thymus for further development. Receptors on T cells recognize ligands on other cells and initiate or regulate immune responses. These cells are thought of as the conductors of the adaptive immune system, the cytokines that are produced by these cells have far reaching implications on other cells of the immune system and the surrounding cells in general (Figure 1) (2). Th1 cells provide protection in response to intracellular pathogens, but

cause autoimmunity and delayed hyper-sensitivity. Th2 cells have been shown to provide protection against extracellular pathogens, and are associated with allergy and asthma. Th17 cells, like Th2 cells have also been associated with extracellular pathogens, autoimmunity and more recently with asthma. Finally T regulatory cells (Treg) are cells that are associated with immunosuppression of cellular responses.

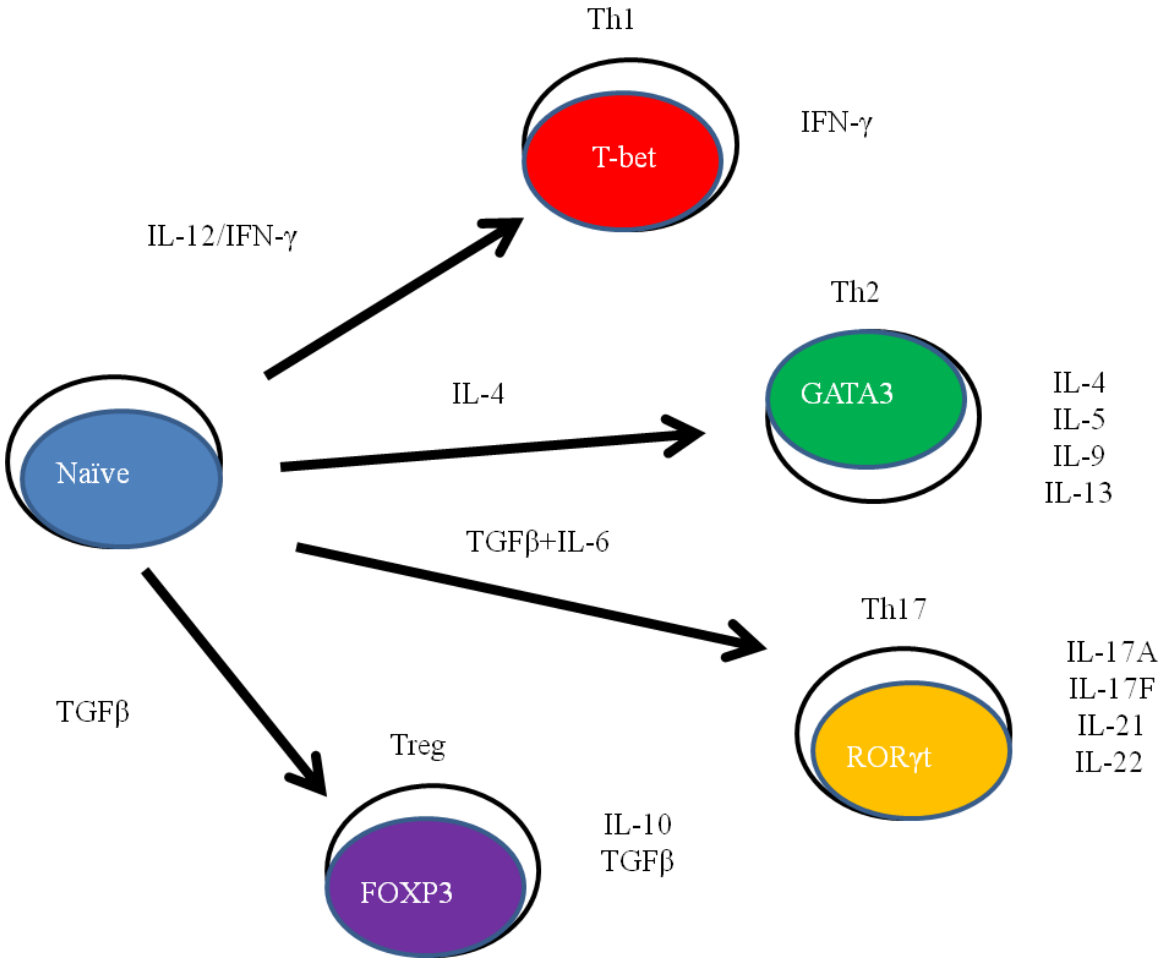

Figure 1. T cell Subsets. Naïve Th cells can differentiate to various Th subsets in the presence of specific cytokines. In the presence of these cytokines and under the control of STAT proteins and various transcription factors these cells are able to differentiate and affect many types of situations. Adapted from O'Shea and Paul; Science, 2010 (2).

B lymphocytes function as APCs by binding native or denatured antigens with their receptors which promotes the internalization of these antigens. Once internalized, these antigens are processed and presented by MHC molecules on the B cell surface. Each of these different cells works together to form overlapping layers of protection for the body. When the entire system is working in harmony, it provides a cellular network of agents that help defend the organism against native or foreign foes.

Allergic asthma

Allergic asthma, is one of the most common long term diseases affecting more individuals annually. In 2009, it was estimated that asthma prevalence was 8.2% of the United States population or about 24.6 million individuals (3). Asthma has been defined as a leading cause of obstructive lung disease, which is characterized by inflammation, mucus secretion, and lung remodeling (4). The classical symptoms of asthma include: airway constriction, cough, dyspena (shortness of breath), and wheezing. The symptoms of asthma are a consequence of a number of physiological changes that occur within the body of the asthmatic. These include: airway inflammation, intermittent airway obstruction, bronchial hyper-responsiveness, mucus hyper-secretion, smooth muscle hypertrophy, and remodeling of peribronchiolar connective tissues (5). The mediators of asthma are varied and include genetic, lifestyle and environmental factors, such as pollutants and microorganisms that work to augment or inhibit allergen sensitization (6). It is also thought that the combination of genetic and environmental factors play an important role, since all individuals are exposed to allergens and other sensitizing agents in the environment.

The classical schema of asthma pathogenesis is highlighted in (Figure 2). Initially, the inhaled allergen is taken in and processed by antigen presenting cells (APC) and presented in the context of major histocompatibility complex (MHC) II. APCs then present this antigen/MHCII complex to naïve T cells which then causes them to differentiate into Th cells, in this case the cells would differentiate mainly into Th2 cells. From there, a complex cascade of cytokines known as interleukins (IL) mediate the disease progression by acting on other cells. IL-4 and IL-13 produced by T cells, promotes antibody generation specifically IgE production from plasma cells. IL-13 that is produced by Th2 cells, acts on epithelial cells that cause the production of mucin proteins. At the same time IL-9 activates mast cells priming them for recruitment and IgE binding to the surface of the mast cells. This allows the inhaled allergen to crosslink the IgE antibodies on the mast cell surface and cause degranulation of the mast cells, releasing various mediators including histamine and leukotrienes which are able to cause smooth muscle contraction. At the same Th2 cells produce IL-5 which activates eosinophils. This causes eosinophilic inflammation and allows for the production of more leukotrienes and major basic protein (MBP) which can cause inflammation and increased airway hyper-responsiveness (AHR) (7).

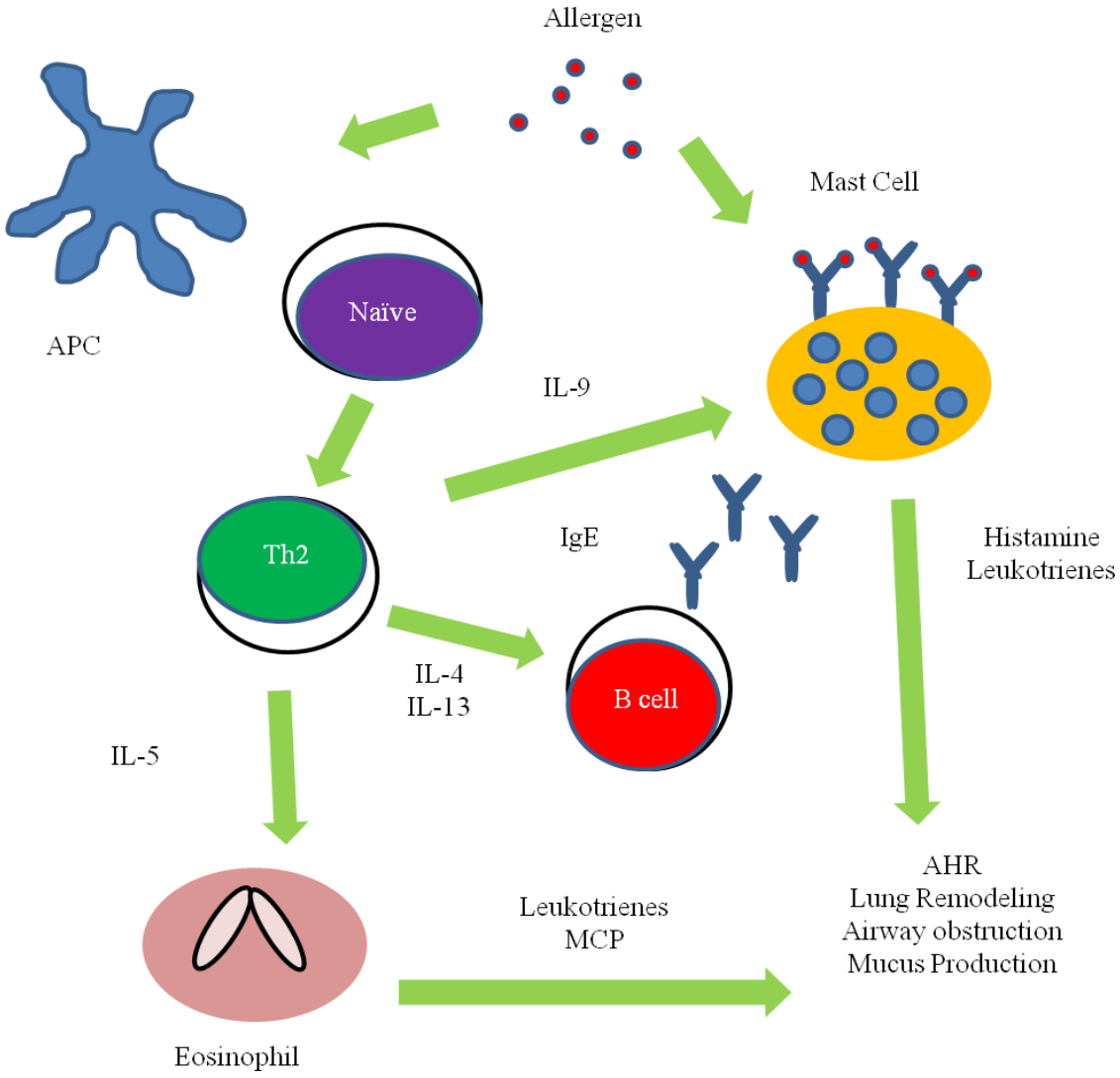

Figure 2. Schematic of Th2 mediated development of allergic asthma. Adapted from Nature Review Immunology, 2008 (7).

Th2 Cells

T helper 2 (Th2) cells are the cell most identified with allergic asthma and its murine model allergic airway disease (AAD). These cells are thought to be one of the first to initiate the disease and continue disease progression in individuals. Th2 cells secrete the cytokines IL-4, IL-5, and IL-13, which are critical in the pathogenesis of asthma. The development of Th2 cells begins with naïve T cells, Th cells, which in the presence of IL-4 and T cell receptor activation differentiate into Th2 cells. IL-4 is required for Th2 differentiation; however, the exact source of IL-4 has not been fully elucidated. Once under the control of IL-4, the cells utilize the transcription factor GATA3. GATA3 is known as the main transcription factor of Th2 development (8, 9). The necessity of GATA3 was shown by its deletion which prevented the production of IL-4 and Th2 differentiation (9, 10). Other important components of T cell differentiation include proteins known as signal transducers and activators of transcription (STAT) proteins. These proteins function in the induction of the major gene regulators and work with those regulators to produce cytokines. In the case of Th2 cells, two STAT proteins, STAT6 and STAT5 have been shown to be involved in Th2 cell development. STAT6 is the major STAT involved in IL-4 generation and in Th2 commitment (11). STAT5 has also been shown to play a role in Th2 differentiation, and also with respect to GATA3 expression (12, 13). Interestingly, because all Th cells develop from naïve cells, it is necessary to prevent them from expressing other cytokines. Studies have shown that GATA3 blocks the expression of Th1 cytokines by committed

Th2 cells (14). Th2 cells have been linked to several diseases, however the most well known are those that involve allergy. These cells also provide protection against extracellular pathogens.

Th17 Cells

Asthma and other inflammatory and autoimmune diseases have been linked to a relatively new class of T helper cell, Th17. Th17 cells secrete a number of cytokines, including IL-17A, IL-17F, IL-21 and IL-22. These cells have been linked to the recruitment of neutrophils, due to the actions of IL-17A and IL-17F (15, 16). Murine studies have shown that Th17 cells are generated in the presence of IL-6 and TGF-β (17, 18). Several other cytokines have been shown to induce Th17 development, IL-21 in cooperation with TGF-β can also induce Th17 cellular differentiation (19, 20). IL-23 was originally thought to promote Th17 generation, however recent research shows a more specific role for IL-23, maintenance of the phenotype of Th17 cells (21, 22). The studies involving IL-23 as a mediator of Th17 development continue to evolve (23). As with other Th subtypes TH17 cells express a major regulator gene, the orphan receptor RORγt (24, 25). Studies have shown in mice that are deficient for RORγt or IL-6, lack Th17 cells (24). Recent studies have highlighted the importance of RORγt to Th17 differentiation, Solt et al. proved that inhibition of RORγt by synthetic ligand prevented Th17 development (26). STAT proteins were described earlier as integral members of T cell signaling pathways, STAT3 functions in that role for TH17 cells. Studies using

STAT3 deficient mice demonstrated RORγt expression was regulated by STAT3 (27). Th17 cells have been linked to a number of diseases that were at one time thought to be regulated by only Th1 or Th2 cells.

Cytokines associated with asthma

IL-4

Interleukin 4 (IL-4) is a type I cytokine that plays an important role in various diseases, especially that of asthma. The main source of IL-4 arises from $CD4^+$ T cells; however, basophils and mast cells also produce quantities of IL-4. The main functions of IL-4 include the differentiation of naïve T cells into Th2 cells, and the induction of class switching by B cells to produce IgE (28, 29). Because of its role in promoting Th2 cell differentiation, IL-4 is also responsible for the production of the other Th2 cytokines. However these are not the only functions of IL-4. Receptors for IL-4 are widely expressed on a number of cell types such as hematopoietic and non-hematopoietic cells including: epithelial, endothelial, muscle, and fibroblasts (30). Studies have shown a link between IL-4 and the hallmark pathological signs of asthma, such as the induction of AHR and goblet cell metaplasia (31, 32). These signs of asthma were shown to be mediated by other cytokines besides IL-4; goblet cell metaplasia was shown to be heavily regulated by IL-13, which will be discussed in the following section. On the front of AHR, Cohn et al. demonstrated the generation of AHR independent of IL-4 (33). However, even with all of this information, IL-4 is still a potent cytokine that functions in inflammation and tissue adhesion and has various other cellular effects (34).

IL-13

Interleukin 13 (IL-13) is a 17kDa glycoprotein that is mainly produced by $CD4^+$ Th2 cells (35). Although IL-13 is primarily produced by Th2 cells, other T cell types have also been shown to produce the cytokine. Wills-Karp et al. demonstrated the role that IL-13 plays in the pathogenesis of allergic asthma. By blocking IL-13, Wills-Karp et al. were able to block AHR in mice and were able to also reverse AHR in established AAD models (36). In relation to asthma in humans or AAD in mice, IL-13 has been shown to be the key mediator in one of the main symptoms of both diseases; that of goblet cell metaplasia and mucus production (37, 38). Much of the focus on IL-13 has been in relation to asthma, however, IL-13 is been shown to be involved in other diseases. As reviewed by Wynn, these diseases include intracellular parasites, various types of cancer, fibrosis and forms of tissue remodeling (39).

IL-17

Interleukin 17 (IL-17) is actually a family of cytokines that includes several members. The family includes IL-17A (which is known as IL-17), IL-17B, C, D, E, and F. IL-17s are mainly produced by Th17 cells; however, their production is not limited exclusively to Th17 cells. The link between IL-17 and diseases is an ever evolving subject; the number of diseases associated with changes in IL-17 production continues to increase. IL-17 has been linked to the following diseases: multiple sclerosis, various skin diseases, and arthritis (40). IL-17 and its relationship to asthma began through its effect on neutrophils recruitment (15, 16). Previously it was shown that IL-17 could alter AHR through other cells, however, new research by Kudo et al. shows IL-17A can alter

smooth muscle contraction directly (41). In severe forms of asthma, IL-17A and IL-17F have been found to be expressed in the airways of asthmatics (42). Recently, studies have been performed that show a link between two cytokines involved in the progression of asthma. Newcomb et al. were able to demonstrate for the first time that a functional IL-13R is expressed on $CD4^+$ Th17 cells (43). These studies prove that IL-13 could down-regulate the ability of Th17 cells to produce IL-17, by using IL-10; interestingly this alteration in IL-17 has now been seen in human Th17 cells also (44, 45). In terms of other diseases associated with interstitial lung remodeling, IL-17 has been shown to be involved in col(V) specific responses in terms of lung allograft rejection (46, 47). Fan et al. also demonstrated that blocking IL-17 prevents lung rejection in minor mismatched animals (48).

Other cytokines associated with asthma

Various other cytokines have been shown to be involved with the pathogenesis and regulation of asthma. Interleukin 5 (IL-5) and interleukin 9 (IL-9) are two cytokines that play roles in asthma. Generally IL-4, -13, and -17 are the main cytokines associated with asthma; however, these cytokine also take part in both asthma and AAD. IL-5 has been shown to be involved in eosinophil development and function. IL-5 is produced by a wide variety of cells, including T cells, mast cells, and eosinophils (49). IL-9 is secreted by cells similar to those that secrete IL-5. The main function of IL-9 as it relates to asthma is that of mast cell function and also augmenting IgE production from B cells (49). Studies have shown a possible role in mucus expression, specifically in up-regulating mucin expression (50).

Type V Collagen

Collagens are a group of proteins that are characterized as the most abundant protein in the human body. Collagen chains are composed of Gly-X-Y sequence, in many instances with X = proline and Y = hydroxyproline. This repetitive amino acid sequence allows the collagen to assume the well-known triple helical structure (51). Type V collagen [col(V)], is a minor fibrillar collagen that is expressed in abundance in the lung, skin, liver and placenta. This distinct form of fibrillar collagen was first isolated in 1976 (52, 53). The collagen molecule has been shown to possibly contain 3 distinct chains; however, other studies have shown that this molecule can exist as several isoforms. These isoforms can manifest themselves in various combinations such as: $\alpha1(V)_2\alpha2(V)$ heterotrimer, $\alpha1(V)\alpha2(V)\alpha3(V)$ heterotrimer, $\alpha1(V)_3$ homotrimer. The main function of col(V) in the body is that of tissue elasticity and compliance (54). In the lung, col(V) is present in the peribronchiolar connective tissue, alveolar interstitium, and capillary basement membrane (55, 56). Type V collagen has become interesting to researchers in relationship to several diseases, which include mutations in col(V) which can possibly lead to abnormalities in the cornea and eyelid (57). Studies have also been conducted that demonstrated that col(V) was able to regulate the expression of apoptotic and stress genes in breast cancer cells (58).

Relationship of col(V) to diseases associated with interstitial remodeling

The recognition of self-peptides as foreign antigens by the immune systems has been demonstrated in several instances of organ transplants for example, hsp60 in relation to skin allografts was shown to increase rejection and also myosin increased the

rejection responses in cardiac transplants (59-61). Antigenic properties have also been demonstrated for several types of collagen molecules, such as the usage of type II collagen [col(II)] in the collagen-induced arthritis model (62-65). Previous studies have demonstrated the involvement of col(V) in the pathogenesis of lung allograft rejection and the development of obliterative bronchiolitis (OB).

The non-reversible obstructive lung disease known as OB is characterized by fibroproliferation and abnormal tissue remodeling. These obstructions by way of the formation of scars in the small airways and thickening and sclerosis of vessels (66). The gradual development of these obstructions leads to decreased airflow through these necessary airway passages (67). Studies have demonstrated the involvement of col(V)-mediated immunity in the pathogenesis of allograft rejection (68, 69). The involvement of col(V) in lung diseases has not been limited to transplant rejection, other studies have shown a relationship between col(V) and idiopathic pulmonary fibrosis (IPF) (46). Recently studies have demonstrated a link between col(V)-immunity and IL-17 in lung transplantation (47, 70). Using mice deficient in apolipoprotein E, researchers were able to show a link between IL-17, col(V), and atherosclerosis (71).

Tolerance

Immunologic tolerance is defined as the unresponsiveness immunologically to a given antigen. This process is one way the body eliminates or neutralizes auto-reactive cells. The concept of tolerance is generally divided into two types: central and peripheral tolerance. Central tolerance occurs in the thymus as T cells are educated and selected based on their ability to respond to T cell receptor (TCR) stimulation. During

the process, double positive cells that are $CD4^+CD8^+$ are stimulated through their TCR; those that do not respond are allowed to die by neglect. Those cells that respond to the signal are then positively selected by responding to a low affinity signal. Finally, the single positive cells, either $CD4^+$ or $CD8^+$ are allowed to undergo negative selection. This is accomplished by APCs; specifically proteins are expressed in the context of MHCII on the surface of cortical thymic epithelial cells (cTEC), medullary thymic epithelial cells (mTEC) and DCs that reside in the thymus. These cells present self peptides and other peptides to developing T cells. Immature T cells that respond to self peptides with high affinity are deleted by apoptosis (72). Another method of tolerance the body uses is that of peripheral tolerance. Reactive cells that escape central tolerance are controlled by peripheral tolerance, which comprises various cells types. These cells include Tregs which produce the inhibitory cytokine IL-10 or function by cell-contact mediated regulation. Another method of regulation is by the use of suppressor DCs and other myeloid derived suppressor cells (MDSC) (73). These cells can function through the regulation of the inhibitory molecules that are present along with co-stimulatory molecules during TCR/MHCII binding (74). Various models of disease have been used to test different methods to induce immune tolerance, these methods include: oral, intranasal, subcutaneous, and intravenous administration of antigen.

Methods of Tolerance Induction

Oral Tolerance

Tolerance to antigens has been utilized by researchers for the study of diseases. One such route of tolerance induction is that of oral tolerance. The gut associated lymphoid tissue (GALT), is the largest immune organ in the body. Because of this the digestive tract is exposed to a multitude of antigens that can elicit various responses [reviewed by(75)]. The mechanism of oral tolerance can be divided into two pathways dependent on the amount of antigen used. Low doses of antigen function through the generation of Tregs that regulate the behavior of other cells through the use of cytokines such as TGF-β or cell-contact mediated regulation. When high doses of antigens are utilized, cellular anergy or deletion is elicited to blunt or block the immune system (76). Various studies have used the oral route of tolerance induction, including the suppression of arthritis (77). In terms of col(V), studies performed using oral tolerance have been able to prevent lung allograft rejection (78-80). These studies lead to analogous approaches using col(V) to promote lung transplant engraftment. In these later studies that $CD4^{+}CD45RC^{high}$ Treg, which are rat $CD4^{+}$ cells that upon stimulation produce IL-2 and IFN-γ and mediate alloreactive cells (81, 82). Tregs which produced TGF-β were one of the cell types that mediated col(V)-induced tolerance (83).

Intranasal Tolerance

Tolerance can be induced by intranasal exposure or nebulization of antigens. Tolerance induced nasally, benefits by using one of the largest mucosal surfaces of the body. The study of nasal tolerance is very interesting with possible application in the

field of asthma research and asthma treatment. Studies have shown, especially in regards to the cells that possibly mediate this tolerance that $CD4^+$ cells may play an essential role in this regulation. Unger et al. and demonstrated using nasal application of OVA, that $CD4^+CD25^-$ Treg cells were able to mediate tolerance that was transferrable to naïve mice (84). Pellaton-Longaretti et al., further demonstrated the usefulness of intranasal tolerance with respect to asthma and Treg cells which express membrane bound TGF-β (85). Tolerance mediated by nasal or IV administration of a variety of antigens may be mediated by induction of IL-10 (86).

Intravenous Tolerance

Intravenous (i.v.) tolerance is another method of tolerance induction. Many times, this is done before a specific treatment or induction of a disease state. Using models of collagen-induced arthritis (CIA) in both mice and rat studies, demonstrated the effectiveness of col(II)-induced tolerance has been demonstrated (87, 88). Gumanovskaya et al. highlighted the fact the col(II)-induced tolerance caused the increase of IL-4 and IL-17 which lead to decreases in disease severity (88). Studies involving multiple sclerosis have also utilized i.v. tolerance. Warren et al. demonstrated the induction of tolerance to myelin basic protein. Long term tolerance was also observed in these patients (89). Many studies using experimental autoimmune encephalomyelitis (EAE), the murine model of multiple sclerosis; have harnessed the usefulness of i.v. tolerance in the study of the disease. Studies by Li et al. and Zhang et al. demonstrated the importance of DCs in tolerance induction and the deleterious nature of IL-12 in disease progression in EAE (90, 91). In the context of col(V), studies have

demonstrated col(V)-induced tolerance can be accomplished using i.v. administration of col(V) (92). Using a mouse model of bleomycin-induced pulmonary fibrosis, researchers were able to utilize i.v. inject col(V) to prevent fibrosis by inhibiting IL-6, -17 production (93).

Complement

The complement system was identified over 100 years ago as a component of the innate immune system. The innate immune system of the human body is involved in the recognition of foreign pathogens through the recognition of pathogen-associated molecular patterns (PAMP) (94). Complement is responsible for recognizing foreign pathogens and facilitating the removal of these pathogens (95). The main components in complement involve more than 30 serum and membrane-bound proteins that aid in the clearance of microorganisms (96). This system is mainly activated by antibodies and mannan-binding lectins on the surface of bacteria (97). Complement itself works through the proteins that function in a cascade, amplifying the function of the previous components of the cascade (98). The majority of complement proteins are synthesized in the liver by hepatocytes, however, some proteins are formed on site in the tissues (99). After formation, most complement proteins circulate in the serum in an inactive form. These complement proteins include: serine protease, and regulatory molecules (100, 101). Complement itself is divided into three separate pathways: the classical pathway, the alternative pathway and the mannose-binding lectin pathway. Each pathway is activated by the binding of the respective receptor to the target pathogen.

Complement Pathways

The classical pathway is activated upon binding of an antibody to the pathogen surface (102). Upon ligand binding, a series of proteins are activated called the C1 complex (this consists of C1q, two molecules of C1r and C1s) that binds to the IgM antibody, in turn bound to the surface of the bacterial cell. The C1 complex cleaves the protein C4 into C4a and C4b; C4b then binds to the surface of the bacteria. The C1complex also cleaves C2 into C2a and C2b. C2a then binds C4b forming C4b2a which is also known as the C3 convertase.

The mannose-binding lectin (MBL) pathway is activated by the binding of mannose residues and other sugars on the surface of bacterial cells. This pathway has similarities to the classical pathway in the fact that the initial protein has a structure that resembles the C1q protein and has similar functions to the C1 complex of the classical pathway. MBL binds to the serine proteases mannose-binding lectin-associated proteases 1 and 2 (MASP1 and MASP2). The proteins C4 and C2 are also present in this pathway; MASP2 is responsible for the cleavage of both proteins. The next step is the binding of C4b and C2a and the formation of the C3 convertase.

The alternative pathway is the final pathway in complement; however, this pathway does not depend on the pathogen-binding proteins or the binding of surface sugars as is required by the other two pathways. The alternative pathway is activated by the spontaneous hydrolysis of C3 into C3a and C3b. C3b then binds to the foreign surface (103). After binding, the serum protein Factor B binds to C3b; Factor B is then cleaved into Ba and Bb by the protease Factor D. The complex of C3bBb is then also known as the C3 convertase.

The convergence point of the three pathways is the assembly of the C3 convertase. All three pathways of the complement system have C3 convertases that are formed by differing enzymatic activities. The function of the C3 convertase is that of cleaving the serum protein C3 into two components, that of C3a and C3b. The components of C3 play very different roles; C3a is known as an anaphylotoxin and is involved in the induction of inflammation, while C3b functions in the complement cascade. C3b joins the C3 convertase formed by the classical, MBL pathways (C4b2a) or the alternative pathway (C3bBb) to form the C5 convertase. The C5 convertase is another serum protease that cleaves the protein C5 into two components, specifically C5a and C5b. Similar to the components of C3, C5a is involved in inflammation while C5b functions in the complement cascade. C5b binds to the serum protein C6 and initiates the formation of the membrane-attack complex (MAC). The MAC forms from the binding of C5/6 to several serum proteins: C7, C8, and C9. The fully formed MAC then forms a pore in the surface of the target bacteria; this pore then allows diffusion of small molecules and water to alter the osmotic pressure within the bacteria (104, 105).

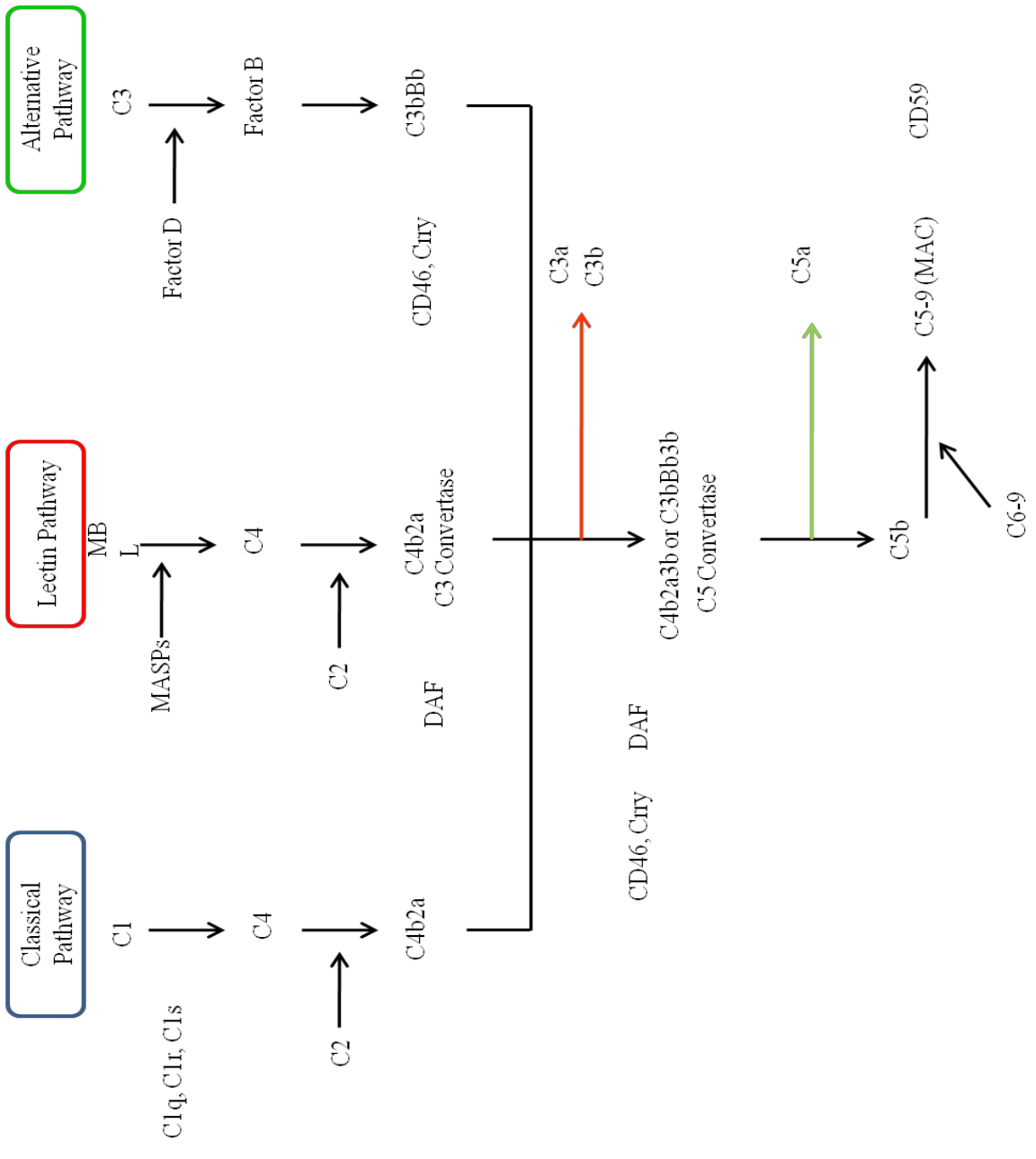

Figure 3.The complement cascade, adapted from Lalli et al.; Frontiers in Bioscience (Scol Ed), 2009 (105).

Complement was once thought of as strictly a component of the innate immune system; however, that idea is beginning to shift. There is mounting evidence that complement plays an increasingly prominent role in adaptive immunity.

Anaphylotoxins (C3a and C5a)

The complement cascade is an important part of the innate immune system; a by-product of the complement cascade is the production of anaphylotoxins. C3a and C5a are glycosylated peptides with an approximate molecular weight of 8,000 kDa (106). C3a and C5a cause inflammation by the activation and recruitment of inflammatory cells (107). As stated earlier, the major site of C3 synthesis is the liver. C3 is also produced in sites outside of the liver including: macrophages, kidney epithelial cells, and umbilical vein endothelial cells (108-110). The serum protein C5, like C3; is produced in various sites in the body such as the bone marrow, spleen and fetal tissues (111-113). The products of the cleavage of C3 and C5 are that of C3a, C3b, C5a and C5b.

The main function of C3a and C5a are carried out by the binding of these peptides to their respective receptors. Receptors for C3a and C5a are found on various cell types including, peripheral mononuclear cells, macrophages, platelets and mast cells (114, 115). These receptors are now known to be expressed on non-immune cells. Chemotaxis of cells has been described as one of the main functions of anaphylotoxins on cells. The effects of C3a and C5a on $CD4^+$ T cells include CD28 co-stimulation signals (116). The main effect of C3a and C5a is that of inflammation, they have however been linked to

many diseases. They have been implicated in: sepsis, acute lung injury, arthritis, inflammatory bowel disease, and chronic obstructive pulmonary disorder (COPD) (117-119).

Complement regulatory proteins

As stated earlier, complement, like many other components of the immune system can affect both infected and healthy cells. Due to the destructive potential that complement possesses is it necessary to control and regulate this process. The complement system has certain regulatory mechanisms built into to help prevent this and control the enzymatic processes involved in the cascade. These regulatory molecules are known as complement regulatory proteins (CRP). These proteins are present throughout the entire complement cascade; however, the majority of them are concentrated around the C3/C5 steps of the pathway. The CRPs include; CD55 (decay accelerating factor), CD46 (membrane cofactor protein).

CD55/DAF

CD55, also referred to as decay accelerating factor (DAF) is a glycosylphosphatidylinositol (GPI)-anchored protein that is tethered to the cellular membrane by the GPI anchor (120). This approximately 70 kDa protein is mainly responsible for the dissociation of the C3 and C5 convertases; this function explains the non-cluster of differentiation name (121). An accelerated dissociation of the C3 and C5 convertases helps regulate their activity in both the classical and alternative pathway convertases by preventing the amplification of the cascade. CD55 also works to shorten

the half-life of the preformed convertases. CD55 is a widely expressed protein, which is present on all blood cells, vascular endothelium and epithelial cells. Although, the main function of CD55 is that of complement regulation, however, other effects of CD55 have been shown. One interesting effect of CD55 is the regulation of T cell immunity; CD55 deficient mice have increased T cell responses following immunization (122).

CD46/MCP

CD46, also referred to as membrane co-factor protein (MCP) is another membrane bound CRP tasked with regulating the complement cascade. Structurally, CD46 varies vastly from CD55, while CD55 is a GPI-anchored protein; CD46 is a type 1 transmembrane protein that is able to signal intracellularly. This 51-68 kDa protein, like DAF is expressed on a wide variety of cells in the human body (123). CD46 functions as a regulatory protein at the level of the C3 and C5 convertases in the complement pathway. Unlike CD55 which functions to accelerate the decay of the convertases, CD46 works to prevent their formation. CD46 functions as a cofactor that binds to constitutively active serine protease known as Factor I (124). The regulation occurs by inactivating the deposited C3b and C4b on the surface of cells.

CD46 is expressed in humans; however, this surface protein is not expressed in murine cells. The only expression of CD46 in mice was observed in the testis (125). There is, however, and orthologue of CD46 in murine systems, termed complement-receptor-related protein-y (Crry). Like CD46, Crry is a transmembrane protein of approximately 65 kDa in mass, which possesses similar regulatory properties of CD46 and is widely expressed in most murine tissues (126, 127). Interestingly, further study

into the function of Crry demonstrated a dual role for the protein; that of both CD46 and CD55 functions in regulating both the C3 and C5 convertases (127, 128).

Role of Complement in Disease

CRPs play a critical role in the regulation of complement; however, links to diseases have been shown to be both protective and deleterious. In terms of transplant immunology, Pavlov et al. demonstrated the lack of DAF accelerated rejection of cardiac allograft in a T cell-mediated dependent manner using DAF deficient cardiac allograft or DAF deficient recipients (129). In relation to allograft rejection, studies have shown C3a and C5a possess the ability to allow CD4 and CD8 interaction to bypass co-stimulatory molecules (130). Other studies using models of organ transplantation such as kidney, trachea, and ischemia/reperfusion have also shown the involvement of complement (131-133). Studies have also shown the involvement of CD55 in relation to tumor cells, CD55 is up-regulated on the surface of tumor cells: breast cancer, colorectal, and thyroid (134, 135). In terms of cellular activity on various cells, CD46 and DAF have been shown to possess the ability to regulate T cell activation (136, 137). The alternative pathway has been shown to initiate antibody-induced arthritis in mice; inhibition of the alternative pathway prevents inflammation in experimental arthritis (138, 139).

Role of Complement in Asthma

The relationship of complement and asthma has been investigated thoroughly through the years. The main portions of complement are involved in host defense against pathogen infections, however, the by-products of the pathway C3a and C5a have potent

inflammatory properties. Initial studies by Stimler et al. demonstrated the potency of both C3a and C5a in relation to pulmonary injury. These studies highlighted the fact that these potent mediators of inflammation caused contraction of the smooth muscle walls (140). Further studies continued to highlight the potential harmful impact of these inflammatory mediators (141). The involvement of C3a and C5a in asthma is explained further as bronchial epithelial and smooth muscle cells express receptor for both C3a and C5a (142). Because of the expression on these two major cells types that are involved in asthma, specifically in the difficulty in breathing, it makes sense that C3a and C5a would be involved. The presence of increased levels of the two peptides in both the BAL and serum of asthmatics and the increased expression of their respective receptors are also signs of the involvement of complement (143-145). Genetic studies of C3a and C5a genes have also shown a potential for increased risk of asthma development (146, 147). Additional work also shows influence from the allergens themselves. Allergens from particulate matter and house dust mite proteases have now been shown to either be directly involved or via by-products of their activities (148, 149). Both anaphylotoxins have been shown to be involved in the murine model of asthma termed allergic airway disease (AAD), specifically in the late phase of AAD (150-153). Previous studies demonstrated that C5a can mediate AHR. Interestingly, several studies have shown other possible roles. In established asthma, C5a may have detrimental effects, however, at earlier time points C5a has been shown to decrease symptoms of asthma depending on mouse strains (154-157).

Research Goals

Previous research by the Wilkes lab has shown the involvement of col(V)-mediated immunity in the pathogenesis of lung allograft rejection and other diseases associated with interstitial lung remodeling. Because of these noted observations, we sought to understand the role of col(V) in another lung remodeling disease, asthma. Our overall goals of this research were to gain an understanding into the possible involvement of col(V) in the pathogenesis of asthma. Specifically we had two goals of our research. The first goal was to determine the expression of col(V) in asthma using a cohort of volunteers. Our second goal was to elucidate the role col(V) may play in the asthmatic response. For this second goal the induction of tolerance was utilized to modulate the asthma response in a mouse model of asthma, allergic airway disease (AAD).

The initial studies also examined the expression of circulating anti-col(V) antibodies in a cohort of diagnosed asthmatics. Further studies more closely examined the effect of asthma on lung morphology and the expression of col(V) within the lungs. Using a model of asthma, AAD, the effect of col(V)-induced tolerance was examined using readouts of lung function and cytokine analysis. Finally, the effect of col(V)-induced tolerance on complement-linked regulation of AAD was assessed to determine the mechanism of col(V)-induced alterations in AAD.

Hypothesis

Col(V) and anti-col(V) antibodies are up-regulated in clinical asthma, and col(V) induced tolerance can modulate allergic lung disease.

MATERIALS AND METHODS

Human subjects

The Human Subject Institutional Review Board at Indiana University School of Medicine approved the study protocol used for sample collection. Blood was isolated from ten non-smoking adult normal volunteers, age 18-55. Adult, non-smoking asthma patients were recruited via the Asthma Clinical Research Center at the Indiana University School of Medicine and asthma was defined by a 12% increase in FEV1 post bronchodilator and positive methacholine challenge test as reported (158). Asthmatics were randomly selected without reference to stability of disease, or medication use, including corticosteroids. Adult, non-smoking asthma patient samples were also obtained from the Asthma Institute at the University of Pittsburgh Medical Center/University of Pittsburgh School of Medicine. Human tissue sections were harvested from a 35 year old female who died as a result of an asthmatic attack with inflammation of both lungs, and occlusion of bronchi and bronchioles by thick mucus plugs. Medications included: prednisolone 30mg twice daily, fluoxetine, conjugated estrogens and medroxyprogestrone tablets 0.625mg daily, alendronate 10mg once daily, albuterol, omeprazole, and calcium. Normal tissue was harvested from an individual who died from intracranial bleeding and whose lungs were harvested for transplantation but were not transplanted. All patient tissue samples were from National Jewish Health, Denver Colorado. These tissue samples were provided by Dr. Sally Wenzel (University of Pittsburgh School of Medicine).

Mice

Balb/c mice, 6-8 weeks old were purchased from Harlan (Indianapolis, IN) and maintained in a pathogen-free environment in the Laboratory Animal Research Care Facility at Indiana University School of Medicine (Indianapolis, IN). All animal studies were done in accordance to established institutional animal care usage guidelines.

Formulation of buffers

Complete and incomplete Freund's adjuvant

Complete Freund's adjuvant (CFA) was formulated using 85% Heavy Mineral Oil (Fisher Scientific, Pittsburgh, PA) and 15% Mannide Monooleate (Sigma Aldrich, St. Louis, MO). Using a mortar and pestle, 4mg/ml of heat killed *Mycobacterium tuberculosis* (Fisher Scientific, Pittsburgh, PA) was ground and added to the oil mixture. Incomplete Freund's adjuvant (IFA) was formulated by mixing 85% Mineral Oil and 15% Mannide Monooleate.

ELISA coating buffer

In a 1mL beaker, 10ml of 10x Phosphate buffered saline (PBS) is added to 90ml of ddH_20, this is the followed by the addition of 0.8401g of $NaHCO_3$. The mixture is then stirred with a stir bar until a pH of 9.0 has been achieved.

ELISA blocking buffer

In a 1L beaker, 50ml of 10x PBS is added to 450ml of ddH_20. To this solution, 5g BSA (bovine serum albumin) (Fisher Scientific, Pittsburgh, PA) is added. The mixture is then mixed with a stir bar and sterilized by filtration.

FACS buffer

In a 1L beaker, 20ml of 10x PBS is added to 150ml of ddH_2O. To this solution, 2g of BSA (Fisher Scientific, Pittsburgh, PA), 0.2g NaN_3 are added. The mixture is then mixed with a stir bar and stored for use at 4°C.

MACS buffer

In a 1mL beaker, 50ml of 10x PBS is added to 448ml of ddH_2O. To this solution, 2ml of 0.5M EDTA is added and stirred with a stir bar, followed by the addition of 2.5g of BSA (Fisher Scientific, Pittsburgh, PA). Following complete incorporation of the previous components, the solution is filter sterilized and degassed for approximately one hour.

Cell lysis buffer

In a 2L beaker, 8.29g NH_4Cl, 1g $KHCO_3$, 0.372g Na EDTA are added to approximately 1 liter of ddH_2O for a total volume of 1L, the buffer is then filter sterilized.

Washing buffer

In a 500ml bottle, 50 ml of 10x PBS is added to 450ml of reverse osmosis (RO) H_2O; to this 250 µl of Tween 20 (Sigma Aldrich, St. Louis, MO) is added and mixed.

Detection of circulating anti-Col(V) antibodies

Flow cytometric detection of anti-col(V) antibodies was performed as previously described (159). In brief, streptavidin-coated beads (5µm, binding capacity 10-20µg, 1×10^7 beads; Polysciences, Warrington, PA) were washed in PBS. Following washing, the beads were suspended in 100µl of bovine col(V) (40µg/ml) and after 60 min of incubation at 4°C, the beads were washed in PBS with 10% FBS and stored at 4°C. Beads were washed 2x in PBS and incubated in 100µl of PBS with 50µl of human or mouse serum and incubated for 30 minutes at room temperature. After incubation, the beads were washed with PBS/10% FCS buffer. The beads were then incubated with anti-human or anti-mouse PE conjugated IgG antibodies (Sigma Aldrich, St. Louis, MO), the beads were washed and analyzed on a FC500 MCL (Beckman Coulter, San Jose, CA).

Col(V) immunization

Six to eight week old Balb/c mice were immunized with either col(V) emulsified in complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA). The CFA/IFA mixture was then drawn up in a 2ml glass syringe (Popper & Sons Inc., New Hyde Park, NY) with an 18-G needle (BD & Co., Franklin Lakes, NJ). At the same time, 2mg/ml of bovine col(V) as the antigen in PBS was drawn up into another glass syringe or PBS alone. The needles were removed and air expelled, each syringe was attached to a double-ended locking hub connector (Popper & Sons Inc., New Hyde Park, NJ). The mixture was then forced back and forth from one syringe to the other repeatedly. Once an emulsified mixture had been achieved, homogenous and white, the syringes were disconnected and the emulsion tested. The emulsion is tested by placing one drop onto the surface of 50ml cold water. A good oil-in-water emulsion will hold together as a droplet. The mice were then sedated using ketamine cocktail, diluted 1:6 in sterile PBS. The area around the base of the mice's tail was then shaved to provide unobstructed access to the skin. Using 100µl of the mixture, a 30-G needle (BD & Co., Franklin Lakes, NJ) was attached to the end of the glass syringe and injected intra-dermally at the base of the tail. The 100µl of emulsion is equal to 100µg of col(V) per animal. Ten days after primary immunization, the mice were injected with IFA/col(V) emulsion or IFA/PBS mixture as the booster immunization. After an additional 10 days, the mice were then ready for use in further studies.

Col(V) intravenous injection

Six to eight week old Balb/c mice were injected intravenously with 100µg of bovine col(V) (A generous gift from ImmuneWorks, Indianapolis, IN) in PBS. Approximately 7-10 days later the mice were then used for further studies.

Induction of allergic airway inflammation

Mice were sensitized using intra-peritoneal (i.p.) injections of OVA (Sigma, St. Louis, MO) absorbed with alum (Sigma Aldrich, St. Louis, MO) at a dose of 20µg OVA/mg alum on days 0 and 7. On day 14, mice were intra-nasally challenged using isofluorane to temporarily sedate the animals, the animals were then treated drop-wise using a pipette to the nostrils with OVA at a dose of 100µg per day for 6 consecutive days. One day 20 or 21, mice were euthanized before blood, BAL, lungs, spleen, mediastinal and inguinal lymph nodes were harvested.

Measurement of airway hyper-responsiveness

Airway responsiveness to inhaled methacholine (10-300mg/ml) was assessed 24hr after final intranasal challenge of OVA. AHR was measured in non-restrained, conscious mice using whole body plethysmography (Buxco Systems, Wilmington, NC) and described as a dimensionless constant called enhanced pause (Penh) to characterize an index of airway resistance in treated animals. In a separate set of studies, AHR was measured by invasive determination of airway resistance and compliance (Buxco Systems, Wilmington, NC).

Mice were anesthetized and the trachea intubated, following which the animals were ventilated and nebulized methacholine was delivered intratrachelly, at doses of 5-200 mg/ml.

Isolation of lung mononuclear cells

Lungs from Balb/c mice were chopped into pieces using scissors and then incubated for 90 minutes at 37°C in deoxyribonuclease I from bovine pancreas (Sigma, St. Louis, MO) and collagenase D (Roche Diagnostics, Indianapolis, IN) diluted in complete RPMI with rotation. After collagenase/DNase digestion, the suspension was washed in RPMI and mononuclear cells isolated by density gradient centrifugation using 1.131g/ml Percoll (GE Healthcare, Piscataway, NJ). Total lung cells were resuspended in 1.075g/ml Percoll, the 1.03g/ml density Percoll was layered on top of the heavier density material. The cells were centrifuged for 20 min at 1,000 rpm with no brake. After centrifugation, the cells at the interface were isolated and washed with RPMI.

Isolation of murine splenocytes and total lymph node cells

Total splenocytes were isolated from the spleens and total lymph node cells were isolated from the mediastinal lymph nodes of 6-8 week old Balb/c mice. Spleens were homogenized with glass homogenizers and lymph nodes teased apart with bent 25-G needles to achieve a single cells suspension, following which red blood cells were lysed using NH_4Cl lysis buffer. After RBC lysis, the cell suspension was

centrifuged at 1,500 rpm for 5 minutes. Once cells were washed, they were resuspended and prepared for usage in experiments. Total splenocytes cultured with lymph node cells as APCs were irradiated at 3,000 rads.

Real-time PCR quantification of mRNA encoding cytokines

Lung mononuclear cells, total lung homogenates or splenocytes were isolated and total RNA extracted using RNeasy RNA extraction kit (Qiagen, Valencia, CA). Total RNA quantity and purity were assessed using the Nano Drop ND-1000 (Thermo Scientific, Wilmington DE). cDNA was synthesized using 500ng of total RNA with qScript^{TM} cDNA Supermix (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. mRNA levels were measured using real-time quantitative PCR and detected with PerfeCTa^{TM} SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD). The samples were run on an Applied Biosystems 7500 (Applied Biosystems) following manufacturer's directions, 1µl of cDNA was amplified for 40 cycles at 95°C for 15 sec, 60°C for 1 min. All samples were run in triplicate and normalized to the expression of murine β-actin and relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Primer	Sequence
IL-4	F: 5'ACAGGAGAAGGGACGCCA 3' R: 5'GAAGCCCTACAGACGAGCTCA 3'
IL-5	F: 5'AGCACAGTGGTGAAAGAGACCTT 3' R: 5'TCCAATGCATAGCTGGTGATTT 3'
IL-13	F: 5'AGACCAGACTCCCCTGTGCA 3' R: 5'TGGGTGCTGTAGATGGCATTG 3'
IFN-γ	F: 5' TGGCTCTGCAGGATTTTCATG 3' R: 5' TCAAGTGGCATAGATGTGGAAGAA 3'
IL-10	F: 5' GGTTGCCAAGCCTTATCGGA 3' R: 5' ACCTGCTCCACTGCCTTGCT 3'
IL-17a	F: 5' CTGTGTCTCTGATGCTGTTG 3' R: 5' ATGTGGTGGTCCAGCTTTC 3'
Muc5ac	F: 5' CCATGCAGAGTCCTCAGAACAA 3' R: 5' TTACTGGAAAGGCCCAAGCA 3'
Gob5	F: 5' ACTAAGGTGGCCTACCTCCAAG 3' R: 5' GGAGGTGACAGTCAAGGTGAG 3'
B-Actin	F: 5' CAAGTGATGACCGGCCGT 3' R: 5' AGAGGGAAATCTCGTGAC 3'

Table 1. Cytokine primer sequences used for qRT-PCR (160).

BAL cell surface staining and analysis using flow cytometry

BAL (bronchoalveolar lavage) cells ($0.5\text{-}1\times10^6$) were placed in flow cytometry tubes. Cells were centrifuged at 1,500 rpm for 5 minutes at 4°C, and washed once in FACS buffer (1% BSA, 0.01% NaN_3 in PBS). The buffer was removed and samples were incubated with FcγIII/II receptor (BD Biosciences, San Jose, CA) for 10 minutes at 4°C. Cells were then stained for MHC class II (clone# 2G9), B220 (clone# RA3-6B2), CD11c (clone# HL3), CD3 (clone# 145-2C11), and CCR3 (cat# FAB729P) using fluorochrome conjugated antibodies (BD Biosciences, San Jose, CA) and (R&D Systems, Minneapolis, MN) for 30 minutes at 4°C. Cells were then washed in 2ml of FACS buffer and resuspended in formaldehyde at a final concentration of 1%. Cells were then analyzed by flow cytometry using a FACS Calibur flow cytometer recording 20,000-30,000 events (BD Biosciences, San Jose, CA). The data was analyzed using BD Cell Quest Pro (BD Biosciences, San Jose, CA) (161).

Measurement of BAL cell number

BAL (bronchoalveolar lavage) cells were centrifuged for 5 minutes at 4°C, and the supernatant aspirated. The cells were then resuspended in 1ml of PBS and cells counted using trypan blue exclusion staining. Approximately, 10µl of cells were resuspended in 190µl of trypan blue, 10µl of the suspension was then placed on a hemocytometer and counted by light microscopy.

Proliferation assays

Total splenocytes were isolated from murine spleens of mice treated intravenously with either PBS or bovine col(V) followed by allergen induced inflammation. Cells were plated in the presence of either 100µg/ 1×10^6cells of OVA, 40µg/well of col(V), or anti-CD3 antibody (0.5µg/ml, BD Biosciences, San Jose, CA) in 200µl of cRPMI at 37°C for 72hours. Cultures were pulsed with ^{3}[H]-thymidine (0.5 µCi/well, Perkin Elmer, Waltham, MA) 18 hours prior to harvest. Plates were harvested using a Filter Mate Harvest (Perkin Elmer, Waltham, MA). ^{3}[H]-thymidine incorporation was measured by a Top Count NXT liquid scintillation counter (Perkin Elmer, Waltham, MA).

Total lymph node cells were isolated from mediastinal lymph nodes of mice treated intravenously with either PBS or bovine col(V) followed by allergen induced inflammation. Cells were cultured in the presences of absence of irradiated Balb/c APCs (3000 rads). Cells were plated in the presence of either 100µg/ 1×10^6cells of OVA, 40µg/well of col(V), or anti-CD3 antibody (0.5µg/ml, BD Biosciences, San Jose, CA) in 200 µl of cRPMI at 37°C for 72hours. Cultures were pulsed with ^{3}[H]-thymidine (0.5 µCi/well, Perkin Elmer, Waltham, MA) 18 hours prior to harvest. Plates were harvested using a Filter Mate Harvest (Perkin Elmer, Waltham, MA). ^{3}[H]-thymidine incorporation was measured by a Top Count NXT liquid scintillation counter (Perkin Elmer, Waltham, MA) (162).

Total IgE ELISA

Serum from mice treated intravenously with either PBS or bovine col(V) followed by allergen induced inflammation were incubated overnight at 4°C in a flat bottom 96 well plate that had previously been coated with 50µg/ml of IgE R35-72 (BD Biosciences, San Jose, CA) in coating buffer. They were then blocked at room temperature for one hour at room temperature. Following incubation, the plates were washed and samples and standards added. Standards were added in serial dilutions beginning with 1µg/ml, samples were added using a 10x dilution, and the entire plate was incubated at 4°C overnight. Following washing, 2µg/ml of biotinylated anti-IgE (BD Biosciences, San Jose, CA) was added to each well and incubated at room temperature for 2.5 hours. The plate was again washed and incubated for 1 hour at room temperature with avidin-alkaline phosphatase at a concentration of 1:2000. The wells are then incubated in phosphatase substrate (Sigma Aldrich, St. Louis, MO) at a concentration of 5mg/ml in substrate buffer. The plate was developed and then read at 405nm on a Spectra Max Plus (Molecular Devices, Sunnyvale, CA). Results were analyzed on Softmax Pro 3.1.2 software (Molecular Devices, Sunnyvale, CA).

OVA IgE specific ELISA

Serum from mice treated intravenously with either PBS or bovine col(V) followed by allergen induced inflammation were incubated overnight at 4°C in a flat bottom 96 well plate that had previously been coated with 50µg/ml of OVA (Sigma Aldrich, St. Louis, MO) in coating buffer and blocked at room temperature for one hour at room temperature. Following incubation, the plates were washed and samples and standards

added. Standards were added in serial dilutions beginning with 1µg/ml, samples were added using a 10x dilution, and the entire plate was incubated at 4°C overnight. Following washing, 2µg/ml of biotinylated anti-IgE (BD Biosciences, San Jose, CA) was added to each well and incubated at room temperature for 2.5 hours. The plate was again washed and incubated for 1 hour at room temperature with avidin-alkaline phosphatase at a concentration of 1:2000. The wells are then incubated in phosphatase substrate (Sigma Aldrich, St. Louis, MO) at a concentration of 5mg/ml in substrate buffer. The plate was developed and read at 450nm on a Spectra Max Plus (Molecular Devices, Sunnyvale, CA). Results were analyzed on Softmax Pro 3.1.2 software (Molecular Devices, Sunnyvale, CA).

Cytokine production by cytometric bead array (CBA)

BAL samples from treated mice were collected and stored and cytokine protein levels were measured using the Mouse Th1/Th2 Cytokine Kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Briefly, Mouse Th1/Th2 capture beads were mixed and 50µl were added to FACS tubes (Falcon 12×75mm 5ml round-bottom polystyrene tubes) (BD Biosciences, San Jose, CA). The sample BAL was then added to the tubes, this was followed by the PE detection reagent. Samples were washed and analyzed on a Beckman Coulter Flow cytometer using BD CBA software (BD Biosciences, San Jose, CA).

Hydroxyproline assay

Lung sections were isolated for the determination of the distribution of collagen which was monitored by hydroxyproline assay. Isolated lungs were weighed for wet weight and then placed in acetone overnight. The next day, the acetone was decanted and lungs placed in a 95°C oven for two days. Once the lungs were dry, the dry weight was determined followed by hydrolyzation in 1ml of 6N HCL overnight at 110°C. Samples were dried in a speed vacuum and brought up in 4ml of RO water. Using a 96 well plate, standards of trans-4-hydroxy-L-proline (0-1.0µg) were diluted and added to the plate. Samples were then added to the plate (final volume is 100µl/well). To the well, 50µl of 2.05M Chloramine-T solution was added and allowed to incubate for 20 minutes at room temperature. To this, 50µl of 3.15M Perchloric acid was added and incubated for 5 minutes at room temperature. Finally 50µl of 20% p-Dimethylaminobezaldehyde was added and the plate incubated at 60°C for 20 minutes. The plate was then read at 550nm and the amount of collagen calculated, 7.5 × (µg hydroxyproline) × (dilution factor if any) = µg of collagen (163, 164).

Histological analysis

Lungs were perfused, inflated, and fixed with neutral buffered formalin. The sections were paraffin embedded, sectioned, and stained with hematoxylin and eosin, Periodic acid-Schiff or Masson's trichrome (performed at Indiana University School of Medicine). Histological sections were visualized and acquired using light microscopy with 20x or 40x magnifications on an Olympus BX41 microscope and an Olympus DP12 camera (Olympus). Histology was scored in a blinded manner based on lung

mononuclear cellular infiltration (46). Mucus production was assessed on the PAS positive cells on the histological sections by measuring the color intensity of the staining of airways and the number of cells using Metamorph Imaging software (Universal) as previously reported (165).

Immunohistochemical analysis of mouse lungs

Mouse lungs were formalin fixed, paraffin-embedded and sectioned at 4µm. The tissue sections were de-waxed, hydrated and incubated in Rodent Decloaker (Biocare Medical, Concord, CA) at 95°C for 40 minutes in a pressure cooker (Biocare Medical, Concord, CA) to unmask the epitopes. The sections were blocked with 3% hydrogen peroxide for 10 minutes, followed by a 30 minute block in Rodent Block M (Biocare Medical, Concord, CA). After blocking, the tissues were incubated with either rabbit anti-mouse Crry (cat# sc-30214) 1.3µg/ml or rabbit anti-human CD55 (cat# sc-9156) 4µg/ml (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour, room temperature. The tissues were washed and incubated with Rabbit on Rodent HRP (Biocare Medical, Concord, CA) for 30 minutes, and developed using ImmPACT DAB peroxidase substrate (Vector Laboratoties, Burlingame, CA) for 3 minutes and then counter-stained with Gills hematoxylin (Vector Laboratoties, Burlingame, CA). Mucus production, CD55 and Crry staining was assessed by measuring the color intensity and number of stained cells of airways using a macro in the Metamorph Imaging software (Universal), as previously reported (166). The results were reported as AU, defined as arbitrary units calculated by dividing the IHC staining intensity by the airway perimeter.

Immunohistochemical analysis of human lungs

The human lung tissue was obtained from Dr. Sally Wenzel, University of Pittsburgh School of Medicine. The tissues were formalin-fixed, paraffin-embedded and sectioned at 4μm. The tissue sections were de-waxed, hydrated and incubated in DIVA Decloaker (Biocare Medical, Concord, CA) at 95°C for 40 minutes in a pressure cooker (Biocare Medical, Concord, CA). The sections were blocked with 3% hydrogen peroxide for 10 minutes, followed by a 30 minute protein block (BioGenex, San Ramon, CA). After blocking, the tissues were incubated with rabbit anti-collagen V (cat# 7046) at 10μg/ml (Abcam, Cambridge, MA) for 1 hour, room temperature. The tissues were washed and incubated with MACH 2 rabbit HRP-Polymer (Biocare Medical, Concord, CA) for 30 minutes and developed using ImmPACT DAB peroxidase substrate (Vector Laboratoties, Burlingame, CA) for 5 minutes and then counter-stained with Gills hematoxylin (Vector Laboratoties, Burlingame, CA).

C3a and C5a ELISA

Serum or BAL harvested from mice treated intravenously with either PBS or bovine col(V) followed by allergen induced inflammation were analyzed for the concentration of C3a and C5a fragments by ELISA. Briefly, wells of a 96-well maxi-sorb plate (Nunc, Gibco BRL) were coated overnight with either anti-mouse C3a or anti-mouse C5a (3μg/ml). After incubation with samples, wells were incubated with either botin rat anti-mouse C3a or anti-mouse C5a (1.25μg/ml). Samples were then incubated

with streptavadin-HRP (1:1,000) and OptiEIA TMB solution (reagents from BD Biosciences, San Jose, CA). Samples were read at 450nm (Molecular Devices, Sunnyvale, CA).

Measurement of tracheal smooth muscle cell contraction

Whole tracheas were immersed in physiological saline solution (PSS) (composition in mM: 110 NaCl, 3.4 KCl, 2.4 $CaCl_2$, 0.8 $MgSO_4$, 25.8 $NaHCO_3$, 1.2 KH_2PO_4, and 5.6 glucose). Six mm long segments were cut from each trachea and were attached circumferentially to Grass force-displacement transducers in a tissue bath at 37°C and bubbled with 95% O_2 and 5% CO_2. Tracheal segments were stretched to a preload tension of 0.2-0.3g and equilibrated for 1 hour. Force generated by the trachea during the equilibration period was determined (Unstimulated Contraction). Tracheal segments were then stimulated with acetylcholine (ACh) from 10^{-9} to 10^{-3} M for 5 minutes at each dose. Force generated by the muscle was measured after each dose. Values are means ± SEM (PBS, n = 2; Col V, n = 3, naïve, n = 2).

Statistical analysis

Data were analyzed by one-way or two-way Analysis of Variance (ANOVA) using Bonferroni Ad hoc test among multiple groups. Two-Tailed Student's *t*-test was used for comparisons between two groups. The non-parametric Mann-Whitney test was used to analyze col(V) antibody levels in defined asthmatics. All statistical analysis was done using Prism 4 (GraphPad Software for Windows, San Francisco, CA). *P* values of less than 0.05 were considered significant.

RESULTS

Part I: Increased col(V) in human asthma

Anti-col(V) antibodies in clinical asthma

Interstitial remodeling that occurs post lung transplantation in patients and rodents leads to the exposure of col(V) and subsequent production of systemic col(V) (68, 159). Although asthma is associated with interstitial remodeling, the production of anti-col(V) antibodies in this condition was untested. To address this question we utilized flow cytometry based bead assay to detect anti-col(V) antibodies in serum from asthmatics and healthy volunteers for anti-col(V) antibodies. These patients were recruited from the Indiana University School of Medicine and (Table 2) according to established criteria and a cohort of patient serum was obtained from the University of Pittsburgh School of Medicine (Table 3). This analysis revealed clinical asthma was associated with the presence of anti-col(V) antibodies. Compared to healthy normal volunteers, asthmatics had significantly higher titers of serum anti-col(V) antibodies (Figure 4A, $p < 0.001$). Using samples from the Univ. of Pittsburgh, no significant titers of anti-col(V) antibodies were observed (Figure 4B). Therefore, circulating anti-col(V) antibodies may suggest an association between col(V) immunity and allergic asthma.

Variable	Asthmatics (mean ± SD)
Age, yr	41.14 ± 14.69
Male, n (%)	8 (50)
FEV_1/FVC	73.11 ± 9.03
FEV_1	91.45 ± 14.32
FVC	102 ± 12.69

Table 2. Patient demographics of 16 Indiana University School of Medicine asthmatic volunteers.

Variable	Asthmatics (mean ± SD)
Age, yr	41.59 ±12.96
Male, n (%)	5 (33.3)
FEV_1	66.8 ± 24.97
Severe Asthmatics, n (%)	8 (53.3)
Not Severe Asthmatics, n (%)	5 (33.3)

Table 3. Patient demographics of the University of Pittsburgh School of Medicine asthmatic volunteers.

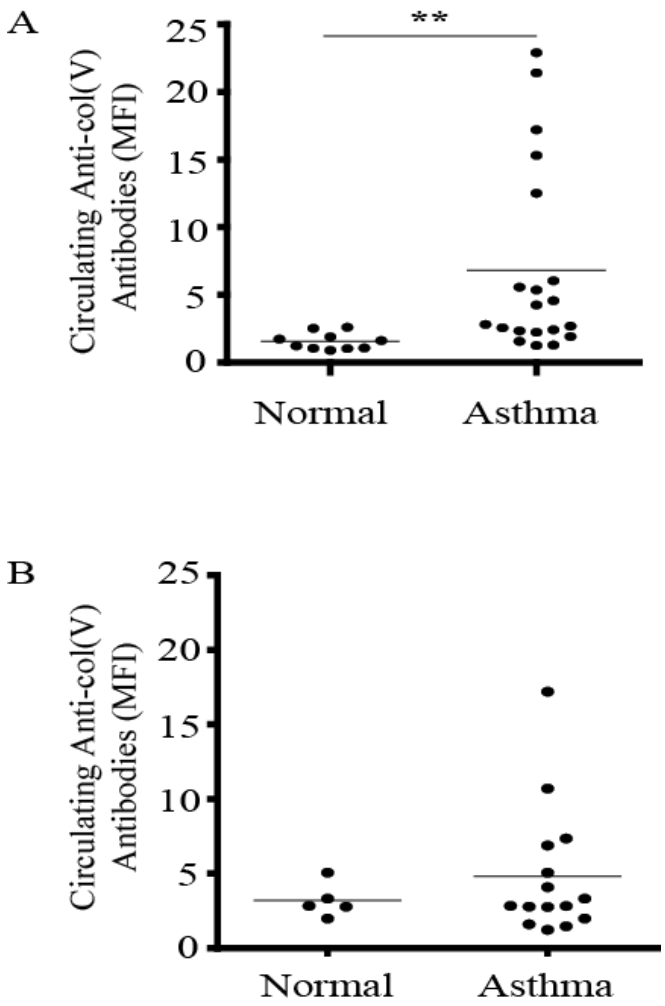

Figure 4. Clinically defined asthmatics produce anti-col(V) antibodies. *A*, Serum was collected from twenty clinically defined asthmatics and ten non-asthmatic from the Indiana University School of Medicine, serum was assayed by the flow cytometry bead assay to detect immunoglobulin Abs bound to col(V) coated beads. *B*, Serum was collected from 15 clinically defined asthmatics from University of Pittsburgh School of Medicine and assayed by the flow cytometry bead assay. Data are shown as Mean fluorescent Intensity (MFI), Data represents the mean ± SEM of either 5-20 individuals per group. **$p<0.01$ using a non-parametric Mann-Whitney test, asthmatics were significantly different from normal individuals. Flow cytometric analysis was performed by Stephanie Case, Indiana University School of Medicine.

Col(V) staining is increased in the asthmatic lung

During fibrosis associated with lung transplantation it is well established that there is increased expression of col(V) in the lung (159). To determine if col(V) is highly expressed in the asthmatic lung, we utilized immunohistochemistry to detect col(V) in the lung of a patient who succumbed to an asthmatic episode. Staining of the normal lungs with our isotype control antibody, we noted no discernible staining of these tissues (Figure 5A). As expected, col(V) was expressed in the sub epithelial tissues and basement membrane and expressed at low levels in adjacent peribronchiolar tissues (Figure 5B). In the fatal asthma case, as in the normal lung when stained with the isotype control we noted little to no staining of the tissue (Figure 5C). By contrast, col(V) was strongly expressed in the thickened basement membrane and adjacent tissues in the fatal asthma case (Figure 5D). Expression of col(V) was also noted in the connective tissues beneath the sub epithelium in the lungs. These data suggest matrix turn-over in the lung was occurring and new collagen deposition was taking place in the lungs in an individual with severe airway disease.

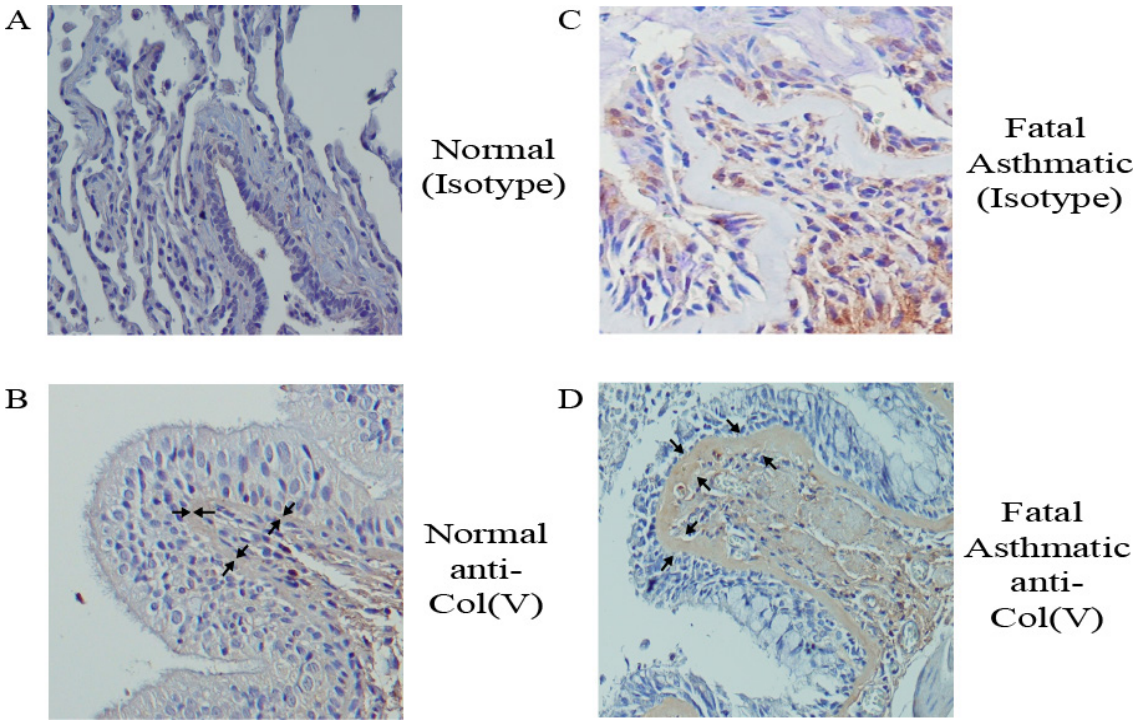

Figure 5. Col(V) staining is increased in the lungs of asthmatics. *A*, Lungs sections from a non-asthmatic patient were embedded in paraffin and stained for the isotype control of the anti-col(V) antibody used in this assay. *B*, Lung sections from a non-asthmatic patient were embedded in paraffin and stained for anti-col(V) expression. The arrows point to small areas of col(V) expression in the sub epithelium. *C*, Lung sections from an individual who succumb to an asthmatic episode were embedded in paraffin and stained for the isotype control of the anti-col(V) antibody used in this assay. *D*, Lung sections from an individual who succumb to an asthmatic episode were embedded in paraffin and stained with anti-col(V), (original magnification 40x) Arrows point to expression of col(V) in sub epithelium. Col(V) is also expressed in the connective tissue of the lungs also visible in the lung sections. Immunohistochemistry was done by Elizabeth Mickler, M.S., Indiana University School of Medicine.

Part II: Col(V)-induced tolerance as a treatment for allergic airway disease

Circulating anti-col(V) antibodies are expressed in mice with allergic airway disease

The expression of antibodies to col(V) has been established in a number of diseases associated with interstitial lung remodeling. To determine if allergic airway disease (AAD), is also associated with anti-col(V) antibodies, we utilized the OVA-induced model of AAD. Allergic airway disease was generated as described in the methods utilizing ovalbumin absorbed in aluminum hydroxide (Figure 6A model). In parallel studies, col(V) tolerance was induced by a single intravenous injection of col(V) (100μg) 7 days prior to OVA/alum as reported in the materials and methods (Figure 6B model). Previous studies by the Wilkes lab have utilized col(V) immunization using intradermal injection in CFA/IFA. Studies were performed to assess the pathogenesis of AAD in the presence of col(V) immunity. In other diseases associated with interstitial lung remodeling, such as lung transplantation, it has been shown in the presence of established col(V)-associated immunity using col(V) immunization by CFA/IFA leads a exacerbate of the induced disease (Figure 6C model).

Initially, to determine if the presence of anti-col(V) antibodies is found in animals with AAD, a disorder triggering lung remodeling, anti-col(V) antibodies were measured in untreated mice and mice following OVA-induced AAD. Serum was collected and used to measure anti-col(V) antibodies in untreated mice and sensitized/challenged mice following OVA-induced AAD. When compared to untreated control animals, the animals that had undergone OVA-induced AAD demonstrated significantly higher levels of systemic col(V) expression when normalized to unsensitized animals and compared to

unsensitized controls (Figure 7A, $p<0.01$). Thus, AAD itself results in the generation of serum anti-col(V) antibodies.

In parallel studies, col(V)-immunity was induced in animals by intra-dermal immunization using CFA and IFA followed by induction of OVA-induced AAD. These animals demonstrated a significant increase in the amount of anti-col(V) antibodies when compared to unsensitized animals (Figure 7B, $p<0.05$). The effect of i.v. injection of col(V) was also assessed by flow cytometry bead assay, interestingly these animals did not show a decrease in anti-col(V) antibodies when col(V) i.v. injection preceded the induction of OVA-induced AAD (Figure 7C). The data show a correlative effect. Thus the generation of AAD in the presence of and established inflammatory environment increased the amount of anti-col(V) antibodies. These data also show that in the context of an inflammatory environment, anti-col(V) antibodies are increased. Taken together, these data indicate that OVA-induced AAD results in anti-col(V) immunity.

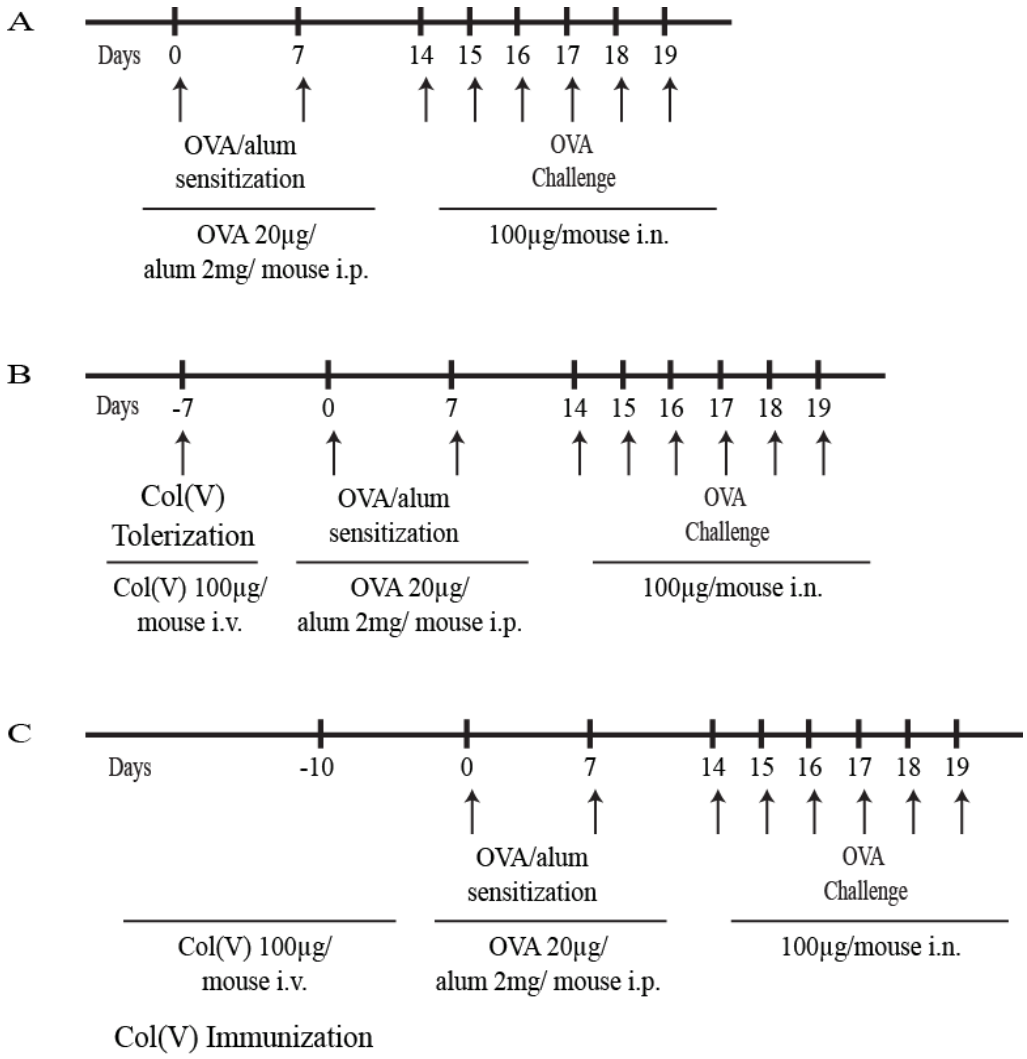

Figure 6. Mouse treatment protocols. *A*, 6-8 week old Balb/c mice were sensitized with 20µg OVA absorbed in 2mg aluminum hydroxide on days 0 and 7. The mice were then challenged intranasally with 100µg OVA for 6 consecutive days, beginning on day 14. *B*, 6-8 week old Balb/c mice were injected with 100µg of bovine col(V) on day -7, mice were then sensitized with 20µg OVA absorbed in 2mg aluminum hydroxide on days 0 and 7. The mice were then challenged by intranasal injection of 100µg OVA for 6 consecutive days, beginning on day 14. *C*, 6-8 week old Balb/c mice were intradermally injected with col(V) in CFA on day -20, on day -10. Mice were intradermally injected with col(V) in IFA on day -10. Mice were then sensitized with 20µg OVA absorbed in 2mg aluminum hydroxide on days 0 and 7. The mice were then challenged by intranasal injection of 100µg OVA for 6 consecutive days, beginning on day 14.

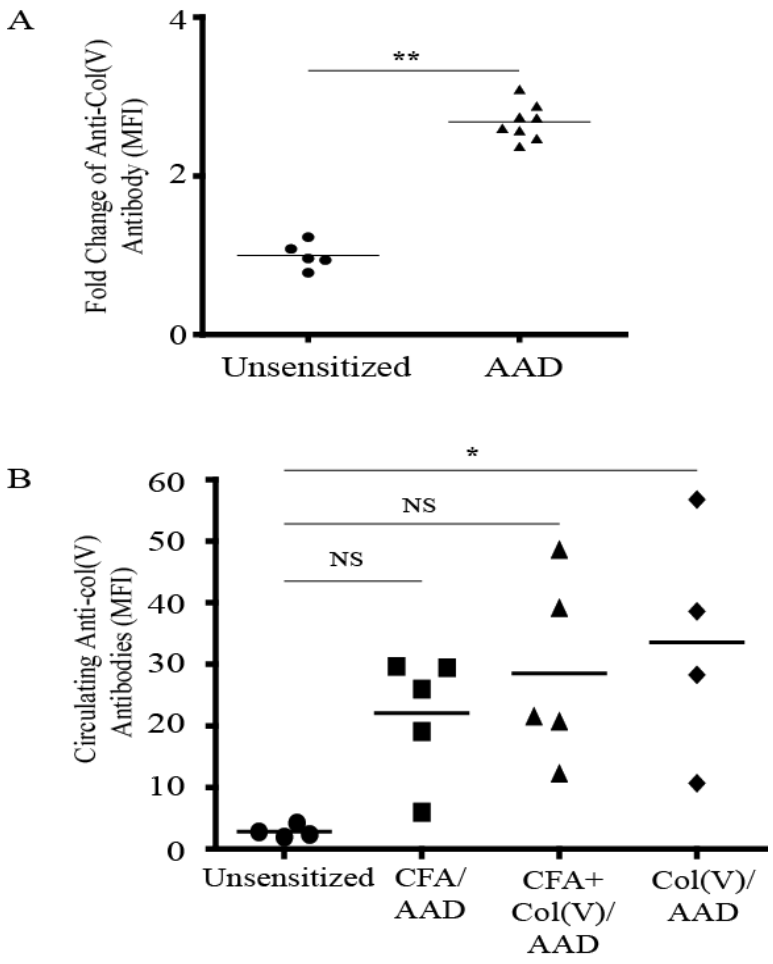

Figure 7. Anti-col(V) antibodies are expressed in mice with OVA-induced AAD. *A*, Serum was collected from five unsensitized and eight mice with allergic airway disease and assayed by flow cytometry to detect IgG Abs bound to col(V) coated beads. *B,* Serum was collected from five unsensitized, five animals immunized with vehicle control or col(V) immunized followed by induction of allergic airway disease. The serum was then assayed by flow cytometry to detect IgG Abs bound to col(V) coated beads. Data are shown as Mean fluorescent Intensity (MFI). Data represents the mean ± SEM of 5-8 mice in each group **$p<0.01$ using a non-parametric Mann-Whitney test, significantly different from untreated animals. *$p<0.05$ using significantly different from unstimulated using two-way ANOVA and Bonferroni *post hoc*, NS, not significant using two-way ANOVA and Bonferroni *post hoc*. Flow cytometric analysis was performed by Stephanie Case, Indiana University School of Medicine.

The effects of col(V)-induced tolerance in allergic airway disease are localized to the lung

To determine if OVA-induced AAD resulted in anti-col(V) cellular immune responses, col(V) tolerance was induced by a single intravenous injection (100µg) of bovine col(V) prior to OVA/alum. Mediastinal lymph node cells were then isolated and cultured in the presence of OVA or col(V). As expected, OVA sensitization, alone, resulted in brisk OVA-induced proliferation (Figure 8A, $p<0.01$). Consistent with detection of anti-col(V) antibodies, OVA-sensitized mice developed a trend towards higher proliferation in response to col(V). Notably, col(V) treatment significantly suppressed OVA-induced proliferation indicating that col(V) administration induced partial tolerance (Figure 8A, $p<0.01$). Treatment with col(V) did not alter the proliferation when cells were culture with bovine col(V). These effects induced by col(V) in OVA-sensitized mice were local and not systemic as splenocytes from treated mice proliferated in response to OVA, but col(V) treatment did not affect this response (Figure 8B).

Local and systemic humoral responses were studied; the levels of total and OVA-specific IgE were assessed in serum and BAL of treated animals. There was a trend towards lower total and OVA-specific IgE in the serum of col(V) tolerized mice when compared to PBS control animals (Figure 9A). In contrast, compared to PBS-treated controls, col(V)-induced tolerance resulted in significant reductions in total and OVA-specific IgE antibodies in BAL fluid (Figure 9B, $p<0.05$). Collectively col(V)-induced tolerance alters local OVA-induced cellular and humoral immune responses.

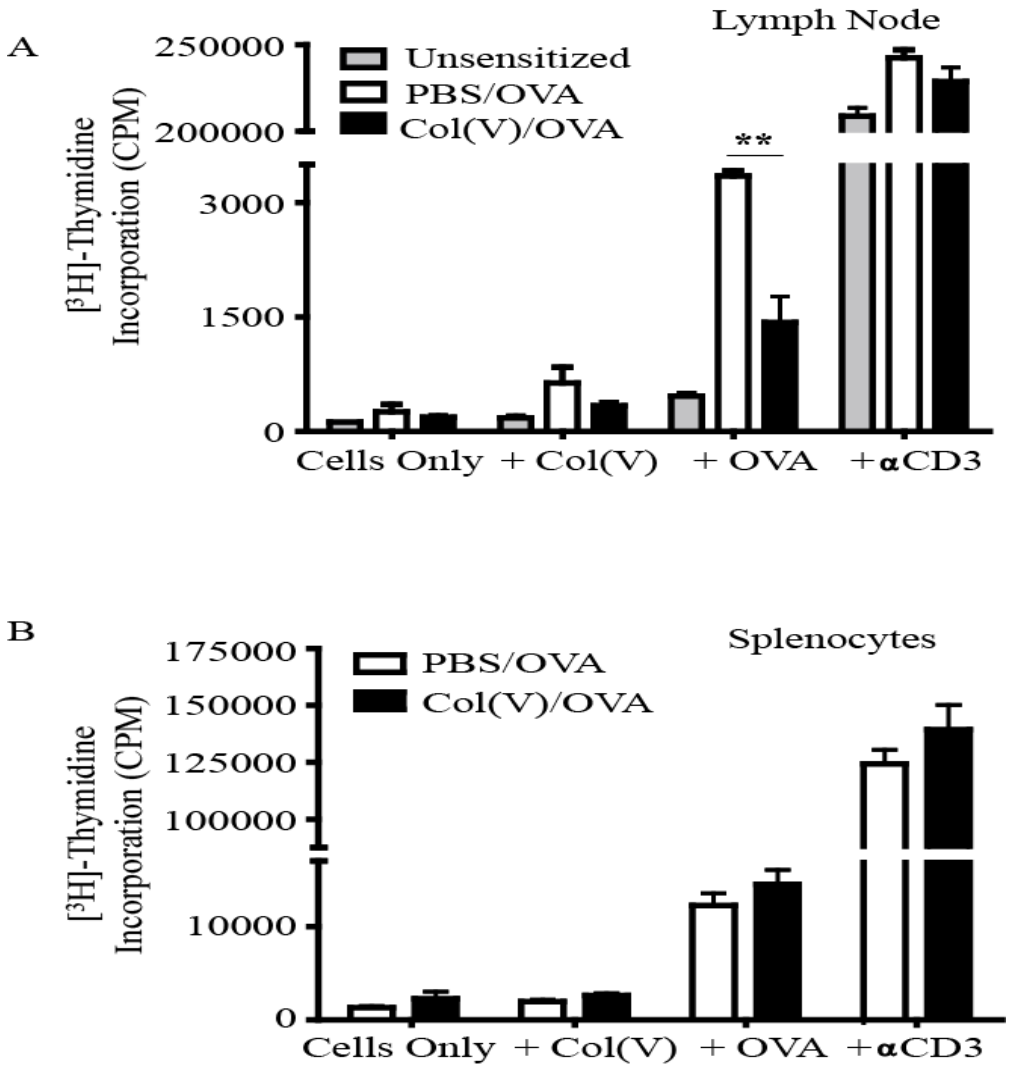

Figure 8. Col(V)-induced tolerance alters localized OVA-specific T cell proliferation. Total cells from the mediastinal lymph nodes of untreated, PBS or col(v) treated animals (3×10^5) were cultured with irradiated splenocytes (3,000 rads) (3×10^5) in the presence of OVA (100µg/ 1×10^6 cells), col(V) (40µg) or anti-CD3 Ab (0.5µg/ml) for 72 hours. Cellular proliferation was measured by [^{3}H]-thymidine incorporation. Results are expressed as mean ± SEM, n = 5 pooled animals and is representative of two independent experiments. Total splenocytes from either PBS or col(V) treated animals (3×10^5) were isolated and cultured in the presence of OVA (50 µg/ml), col(V) (40µg) or anti-CD3 Ab (0.5µg/ml) for 72 hours. Cellular proliferation was measured by 3H thymidine incorporation. Results are expressed as mean ± SEM, n = 5 and are representative of three independent experiments. **p<0.01 using one-way ANOVA and Bonferroni *post hoc*.

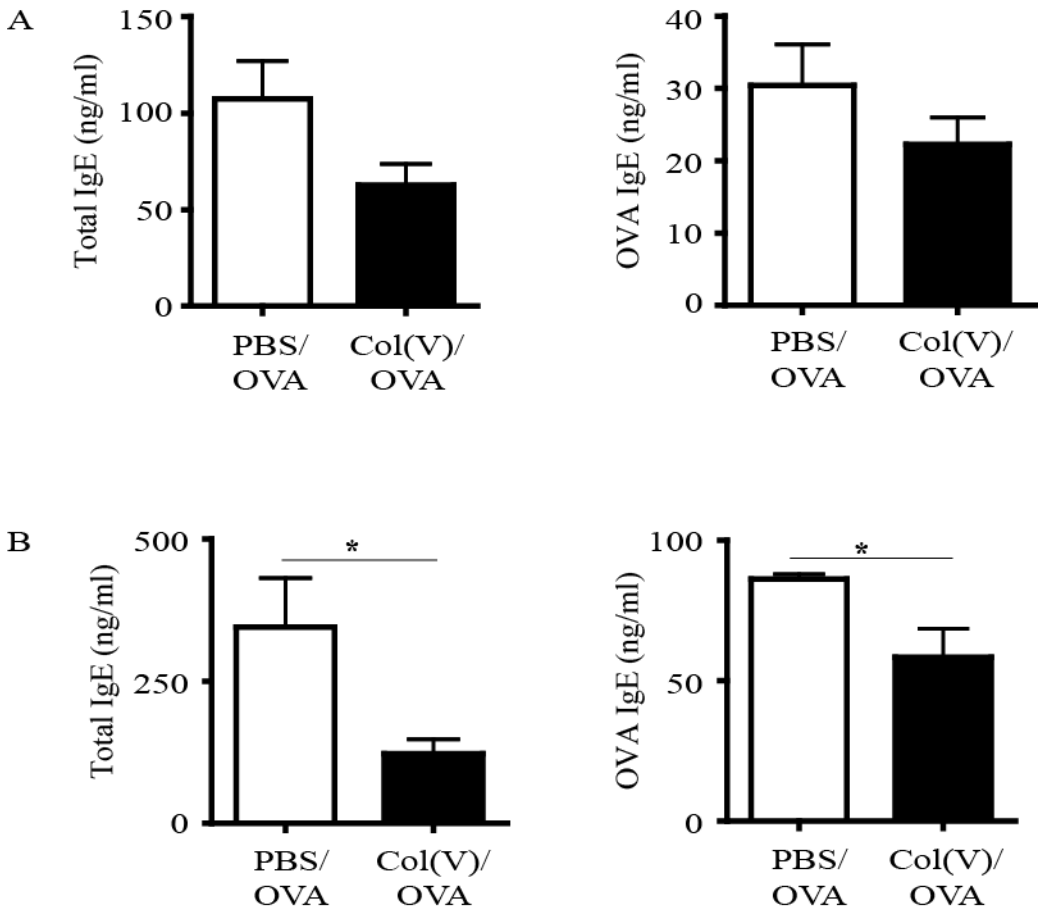

Figure 9. Col(V)-induced tolerance decreases localized total and OVA-specific IgE levels. *A*, Total and OVA specific IgE levels were quantified in the serum of OVA-sensitized and challenged PBS and col(V) animals by ELISA. *B*, Total and OVA specific IgE levels were quantified in BAL fluid of OVA-sensitized and challenged PBS and col(V) animals by ELISA. Results are expressed as mean ± SEM of 5 mice in each group, *$p<0.05$ using two-tailed Student's *t*-test.

Col(V)-induced tolerance does not alter lung remodeling and collagen deposition in allergic airway disease

The effect of col(V)-induced tolerance on lung remodeling was studied in both vehicle control (PBS) treated animals and col(V) tolerzied animals. To do this, lung histological sections from each treatment group were stained for collagen deposition by tri-chrome staining. Surprisingly, each group demonstrated lung remodeling by new collagen deposition when observed by light microscopy (Figure 10A&B). The collagen content of the animals was further analyzed when lungs were measured for hydroxyproline content. As expected when compared to the tri-chrome staining; the analysis of total collagen content demonstrated no difference between either treatment groups (Figure 10C). Together, these data demonstrate that col(V)-induced tolerance did not change the level of lung remodeling in allergic airway disease.

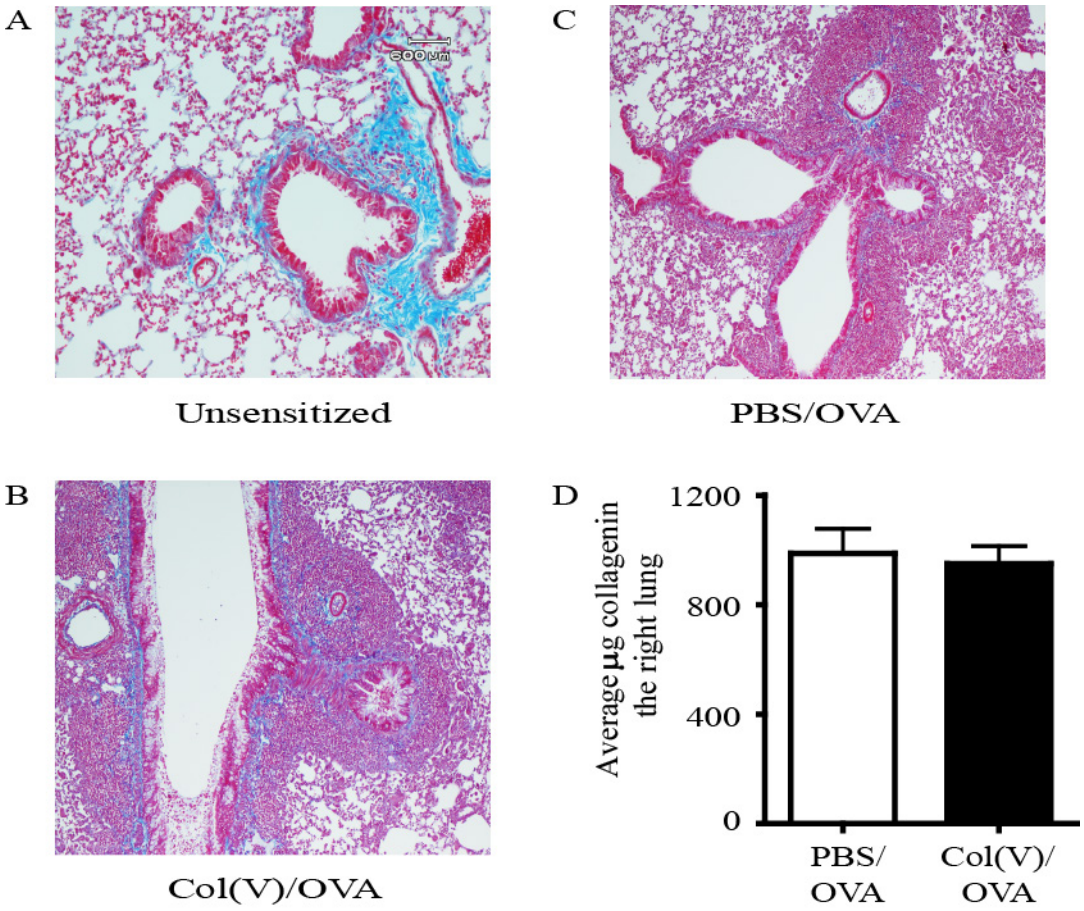

Figure 10. Col(V)-induced tolerance does not alter collagen content in the lung. *A-C*, Lung histological sections were harvested from either unsensitized animals or animals treated with either PBS or col(V)-induced tolerance before induction of OVA-induced allergic airway disease. The lung sections were then stained with Masson's trichrome stain to highlight connective tissue. *C*, Total collagen content was measured in lungs by hydroxyproline measurement of total collagen content. Data represents the mean ± SEM of 5 mice in each group. Measurement of hydroxyproline was performed by Elizabeth Mickler, M.S., Indiana University School of Medicine.

Col(V-induced tolerance decreases airway hyper-reactivity

To study the effects of col(V)-induced tolerance on AHR we assessed lung function both by non-invasive (Penh); and invasive methods of measuring airway resistance and lung compliance. As expected OVA sensitization and challenge resulted in dose-dependent increases in Penh in response to methacholine in PBS-treated (control) mice (Figure 11A). In contrast, Penh gradually increased in col(V)-tolerized mice at low methacholine concentrations, but plateaued with higher concentrations at values which were significantly lower than PBS-treated controls (Figure 11A, $p<0.001$). The AHR of animals immunized to col(V) were also assessed by measuring Penh. The animals immunized either with adjuvant alone or with col(V) showed marked increases in AHR which were very similar to the PBS control animals (Figure 11B).

The measure of airway hyper-responsiveness was further quantified by measuring airway resistance and dynamic compliance. Resistance, a measure of airway obstruction, was also significantly reduced in col(V)-tolerized mice compared to controls at the highest methacholine concentrations (Figure 12A, $p<0.01$). Compliance in terms of lung function refers to the stiffness of the lungs. These data showed there was no statistically significant increase in the compliance of the col(V) group compared to the PBS group (Figure 12B). Taken together, these data further illustrate the fact that col(V)-induced tolerance decreases AHR in mice with OVA-induced AAD.

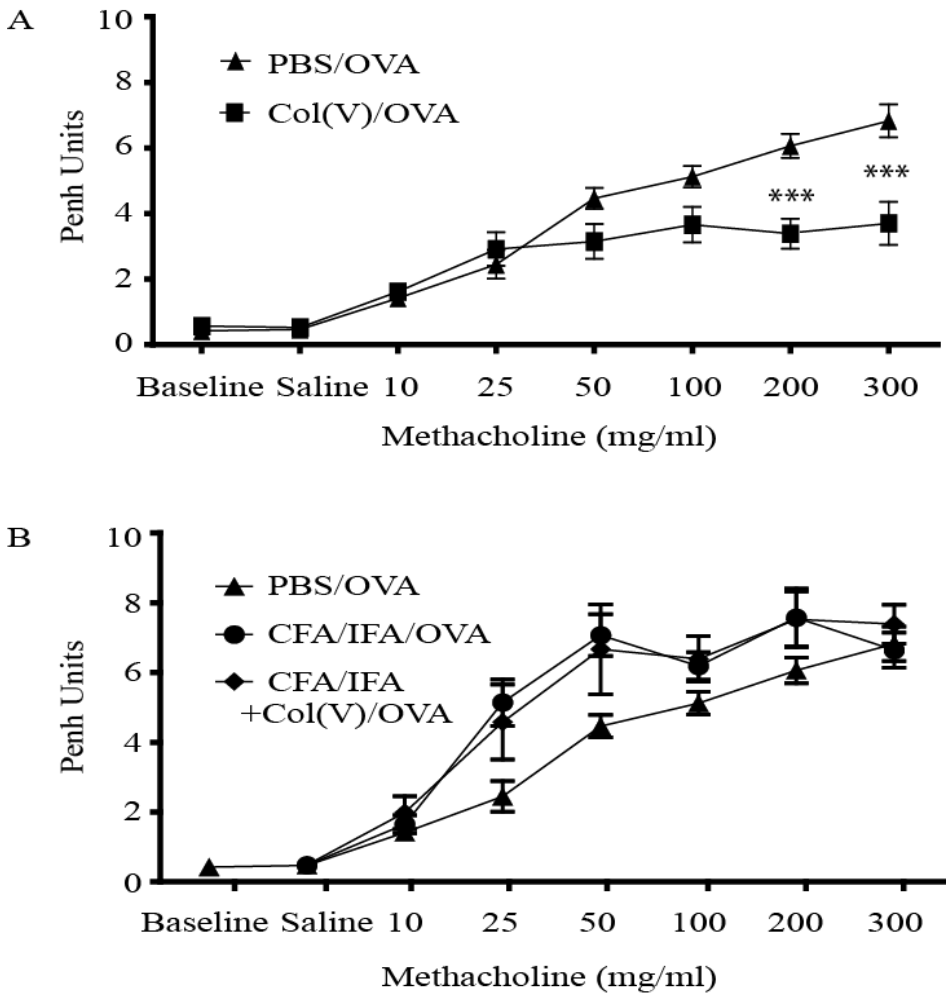

Figure 11. Col(V)-induced tolerance decreases airway hyper-responsiveness. AHR was measured 24 hours after the final intranasal challenge. *A,* Twenty-four hours after the final challenge, airway hyper-responsiveness to aerosolized methacholine was measured in conscious PBS/OVA or Col(V)/OVA. *B*, Twenty-four hours after the final challenge, airway hyper-responsiveness to aerosolized methacholine was measured in conscious PBS/OVA, CFA/IFA/OVA or CFA/IFA+Col(V)/OVA mice. AHR was measured using unrestrained, whole body plethysmography (Penh). The mice were first treated with saline then exposed to increasing doses of methacholine from 10 to 300mg/ml. Methacholine was nebulized for 2 minutes; readings were then measured for 5 minutes following nebulization. Data represent mean ± SEM from 6 mice and are representative of three independent experiments. ***$p<0.001$, significantly different from PBS using two-way ANOVA and Bonferroni *post hoc*. Measurement of penh was performed in collaboration with Sarita Sehra, Ph.D., Indiana University School of Medicine.

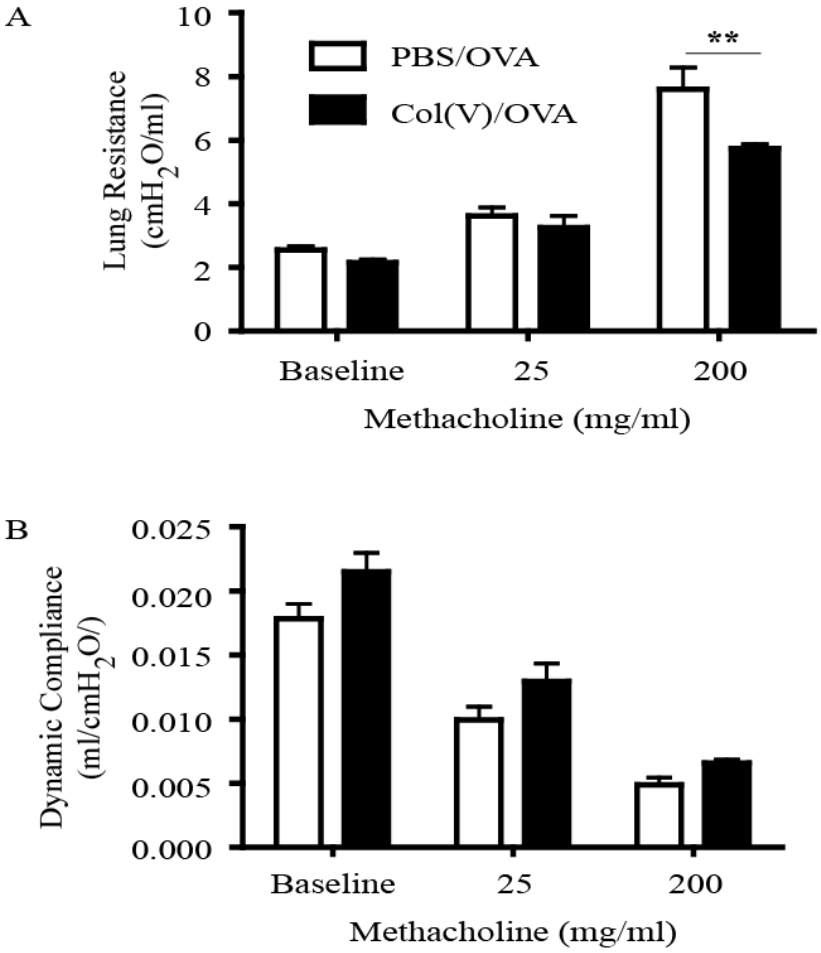

Figure 12. Col(V)-induced tolerance decreases lung resistance. R/C was measured 24 hours after the final intranasal challenge. *A*, Twenty-four hours after the final challenge, lung resistance was measured in anesthetized and intubated mice, delivering nebulized saline and increasing doses of methacholine, from 10 to 200mg/ml. *B*, Twenty-four hours after the final challenge, dynamic compliance was measured in anesthetized and intubated mice, delivering nebulized saline and increasing doses of methacholine, from 10 to 200mg/ml. Data represent mean ± SEM. Data represent mean ± SEM from five mice **$p<0.01$, significantly different from PBS using two-way ANOVA and Bonferroni *post hoc*. Measurement of resistance and compliance was performed with Purvi Mehrotra, Ph.D., Indiana University School of Medicine.

Col(V)-induced tolerance decreases tracheal smooth muscle contraction

A major component in allergic airway disease is that of smooth muscle contractions of the airways of the lung. Previous studies have shown that smooth muscle cells express receptors to both C3a and C5a; these have also demonstrated that C3a can cause smooth muscle contraction. To determine the effect of col(V)-induced tolerance has on smooth muscle contractions, tracheas were harvested from the treated animals and isometric force measured after exposure to acetylcholine. The tracheas were attached to grass force-displacement transducers and stretched to a force of 0.2-0.3g and allowed to equilibrate for 1hr. After equilibration, the tracheas were exposed to acetylcholine (ACH) for 5 minutes, isometric force was then measured. As expected, PBS/OVA animals had higher levels of isometric force when compared to unsensitized control animals. When the col(V)/OVA tracheas were treated with various doses of acetylcholine, the isometric force was decreased when compared to PBS/OVA control animals (Figure 13).

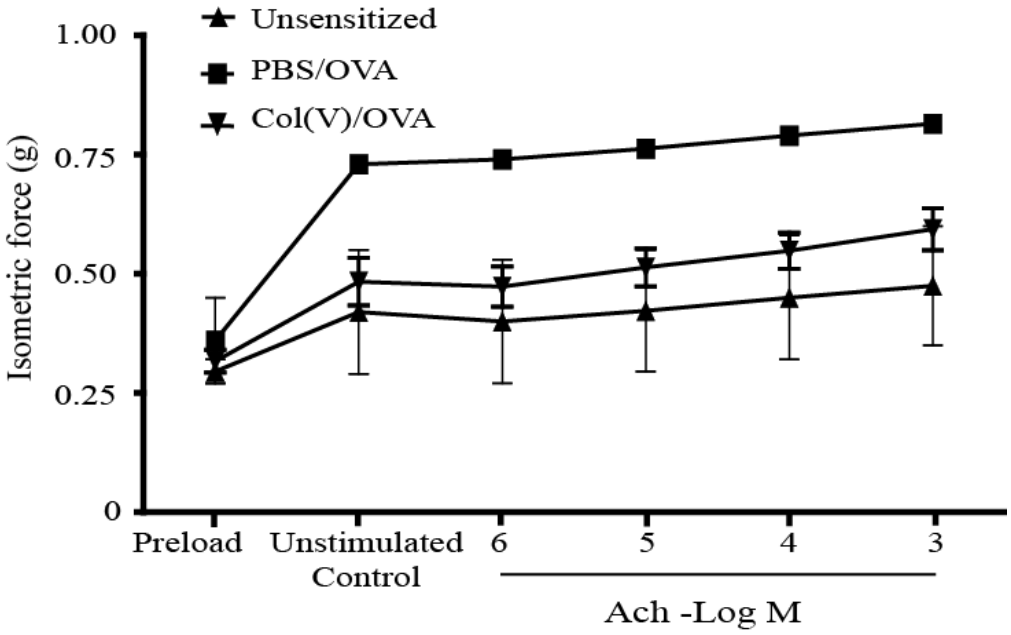

Figure 13. Col(V)-induced tolerance decreases smooth muscle contraction in OVA-induced AAD. Smooth muscle contraction to acetylcholine was measured in tracheas of treated mice; 6mm long segments were cut from the tracheas. Tracheal segments were then stimulated with acetylcholine (ACh) from 10^{-6} to 10^{-3} M for 5 minutes at each dose. Force generated by the muscle was measured after each dose. Data represent mean ± SEM (unsensitized n = 2, PBS/OVA n = 2, Col(V)/OVA n = 3). Measurement of smooth muscle contraction was performed by Wenwu Zhang, Ph.D., Indiana University School of Medicine.

Col(V)-induced tolerance does not alter mononuclear cell infiltration of the lung

The effect of col(V)-induced tolerance on allergic airway disease-associated histopathology was also assessed. Mononuclear cell infiltration is a hallmark of allergic airway disease. When compared to the unsensitized animals (Figure 14A), both the PBS/OVA (Figure 14B) and the col(V)/OVA (Figure 14C) exhibit severe mononuclear cell infiltration around airways and vessels. Aggregates of mononuclear cells are present in the lung parenchyma. Blind scoring of histopathology revealed no difference between treatment groups in the terms of cellular infiltration (Figure 14D).

BAL cellularity was assessed in all treatment animals to determine the number of cells following col(V)-induced tolerance. When compared to unsensitized or PBS/OVA controls, col(V)/OVA BAL cell numbers were unaltered (Figure 15A). The cellular composition of BAL was also assessed by flow cytometric analysis. The cells were stained for the cellular markers of major cell types commonly associated with AAD. Col(V)-induced tolerance did not alter cellular composition of BAL when compared to PBS/OVA treated animals (Figure 15B). Notably, col(V)-induced tolerance did not affect cellular infiltration into the lung or composition of cells in BAL. Taken together, col(V)-induced tolerance was unable to alter cellular infiltration in the lung.

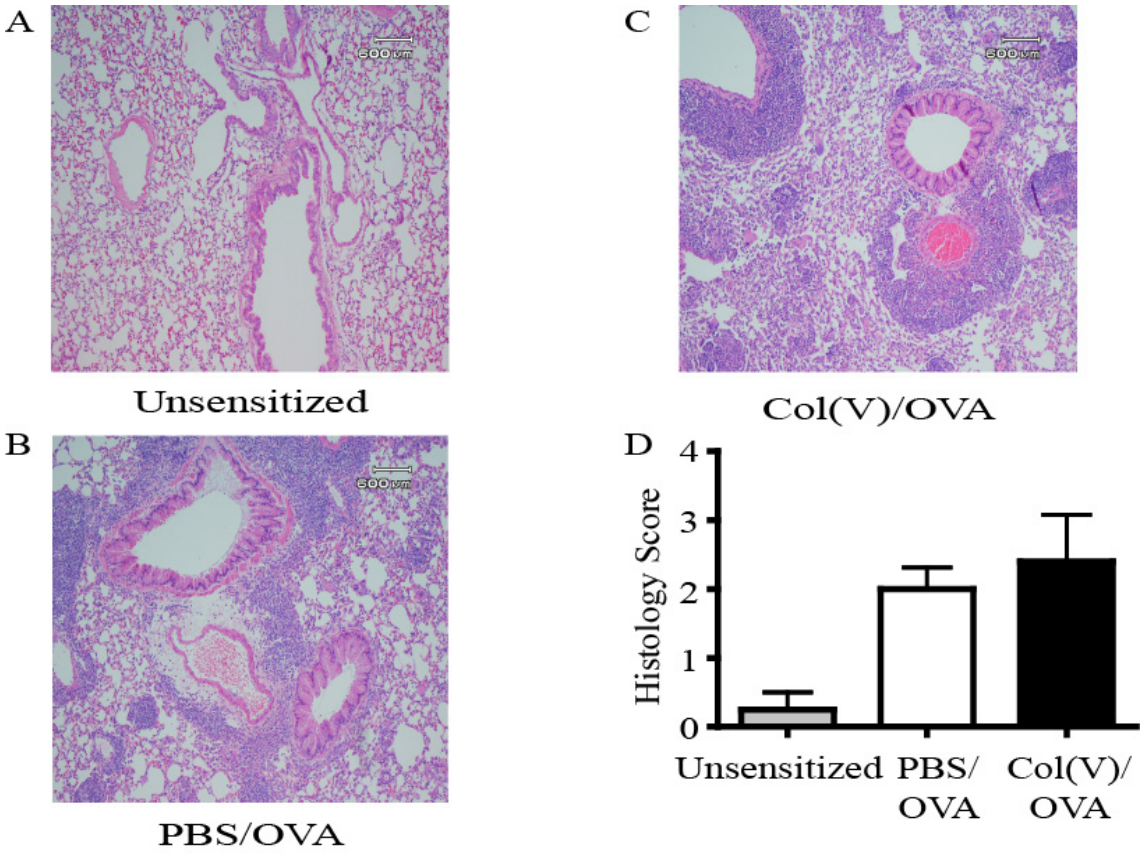

Figure 14. Col(V)-induced tolerance does not reduce lung mononuclear cell infiltration in OVA-induced AAD. *A-C*, Lungs of untreated and sensitize and challenged mice were fixed and embedded in paraffin and stained with H&E (original magnification 10x). Photomicrographs are representative of the histology of five animals in the PBS and Col(V) groups. *D,* H&E stained Photomicrographs were scored blinded for cellular infiltration based on a 0-4 scale, scores represent the mean ± SEM of the pathologic score of 5 mice in each group. Lung sections were scored blinded by David S. Wilkes, M.D.

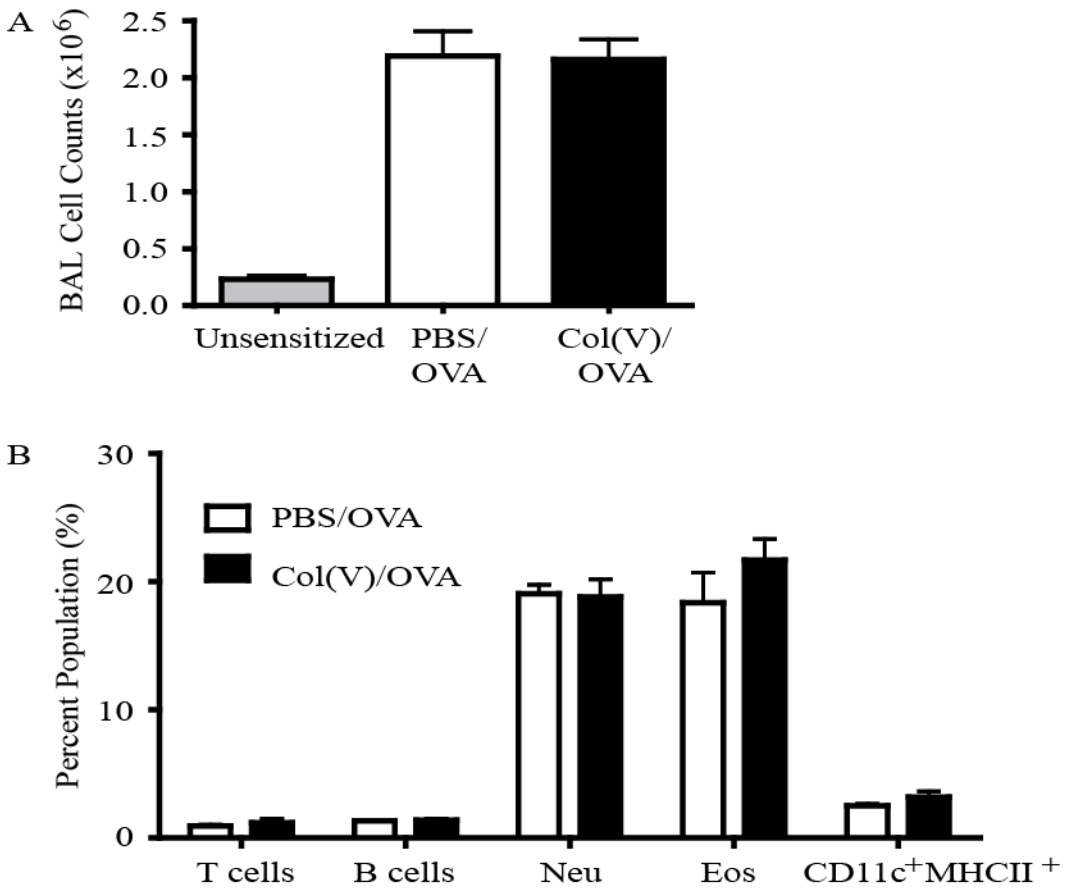

Figure 15. Col(V)-induced tolerance does not alter BAL cellular composition in OVA-induced AAD. *A*, BAL was isolated from treated animals and cells were counted using trypan blue exclusion; counts represent mean ± SEM of 5 animals in each group and are representative of three experiments. *B,* Cell populations in BAL were stained with antibodies to identify, T cells, B cells, neutrophils, eosinophils and mononuclear cells. Results are expressed as mean ± SEM from 5 mice and are representative of three experiments. Analysis of BAL cellular populations was performed by Sarita Sehra Ph.D.

Col(V)-induced tolerance does not prevent goblet cell metaplasia and mucus production

Goblet cell hyperplasia and mucous production are other hallmarks of AAD. Metaplasia is defined as the replacement of one differentiated cell type with another cell type, in the case of asthma replacing one type of epithelial cell with another type (167). Since col(V)-induced tolerance limited AHR, we next determined if this effect was related to fewer goblet cells or mucus expression. As determined by histopathology of mucin positive cells stained by periodic acid-Schiff (PAS); visual analysis revealed no difference between treatment groups (Figure 16A-C). Because of the lack of a definitive visual difference, histopathology of PAS positive airway cells was analyzed by metamorphic software. No difference was seen in the PBS/OVA and col(V)/OVA treatment groups (Figure 16D). To further study the effect of col(V)-induced tolerance on mucus production, the expression of the mucin genes *Muc5ac* and *Gob5* were also assessed by RT-PCR. *Muc5ac* has been identified as one of the main mucin genes and proteins expressed by goblet cell. *Gob5* has been categorized as a calcium chloride activated channel responsible for regulating mucin production. When normalized to beta actin and unsensitized animals, *Muc5ac* and *Gob5* were expressed in PBS/OVA and Col(V)/OVA at comparable levels (Figure 17A-B).

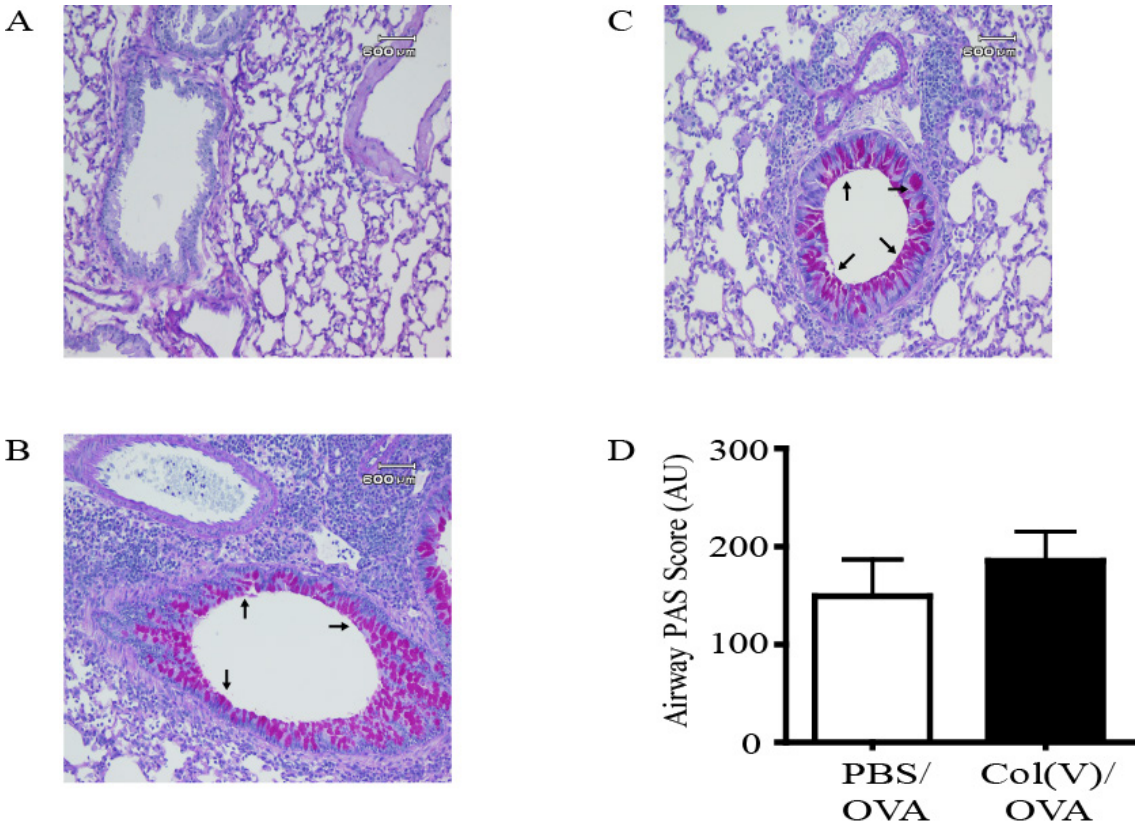

Figure 16. Col(V)-induced tolerance does not alter goblet cell metaplasia in OVA-induced AAD. *A-C*, Lungs of untreated and sensitized and challenged mice were fixed and embedded in paraffin and stained with Periodic-Acid Sciff (PAS) (original magnification 20x). Arrows point to PAS positive goblet cells. Photomicrographs are representative of the histology of five animals in the PBS and col(V) groups. *D,* The staining intensity positive cells for PAS staining per mm of airway epithelial cells, measured using coded slides by Metamorph software. Data represents the mean ± SEM of 5 mice in each group. Metamorphic analysis of PAS positive cells was performed by Irina Petrache, M.D.

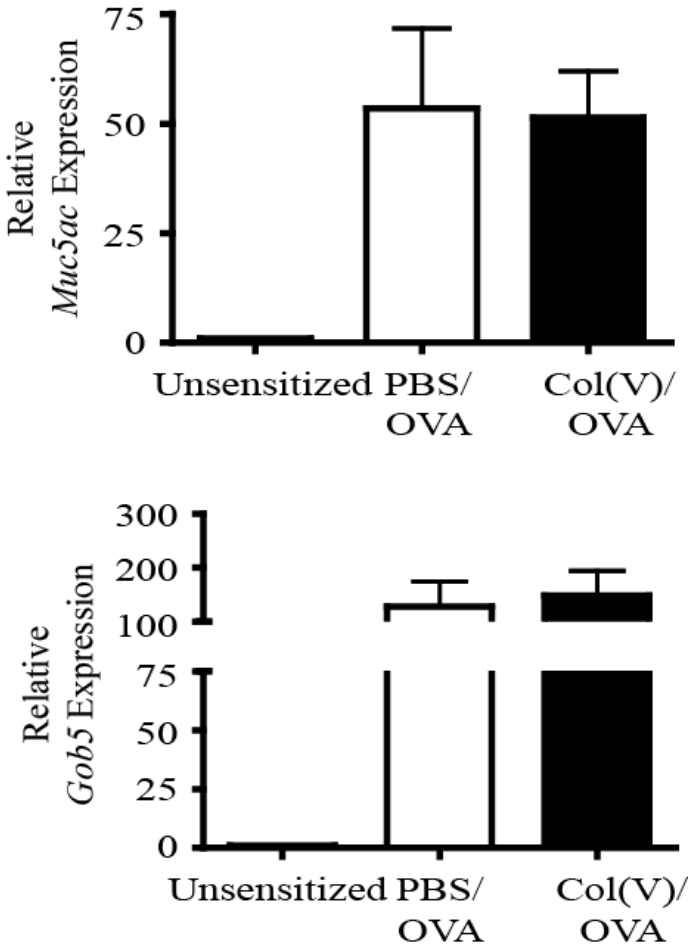

Figure 17. Col(V)-induced tolerance in OVA-induced AAD does not alter mucin gene expression. Lungs of unsensitized and sensitized and challenged mice were homogenized and total RNA isolated. mRNA expression of mucin associated genes was measured by quantative RT-PCR. Gene expression was normalized to beta-actin and then to Unsensitized animals. Data represents the mean ± SEM of 10 mice in the treatment groups group.

Col(V)-induced tolerance alters cytokines associated with allergic airway disease

The hallmark cytokines of allergic airway disease include those produced by Th2 cells, mainly IL-4, 5, 9, and 13. We next examined the effect col(V)-induced tolerance had on Th2 cytokines within the lungs by studying both the total lung population and the lung mononuclear cell population itself. When the mRNA expression of several genes including Il*4, 5, 13, 10, 17* and *Ifng* were measured in the lung mononuclear cell population we found wide variability among the separate experiments that were performed (Figure 18). The trends were much clearer when we studied similar genes with the inclusion of *Tgfb* as another marker of cellular suppression in total lung tissue. These results gave us a more accurate picture of the cytokine environment in the lung during allergic airway disease following col(V)-induced tolerance (Figure 19, $p<0.05$, $p<0.01$). We finally utilized quantitative-Real Time PCR once again to measure mRNA expression in the spleen. The effects of col(V)-induced tolerance have been limited to the lung; however, the systemic effects on cytokine expression were not known. Unlike in the lung, we did not see marked changes in any of the genes systemically; even those involved in allergic airway disease (Figure 20). These data demonstrate the results seen in our splenocytes proliferation data which demonstrated that col(V)-induced tolerance was a localized response.

Protein expression of cytokines was also analyzed by CBA analysis. Serum cytokine levels of Th2 and Th1 proteins did not change (Figure 21). This data were suggestive of a decrease in the Th2 environment associated with AAD, however, the changes were not statistically significant. BAL cytokine protein levels were also analyzed, however, the levels were below the level of detection of the kit being used.

Taken together, these data show that col(V)-induced tolerance does not change the overall cytokine environment associated with allergic airway disease.

Another factor involved in the pathogenesis of allergic airway disease is IL-17. IL-17 has been linked to asthma and anti-col(V) immunity (46, 140); and we reported that col(V)-induced tolerance abrogates alloantigen-induced IL-17 production. Therefore, we next determined if col(V)-induced tolerance resulted in decreased Il17 expression in OVA-induced AAD. As expected, *Il17* transcripts were increased in lungs of PBS-treated mice that developed AAD (Figure 22A). Notably, col(V) induced tolerance abrogated this increase (Figure 22A, $p<0.05$). We further studied the effect of col(V)-induced tolerance on the protein expression of IL-17 in the serum of mice with allergic airway disease. Similar to the lungs, there was a trend towards a decrease in IL-17a expression in the serum of the col(V)/OVA animals, however, the decrease was not statistically significant (Figure 22B).

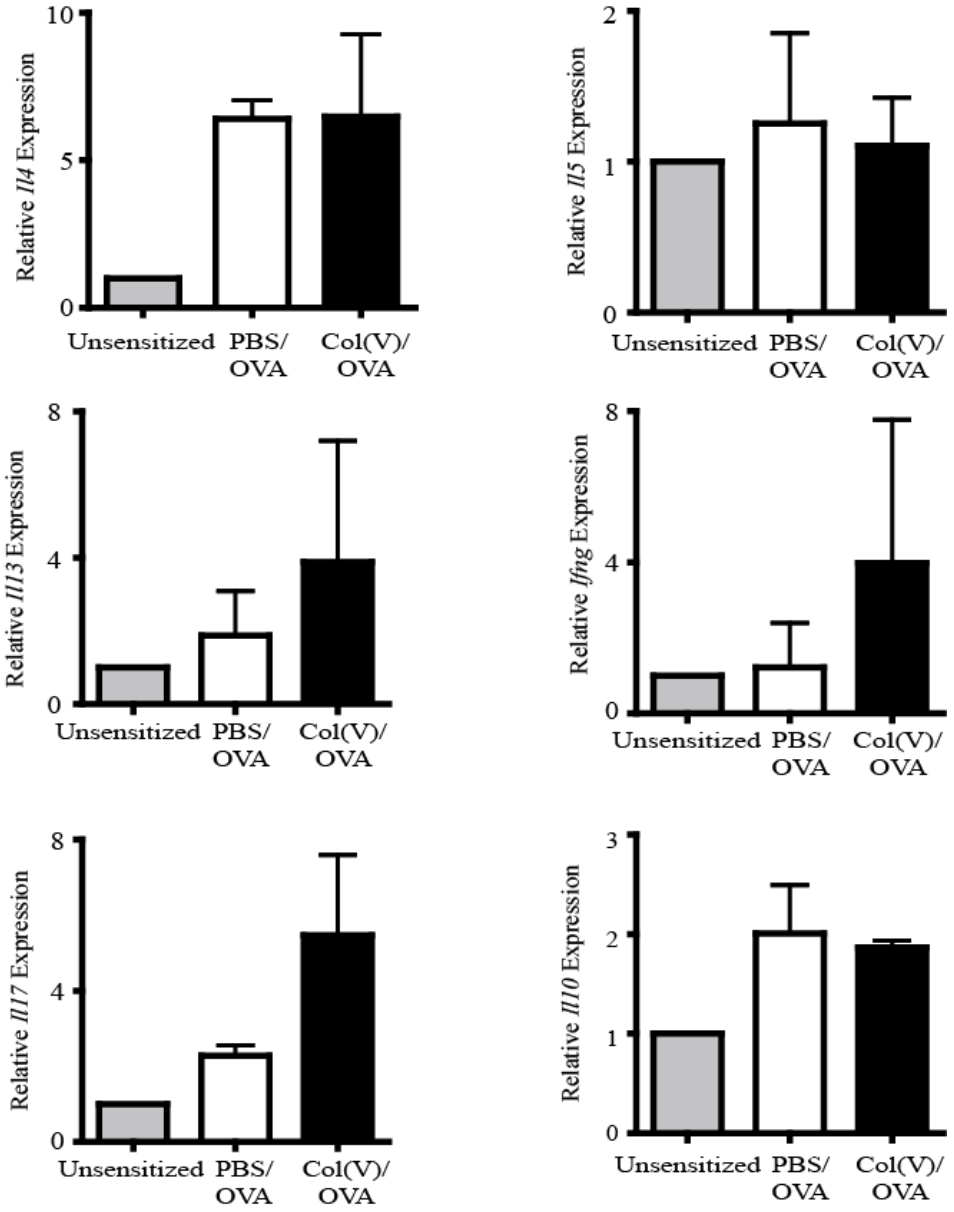

Figure 18. Cytokine mRNA expression of lung mononuclear cells is unchanged after col(V)-induced tolerance in OVA-induced AAD. Lung mononuclear cells of unsensitized and sensitized and challenged mice were homogenized and total RNA isolated. mRNA expression of various cytokine genes were measured by quantative RT-PCR. Gene expression was normalized to beta-actin and then to unsensitized animals. Data represents the mean ± SEM of 5 animals pooled.

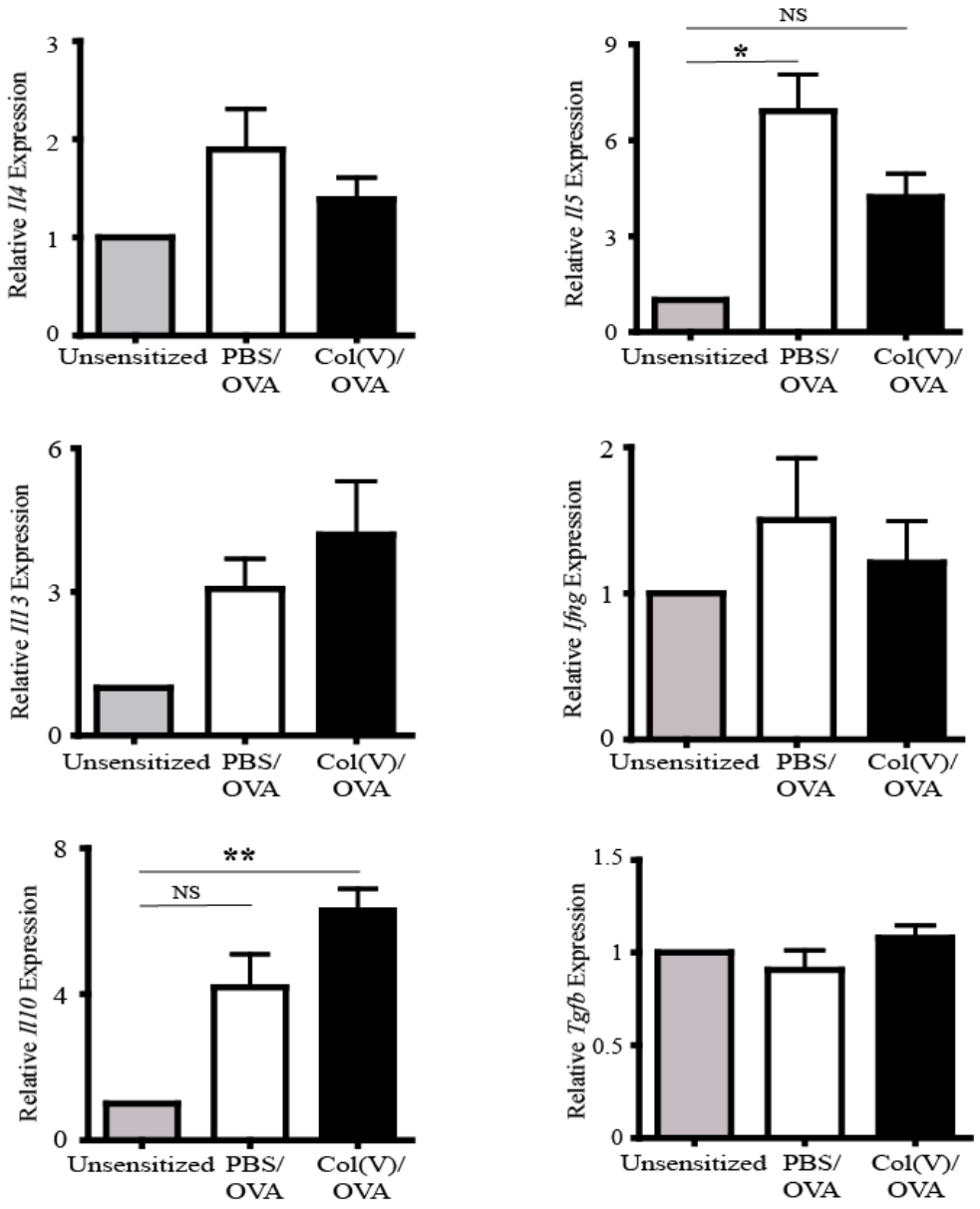

Figure 19. Cytokine mRNA expression of total lung cells is unchanged after col(V)-induced tolerance in OVA-induced AAD. Lungs of unsensitized and sensitized and challenged mice were homogenized and total RNA isolated. mRNA expression of various cytokine genes were measured by quantative RT-PCR. Gene expression was normalized to beta-actin and then to unsensitized animals. Data represents the mean ± SEM of 5 mice *$p<0.05$, **$p<0.01$, significantly different from unstimulated using two-way ANOVA and Bonferroni *post hoc*, NS, not significant using two-way ANOVA and Bonferroni *post hoc*.

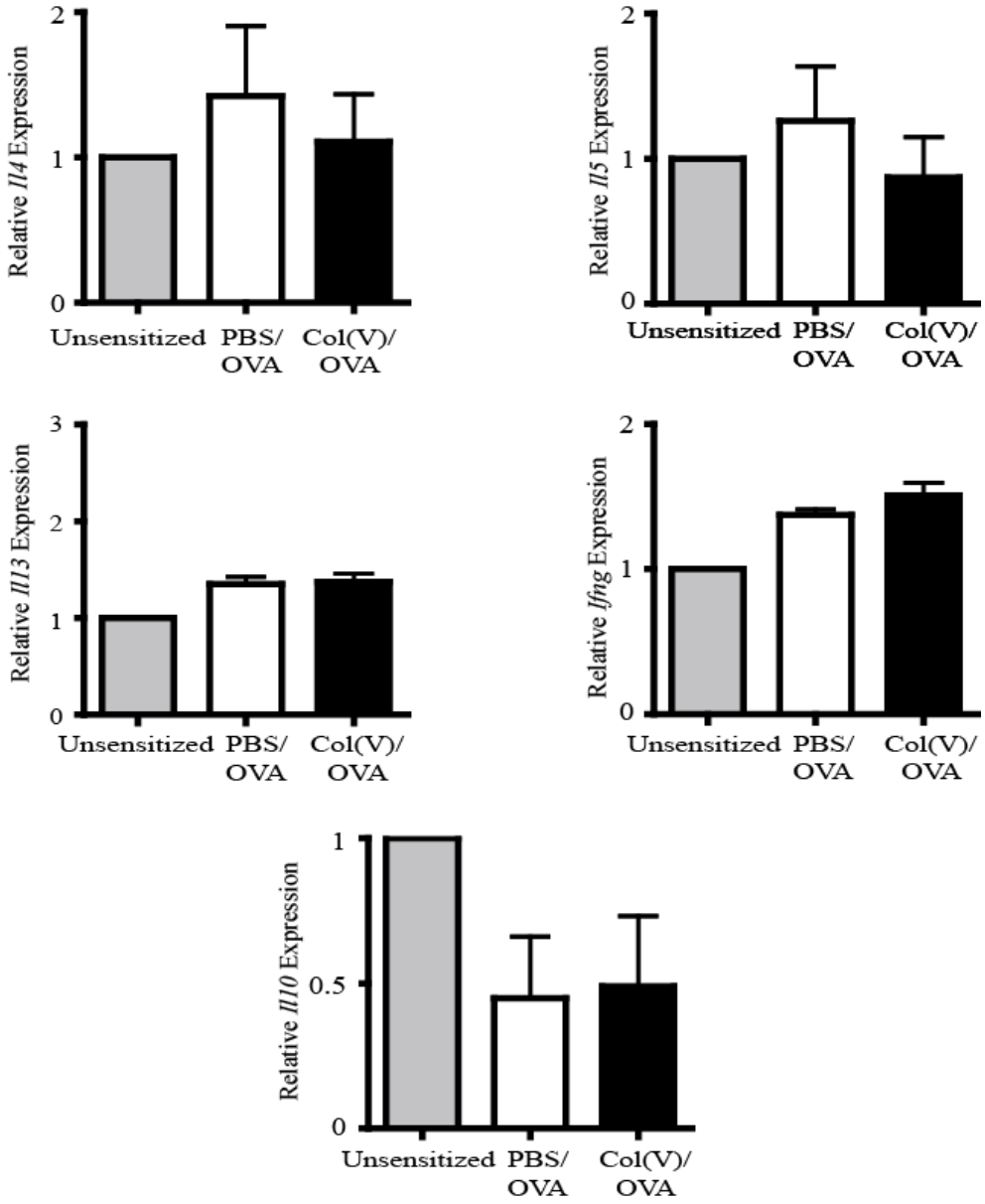

Figure 20. Cytokine mRNA expression of total splenocytes is unchanged after col(V)-induced tolerance in OVA-induced AAD. Total splenocytes of unsensitized and sensitized and challenged mice were homogenized and total RNA isolated. mRNA expression of various Th1, Th2, Treg genes were measured by quantative RT-PCR. Gene expression was normalized to beta-actin and then to unsensitized animals. Data represents the mean ± SEM of 5 animals.

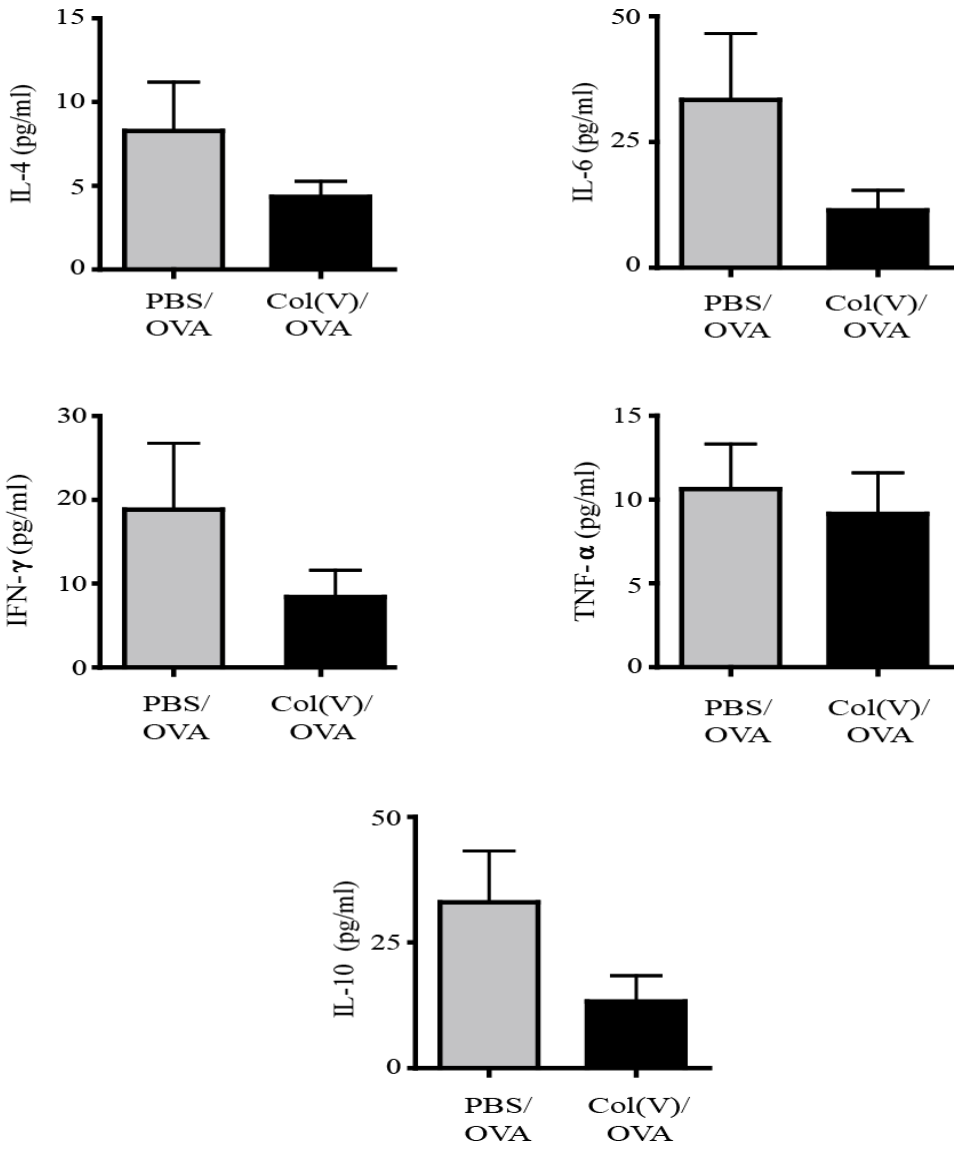

Figure 21. Col(V)-induced tolerance alters serum cytokine expression in OVA-induced AAD. Serum from unsensitized and sensitized and challenged or col(V)-induced tolerance and sensitized and challenged mice were collected and analyzed for various cytokines were measured by CBA. Data represents the mean ± SEM of 5 animals. CBA analysis was performed by Patricia Smith, Indiana University School of Medicine.

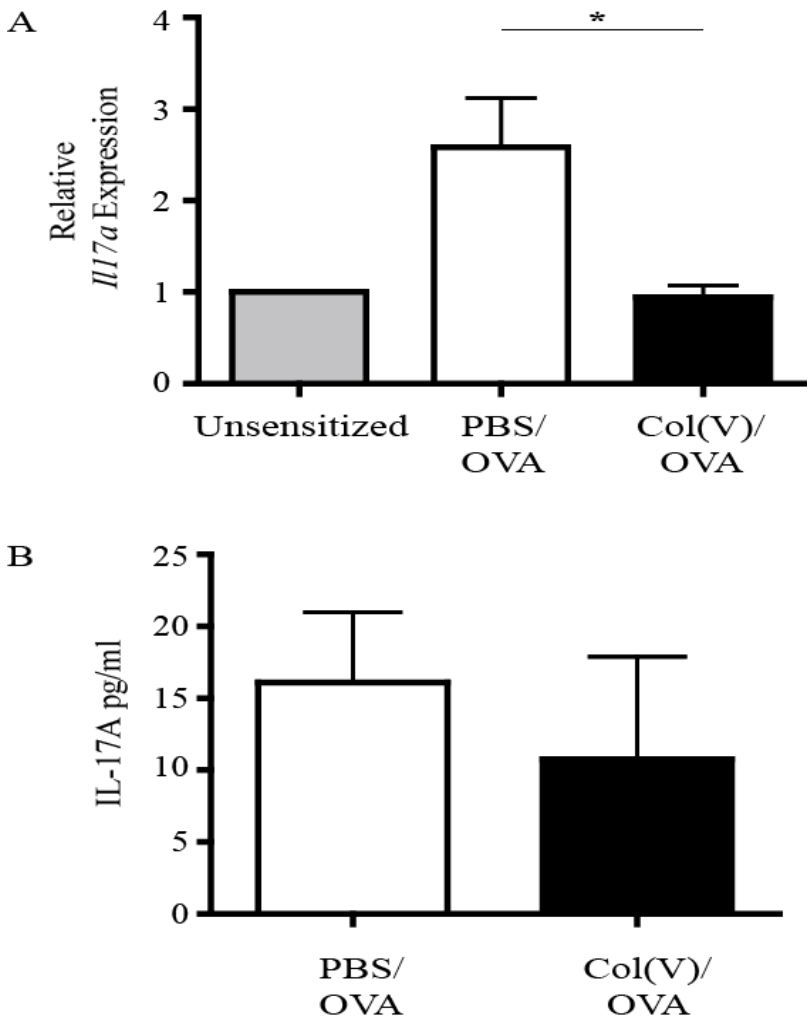

Figure 22. Col(V)-induced tolerance alters IL-17 expression in OVA-induced AAD. *A,* Lungs of unsensitized and sensitized and challenged mice were homogenized and total RNA isolated. mRNA expression of Il17 was measured by quantative RT-PCR. Gene expression was normalized to beta-actin and then to unsensitized animals. B, Serum of unsensitized and sensitized and challenged mice was collected and analyzed for IL-17A protein expression by CBA. Data represents the mean ± SEM of 5-6 animals. *$p<0.05$ using one-way ANOVA and Bonferroni *post hoc*.

Part III: Regulation of complement in allergic airway disease by col(V)-induced tolerance

CD55 and Crry are differentially expressed in allergic airway disease airways

Complement activation in asthma has been well documented and may result from deficiencies in the expression of certain complement regulatory proteins such as CD55 and/or Crry/CD46. Although each is expressed in many tissues, including the lung, a recent report indicates that CD46, the human homologue of Crry, may be down regulated in clinical asthma (168). Interestingly, CD55 is not highly expressed, if at all, in mucin secreting intestinal cells (169). However, the expression of CD55 on airway goblet cells is unknown. Since goblet cells are a hallmark of AAD, and may be the predominant cell type in the asthmatic airway, we next determined the expression of CD55 and Crry in goblet cells during AAD in controls and col(V)-tolerized mice. We first measured *Cd55* and *Crry* transcript expression in the lungs of both PBS/OVA and col(V)/OVA animals. The mRNA levels for *Cd551* and *Cd552* were unchanged when compared to each other or to the unsensitized control animals (Figure 23A-B). The mRNA expression of *Crry* was also analyzed by real-time PCR. The mRNA levels in both PBS/OVA and col(V)/OVA animals seemed to rise; however there was no difference between either group (Figure 23C).

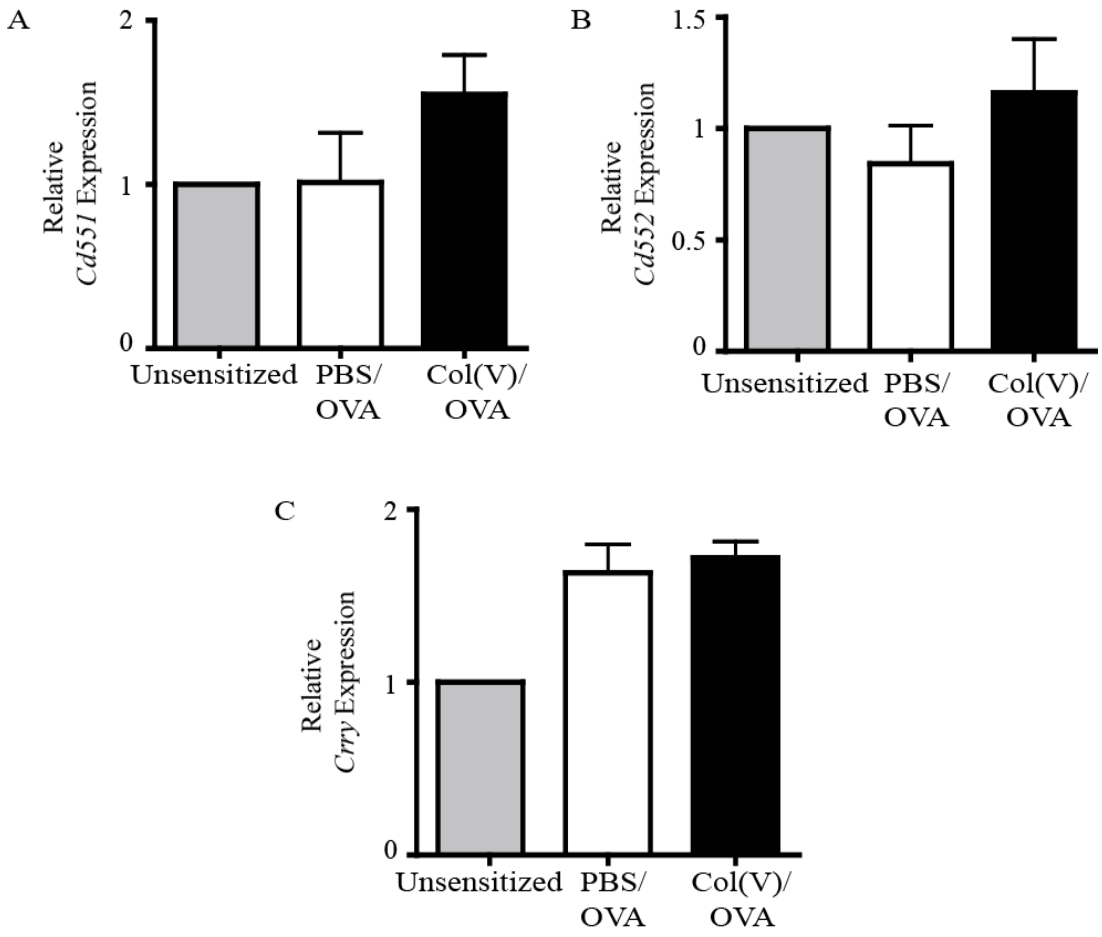

Figure 23. Col(V)-induced tolerance does not alter CRP mRNA expression in OVA-induced AAD. Total lungs of unsensitized and sensitized and challenged mice were homogenized and total RNA isolated. mRNA expression of several CRP genes was measured by quantative RT-PCR. *A*, Relative *Cd551* expression. *B*, Relative *Cd552* expression. *C*, Relative *Crry* expression. Gene expression was normalized to beta-actin and then to unsensitized animals. Data represents the mean ± SEM of 5 animals.

We next determined the protein expression of CD55 and also where the expression was localized in the lung. In the airways of both the PBS/OVA and col(V)/OVA animals that have not undergone goblet cell metaplasia the expression of CD55 remains intact (Figure 24A, C). In contrast, CD55 expression was down-regulated on cells that have undergone goblet cell metaplasia in both PBS-controls and col(V)-tolerized groups (Figure 24B, D). As expected, CD55 is highly expressed on normal airway epithelium (Figure 24A). The images were also analyzed by metamorphic analysis to compare the expression of CD55 in treated groups to unsensitized controls. The analysis demonstrated that in cells that undergone goblet cell metaplasia, CD55 expression is decreased in both PBS/OVA and col(V)/OVA animals when compared to unsensitized control animals (Figure 24F, $p<0.01$).

The results were different when Crry expression was analyzed by immunohistochemistry. Crry expression was maintained in the airways that expressed normal epithelium (Figure 25A, C). The expression of Crry, visually was decreased on airway epithelial cells that had undergone goblet cell metaplasia (Figure 25B, D). Notably, the expression of CD55 and Crry were maintained on stratified epithelial cells during AAD. Metamorphic analysis revealed no statistical significant change in Crry expression on goblet cells when compared to unsensitized control animals (Figure 25F).

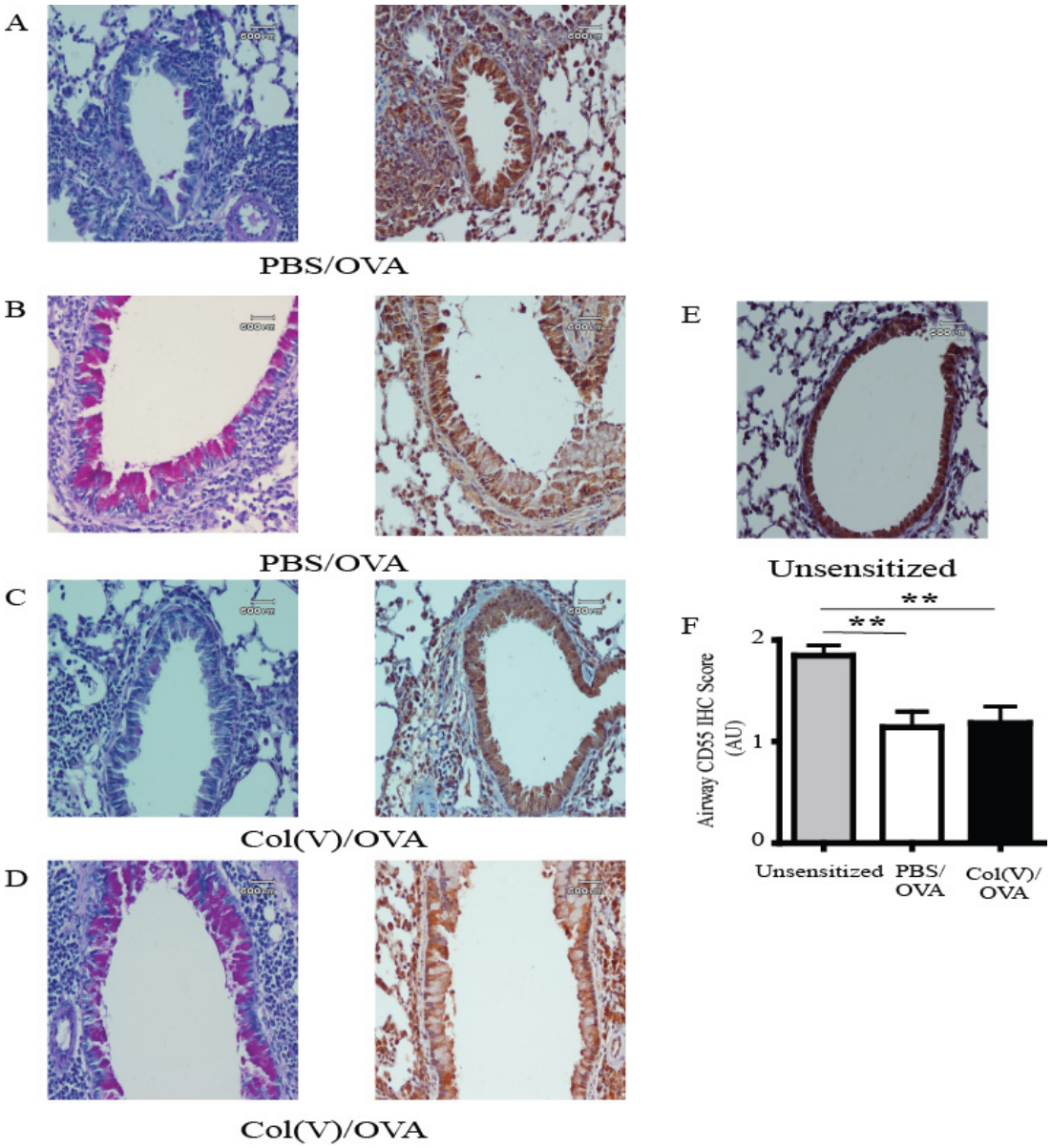

Figure 24. AAD decreases CD55 expression in the epithelium of airways. Lungs of untreated and sensitized and challenged mice with and without col(V) treatment were fixed and embedded in paraffin and stained for the expression of CD55. *A&B*, PAS staining and CD55 expression in an airway of a PBS treated animal. *C&D*, PAS staining and CD55 expression in an airway of a col(V) treated animal. *F,* CD55 expression in an airway of an untreated animal, (original magnification 20x & 40x). *G*, The number of CD55 positive cells per mm of airway epithelial cells, measured using coded slides by Metamorph software. Photomicrographs are representative of the histology of ten animals in each group and are representative of two independent experiments. Metamorph analysis n = 4 in each group. **$p<0.01$ using one-way ANOVA and Bonferroni *post hoc*. Staining performed by Elizabeth Mickler, M.S., analysis performed by Irina Petrache, M.D., Indiana University School of Medicine.

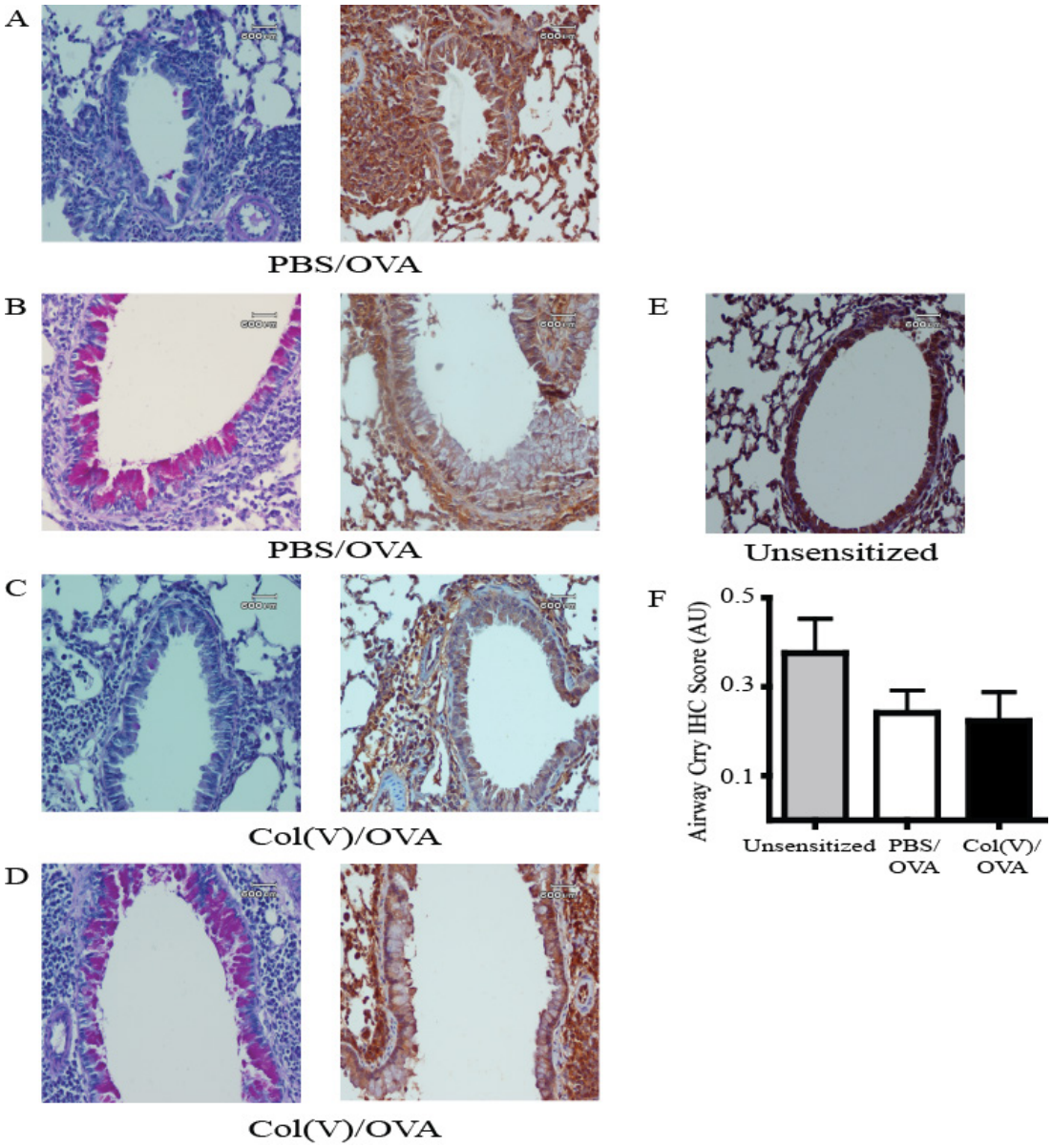

Figure 25. AAD does not change Crry expression in the epithelium of airways. Lungs of untreated and sensitized and challenged mice with and without col(V) treatment were fixed and embedded in paraffin and stained for the expression of Crry. *A&B*, PAS staining and Crry expression in an airway of a PBS treated animal. *C&D*, PAS staining and Crry expression in an airway of a col(V) treated animal. *F,* Crry expression in an airway of an untreated animal, (original magnification 20x & 40x). *G, T*he number of Crry positive cells per mm of airway epithelial cells, measured using coded slides by Metamorph software. Photomicrographs are representative of the histology of ten animals in each group and are representative of two independent experiments. Metamorph analysis n = 4 in each group. Staining performed by Elizabeth Mickler, M.S. analysis performed by Irina Petrache, M.D., Indiana University School of Medicine.

Col(V)-induced tolerance modulates local C3a and C5a expression

Data showing down-regulated expression of CD55 and Crry suggested increased local complement in AAD. C3a has been implicated in AHR by inducing contraction of airway smooth muscles (140), and C3a may promote antigen-induced IL-17 production (140). In contrast, C5a has been linked to inhibiting AAD (154, 155). To determine the potential effect col(V) may have on these mediators of inflammation towards the asthmatic response, C3a and C5a levels were quantitated in BAL fluid of PBS and col(V) treated mice. As expected, C3a was increased in BAL fluid of PBS-treated mice compared to untreated normal controls (Figure 26A, $p<0.001$), however, col(V)-induced tolerance resulted in significantly lower C3a levels compared to PBS-treated mice (Figure 26A, $p<.001$). Interestingly, C5a levels were increased significantly in col(V)-induced tolerized mice relative to PBS-controls (Figure 26B, $p<0.05$). Therefore, col(V)-induced tolerance may act upon components of complement to decrease airway hyper-responsiveness in treated animals.

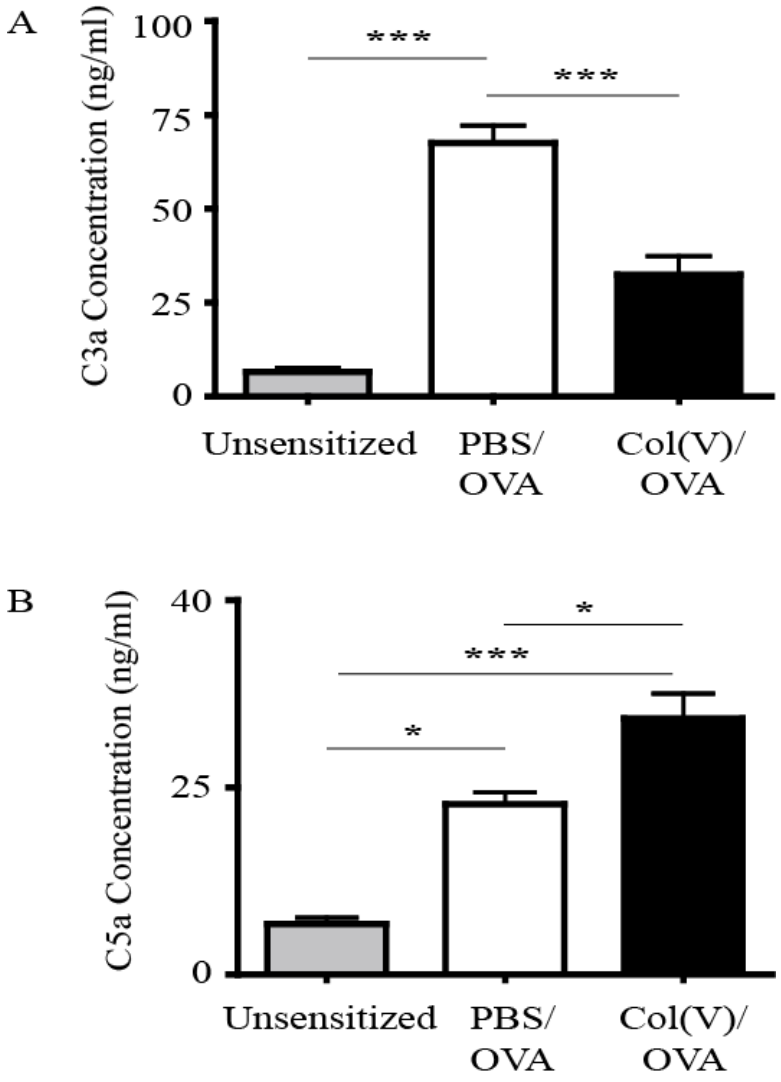

Figure 26. Col(V)-induced tolerance alters pulmonary C3a and C5a in OVA-induced AAD. *A,* C3a protein levels were determined in BAL fluid of untreated and OVA-sensitized and challenged PBS and col(V) animals by ELISA. *B,* C5a protein levels were determined in BAL fluid of untreated and OVA-sensitized and challenges PBS and col(V) animals by ELISA. Results are expressed as mean ± SEM of 6 mice in each group, *$p<0.05$, ***$p<0.001$ using one-way ANOVA and Bonferroni *post hoc*.

DISCUSSION

Summary

In this study we demonstrate a correlation between the development of anti-col(V) immunity in the pathogenesis of AAD and clinical asthma. We utilized a murine model of AAD and identified a possible role for col(V)-induced tolerance in the regulation of AAD. In part I, a correlation between asthma and the presence of antibodies to collagen V was assessed. We were able to show that individuals suffering from allergic asthma have circulating anti-col(V) antibodies. Individuals undergoing lung remodeling wherein lung collagen turnover is occurring perhaps leading to an increase of the expression of col(V).

In part II, using a murine model of AAD, we have defined a role for col(V)-induced tolerance in the modulation of symptoms of the disease. Col(V)-induced tolerance decreased airway hyper-responsiveness in treated animals as compared to vehicle control animals. When common markers of the phenotype of allergic airway disease were studied, we found that col(V)-induced tolerance did not alter the infiltration of cells into the lung parenchyma nor the up-regulation of mucin associated genes or mucus production in these animals. Many of the cytokines that are normally associated with AAD were unchanged or the results were inconsistent in the animals where col(V)-induced tolerance had been generated. However, decreases of IL-17 led to subsequent studies.

In part III, the ability of col(V)-induced tolerance to alter AHR was associated with alterations in IL-17 levels, which could have lead to the observed decrease in C3a

expression. These changes in C3a levels could have lead to the decrease observed in smooth muscle contractility in the tracheas of the treated animals.

Anti-col(V) expression in asthma

There have been several reports showing the presence of humoral responses including anti-col(V) antibodies associated with different human diseases. Human heart transplantation, and the development of acute antibody-mediated rejection (AMR) have an association with the presence of anti-col(V) antibodies (170). Additional studies revealed the presence of anti-col(V) antibodies associated with the pathogenesis of lung transplantation (171-173). The detection of antibodies to col(V) have also been observed in rat transplant models of lung transplantation which have allowed for the study of the mechanisms of the development of alloimmunity to col(V) (159).

In the current study, we examined the expression of anti-col(V) antibodies associated with patients with asthma. We demonstrate that some but not all asthmatics express anti-col(V) antibodies, however not all asthmatics have detectable titers. We show that populations of individuals in the asthmatic populations have antibodies to varying degrees. There was also a cohort of patients that were below the established cutoff point of the assay, that we concluded did not have anti-col(V). This variability of the titers may be due to the varying nature of asthma itself as the disease can be classified into mild, moderate and severe asthma. These three differing classes of asthma may be the three groupings that we noted in the patients from IU School of Medicine (Figure 4A). We are unable to fully explore this possibility because of the lack of data showing the severity of disease associated with the patients that were recruited for the study. The

patients that were represented from the University of Pittsburgh School of Medicine also demonstrated a trend towards higher anti-col(V) antibodies. However, the titers of anti-col(V) antibodies using the University of Pittsburgh samples, these individuals were not statistically significant when compared to normal individuals (Figure 4B). This comparison of individuals shows that not every asthmatic has col(V) antibodies. A recent study showing a relationship between severity of asthma and the presence of anti-col(V) antibodies was published after these studies had begun. As the severity of the disease increased, the amount of anti-col(V) antibodies were increased when compared to normal individuals and the less severe diagnosed individuals (174). The link between col(V) and asthma was further strengthened by the over expression of col(V) in the lungs of a fatal asthmatic. The presence of circulating anti-col(V) antibodies in clinical asthma is consistent with our prior studies showing that lung remodeling and inflammation may be associated with loss of self tolerance to col(V) and development of anti-col(V) immunity (69).

The ability of asthma to generate anti-col(V) antibodies was further studied using a mouse model of OVA-induced AAD. Our data is the first to demonstrate that after induction of AAD in mice, circulating anti-col(V) antibodies are present in the treated animals (Figure 7A). However, one issue that was highlighted was the level at which the antibodies were being detected. As compared to the human subjects, the mouse serum levels of anti-col(V) antibodies were much lower. This may be in part due to the length of time asthma persisted or re-occurred. In humans, asthma is an ongoing one that involves many years of lung remodeling. In our mouse model of AAD, this process has only occurred in a 20 day period (Figure 6A). We further studied the expression of anti-

col(V) antibodies, but associated with an inflammatory environment that was established before the induction of AAD (Figure 6B). Using these data suggest that col(V) immunity in response to AAD may be augmented when coupled with pre-existing inflammation. Inducing tolerance to col(V) before the generation of OVA-induced AAD did not alter the level of anti-col(V) antibodies in treated animals (Figure 6B). Are the anti-col(V) titers detected in asthma and AAD immunogenic? Previous studies using lung transplant patients and rodent models of lung allograft have shown the immunogenicity of anti-col(V) antibodies, however that is not known about the antibodies detected in asthma or AAD. It is necessary to further study these antibodies to determine the effect on asthma and AAD that these anti-col(V) titers have on the diseases.

The issue of lung remodeling is one of the main hallmarks of persistent asthma. In relation to collagen deposition in relationship to asthma, studies have shown that col(V) is up-regulated in response to lung remodeling (175, 176). In cases of repeated allergen exposures as an individual would come in contact with over the course of their lives studies have shown using rat models that repeated allergen exposures alter collagen composition in the lung of animals (177). Recent studies using human subjects have demonstrated that increased col(V) in the lungs of asthmatics can occur outside of the context of inflammation. This increase was caused by the bronchoconstriction of airways only (178). These studies demonstrate a link between changes of collagen composition associated and the pathology of asthma.

Currently, we demonstrate a possible association between lung remodeling that occurs during asthma to possible changes in collagen composition of the lung. We illustrated that the lung structure of the non-asthmatic did not show increased staining for

col(V). The col(V) that was present was limited to a small portion of the sub epithelium and basement membrane (Figure 5B). These are areas that are known to express col(V) in normal lung function related to lung elasticity. When the asthmatic lung was stained for col(V), we found increased col(V) in the sub epithelium which is consistent with previous studies of other diseases associated with interstitial lung remodeling (Figure 5C). We also noted the expression of col(V) in the connective tissue. Although it was not as intense as that in the sub epithelium. Therefore, asthma involves lung remodeling which leads to the unmasking of col(V) and the increase of the possible auto-antigen. While lung remodeling is a well known complication of asthma, to the best of our knowledge, the current study is the first to report that immune responses to the autoantigen, col(V), may correlate with asthma pathogenesis.

Localized effects of col(V)-induced tolerance

In assessing the effect of col(V)-induced tolerance we also found that tolerance had a localized effect in the treated animals rather than a robust systemic response as we had expected. The col(V) was given by tail vein injection and a global response was expected as a result of this treatment. However, most of the changes in responses to OVA were observed to be localized to the area of the lung itself. This pattern was observed in the proliferation of total cells from the mediastinal lymph node, the draining lymph nodes of the lung (Figure 8). This effect of tolerance was not observed when total splenocyte proliferation to either OVA or col(V) was assessed. The proliferation of these cells was not altered in response to either antigen. This could be explained by several possibilities, one being that because the exposed col(V) was in the lung; the cells that

were responsible for generating tolerance migrated to the lung and surrounding area. Another possibility is that the effect was not only localized to the lung, however, but most of the detectable changes were seen in the lung. This possibility is further supported by the previous murine antibody data that was low associated with vehicle control only. This however, cannot explain the slight changes in cytokine protein levels observed in the serum. Cytokine levels in the serum, which will be discussed in detail later in later sections, were found to have changes in protein expression. The tolerigenic effect of col(V) was further demonstrated in the measurement of IgE levels, which were shown to be different in the BAL. However, serum IgE showed a trend towards lower levels that were not statistically different (Figure 9). The actual role that IgE plays in the murine model is under debate. Studies have shown that in the mouse, IgE is not necessary for the development of AHR (179). In the human disease, there is clear evidence the IgE is pathogenic and has a strong relationship with AHR (180). The alteration in IgE responses may not play a role in the mechanism of col(V)-induced tolerization because of this fact. However, why this change occurs may still warrant further study. The effects of local and systemic tolerance were somewhat expected, tolerance is always observed less in the spleen than in lymph nodes. This is because of the presentation of the antigens to respective T cells in lymph nodes versus to the cells of the spleen. The change in T cell responses to OVA and col(V) and the apparent lack of change in anti-col(V) antibody levels shows an uncoupling of T cell and B cell tolerance in the treated animals. These data highlight the intricacies of inducing tolerance and the effects that may come about from the generation of tolerance.

AHR is decreased following col(V)-induced tolerance

In assessing the effect of col(V)-induced tolerance on AAD, specifically the effect on AHR, we found that the induction of tolerance to col(V) caused a decrease in AHR compared to the PBS animals, by measuring Penh (Figure 11A). This trend was observed at lower doses of methacholine. Although the trend did not become significant until higher doses of methacholine were used. This becomes difficult to explain at the highest dose of 300mg/ml of methacholine, at this high dose the lungs may be overloaded and can no longer respond to the methacholine. However, changes at the lower concentrations, specifically 100 and 200mg/ml suggest responses are not saturated. Also if the animals are no longer able to respond, this would not explain the continued rise of Penh and AHR in the vehicle control group. In separate studies, when mice were immunized with col(V) to develop col(V) immunity the results were similar to the antibody data generated earlier. Animals that were immunized with either CFA/IFA alone or CFA/IFA with col(V) had similar penh levels as PBS animals (Figure 11B).

One possible explanation for the disparity that exists between the tolerized and immunized animals may be the mechanism in which tolerance and immunization function. Generally, when tolerance is induced, there is either an increase of Treg cells or a decrease in the ability of T cells to respond through T cell anergy. Associated with immunization; an immune response associated with an inflammatory environment was being generated. We have shown in previous studies that immunization with CFA/IFA with col(V) generates col(V) reactive T cells (70). Irrespective of whether col(V) was present, we saw an increase in AHR that is thought to be due to the CFA/IFA treatment. Previous studies have shown that OVA immunized in the context of CFA will generate

AHR (181). The generation of the inflammatory environment that comes about through immunization could lead to increases in inflammatory cells which contribute to AHR in the treated animals. This does not answer the overall question about CFA/IFA and AHR; however, it does provide an interesting point of discussion.

After establishing that col(V) altered AHR using penh, we wanted to measure lung functions using more direct measurements; including resistance and compliance. This was done specifically because of the lack of other changes in the animals to fully support the Penh data. The results of the resistance and compliance demonstrated that our initial conclusions with Penh were valid (Figure 12A). The col(V) tolerized mice had lower resistance and higher airflow, and they also had a trend towards increased compliance, meaning the lungs were less stiff and easier to inflate (Figure 12B). These data suggest that col(V)-induced tolerance did result in decreased AHR, which could provide protective effects in response to AAD.

A possible correlation between col(V)-induced tolerance and a mechanism by which the decrease in AHR might be explained was studied by measuring smooth muscle contraction. The levels of smooth muscle contraction, as measured by isometric force to Ach showed a decrease in col(V) tolerized animals when compared to PBS treated animals (Figure 13). Interestingly, changes to muscle contraction were not observed when various concentrations of Ach as would normally be expected. The binding of Ach to cholinergic receptors causes smooth muscle contraction as levels higher than what was observed in this study. However, one possible explanation in relation to the current study would be the exposure of the treatment animals to methacholine 24hours prior to isometric force measurement. The tracheal muscles could have been contracted so they

could not respond to the increased levels of Ach. This fact could explain the lack of change in isometric force when tracheas were exposed to Ach, however, we did note a difference in the pre-stimulation isometric force. The PBS animals were contracted more than those in the col(V) or unstimulated groups.

Determining the effects of col(V)-induced tolerance on the AAD phenotype

Along with AHR, other main hallmarks of AAD include cellular infiltration of the lung and goblet cell metaplasia. Crimi et al. demonstrated in human patients that it is possible that AHR and airway inflammation might discriminate between the two factors (182). Studies have also shown that differing mouse strains have separate responses in relation to AHR and inflammation responses (183, 184). This difference has also been shown in mice lacking IL-10 that do not develop airway inflammation while still demonstrating increased AHR (185). Recently at the level of transcriptional regulation, HDAC inhibitors have been shown to regulate airway constriction and not inflammation in both humans and murine models (186). In assessing the effects of col(V)-induced tolerance on lung mononuclear cell infiltration we found that although we decreased AHR in the treated animals the amount of cellular infiltration remained the same. Interestingly we found that the amount of cellular infiltration remained unchanged between treatment groups (Figure 14). This fact was further emphasized when the cellular populations of the BAL were analyzed (Figure 15A). One important cell involved in asthma and AAD are eosinophils (187, 188). The number of eosinophils was virtually the same in PBS or col(V) treated mice, and clearly increased among both of these groups when compared to mice without AAD (Figure 15B).

Lung sections were also studied for the presence of mucus, another hallmark of AAD. Mucin expression in the lungs of treated animals had a similar trend that was observed when mononuclear cell infiltration was studied. Histologically, both treatment groups were positive for mucin expressing cells as shown by PAS staining. The images were quantified and were found to not be statistical different between the PBS/OVA and col(V)/OVA groups (Figure 16A). The lack of alteration of mucus production also was shown in the relative expression of mucin genes which did not change with col(V)-induced tolerance (Figure 16B). Kibe et al. were able to also show a disconnect between goblet cell metaplasia and other factors of AAD after glucocorticoid treatment (189). We illustrate using col(V)-induced that col(V)-induced tolerance can selectively alter individual parts of AAD. These studies demonstrate very clearly that col(V)-induced tolerance uncouples AHR and inflammation or infiltration acting on each independently of each other.

Cytokine changes in response to col(V)-induced tolerance

The expression of cytokines is a hallmark of AAD including cytokines produced by the Th2 subset. Th1 and Treg cytokines have also been shown to be present in the disease (190). Th1 cells secrete IFN-γ, IL-2, and TNF-α; Th2 cells produce IL-4, -5, -13, and -10; and Tregs also produce IL-10. The data presented herein showed gene expression of *Il4*, *Il5*, *Il13*, *Il17*, *Il10* and *Ifng* in lung cells, including lung mononuclear cells and total cells had no consistent pattern of change in col(V)-induced tolerized animals (Figures 19&20). A similar pattern was observed when mRNA expression of the

cytokines was examined in the spleens of these animals. The lack of change in the spleen was not unexpected, since other data presented show that the effect of col(V)-induced tolerance may be localized to the area of the lung.

The dissociation between the decrease of AHR and the other factors of AAD was made more interesting when serum cytokines were analyzed from treated animals. A review by Greenbaum et al. highlighted studies that have shown a dissociation between mRNA and protein expression (191). Serum proteins after col(V)-induced tolerance showed a general trend to lower Th1 and Th2 cytokine levels when compared to PBS control animals. In relationship to our cytokine findings and changes in AHR; Perkins et al. demonstrated that the IL-4 receptor, IL-4Rα was able to promote AHR in animals through multiple mechanisms (31). The trend towards lower cytokine expression, was however not statistically significant when compared to PBS control animals. As shown in earlier data, changes in AHR or other symptoms can occur without complete abrogation of the disease. We hypothesize that col(V)-induced tolerance only slightly alters the immunologic environment in the animals. The changes to IL-4 levels could lead to the production of IgE by plasma cells by the promotion of class switching in these cells that were discussed earlier; however, those changes were only minor. The same can also be said for IL-13 levels and mucin expression. The changes were not dramatic enough to have a noticeable effect on the phenotype. It is possible that col(V)-induced tolerance may be acting on a factor that can directly alter AHR and not need to change other components. These decreases, slight as they might be may provide some insight in

the future about the regulation of AHR by col(V)-induced tolerance. The analysis of cytokine expression demonstrates the uncoupling effect that was stated earlier in the discussion.

Alterations to IL-17 expression following col(V)-induced tolerance

As stated previously, studies have suggested that IL-17 may play a role in asthma and AAD, specifically in neutrophil recruitment. While investigating the possible role of cytokines in the regulation of AHR by col(V)-induced tolerance we focused on IL-17. In our prior studies examining anti-col(V) immunity in lung transplantation and pulmonary fibrosis, a common theme emerged showing a clear role for IL-17 in anti-col(V) responses (92). Following col(V)-induced tolerance and the induction of AAD we found that *Il17a* mRNA expression was decreased in the lungs of the treated animals. A possible association was demonstrated when serum IL-17a protein expression was analyzed and trend towards lower IL-17A levels were found. One correlation between the levels of IL-17A could be due to the levels of IL-6 that were measured. As previously stated, IL-6 is one of the cytokines involved in Th17 polarization. These decreases demonstrated another link between alterations of IL-17 levels in response to col(V)-induced tolerance. Prior studies have shown a clear role $CD4^+$ T cells as the source of IL-17 in other autoimmune-related lung diseases (70). We speculate, similar to prior reports, that $CD4^+$ T cells may be the source of IL-17 in the current study but the exact cellular source is unknown and will be investigated in future experiments.

Taken together these finding suggest that IL-17 is involved in the process by which col(V)-induced tolerance regulates AHR. This could be brought about by several factors; one could be IL-17 directly acting on cells themselves. Recently, Kudo et al.

demonstrated using mice deficient with IL-17a that these mice do not develop AHR. They also showed that Il-17A may have direct actions on smooth muscle cells (41). Another possibility may be the ability of IL-17 to act on components of innate immunity. Lajoie et al. showed that IL-17A can up-regulate the production of C3a which in turn can cause more IL-17A production (192). These data demonstrate the complexity of IL-17A biology in allergic asthma and AAD and suggest that col(V)-induced tolerance can regulate IL-17A expression which can alter the pathogenesis of AAD.

Decrease of CD55 and Crry following AAD induction

Observations of CRP expression in asthma or AAD have not been reported on airway epithelial cells. Activation of local complement activity in asthma may be due to allergen-antibody complexes, recognition of antigen-derived polysaccharides, or proteases derived from allergens or inflammatory cells that cleave complement products directly (193). However, another potential mechanism could be due to down regulation of key CRPs, CD55 and Crry (or CD46 in humans) on airway epithelial cells. Varsono et al. were first to show that under normal conditions CD55 and CD46 were expressed highly on epithelial cells of the lower airway tract (194), while Matsuo et al. reported that blocking these CRPs resulted in spontaneous pulmonary inflammation (195).

Under normal conditions, goblet cells represent a minority of cells in the bronchi/bronchioles. However, Ordonez et al. reported that these cell types can triple in number in mild asthma (196) and extensive goblet cell hyperplasia is occurs in severe asthma. The expression of CD46/Crry and CD55 on goblet cells in the lower respiratory tract is unknown. However, intestinal goblet cells do not express CD55 (169). These

data are interesting since a recent study of asthmatics and patients with COPD reported that local CD46 expression was down-regulated markedly decreased (197, 198). However, the cells expressing CD46 were not reported in those studies. Goblet cells may become a major constituent of epithelium in asthma, and unlike stratified squamous epithelial cells, the current data and a prior study suggest that goblet cells may not express CRPs or do so at much reduced levels. We hypothesize that the balance of cells that do and do not express CRPs in the lower respiratory tract could impact local complement activation. Therefore, more goblet cells that do not express CRPs could perturb that normal airway homeostasis and lead to more complement activation, and release of anaphylotoxins, which exacerbate the asthmatic response. These data suggest a need for further investigation of the mechanisms that regulate CRP expression on airway epithelial cells.

Regulation of complement by col(V)-induced tolerance

IL-17, which has been suggested to be important in neutrophillic asthma (16), has been linked recently to the expression of the complement products, C3a and C5a, in murine AAD (192, 199). Katz et al. demonstrated using skin fibroblasts, that IL-17 regulated the expression of C3 (200). Specifically, C3a has been shown to exacerbate AHR while C5a may be protective of this response (153, 154, 156, 157, 201). Indeed, the current study reports that C3a was up regulated locally nearly threefold more than C5a in control mice (PBS-treated). In contrast, col(V)-induced tolerance abrogated the increase in C3a and IL-17 while further augmenting local levels of C5a. These results may seem paradoxical in that both C3a and C5a have been implicated in exacerbating the asthmatic

response in humans as both are anaphylotoxins able to amplify inflammatory responses (154). Therefore it is unclear exactly why C5a was increased in tolerized mice when C3a was decreased. One possibility could be related to the possibility that up regulated C5a down regulated the quantity of IL-17 producing cells as shown in mice (192), and less IL-17 resulted in less C3a. We speculate that col(V)-induced tolerance may have not only suppressed IL-17 via regulatory cytokines but also via induction of C5a that limited IL-17, and subsequent C3a expression. Our findings that decreased Il-17a was associated with decreased C3a levels were consistent with previous research that highlighted the regulation of C3a by IL-17 (200). Recent studies have revealed an additional link of C3a between IL-17 in that IL-17a can induce its own production through the production of C3a (192).

There are some limitations to our current study. Although we have shown an association between col(V)-induced tolerance and a decrease of airway smooth muscle contraction, decreases antigen-induced T & B cell activity, the exact mechanism by which tolerance induced theses changes has not been specifically elucidated. Previous research by our laboratory has demonstrated the ability of col(V)-induced tolerance to prevent acute and chronic lung allograft rejection in rodent models. In those reports, col(V)-induced tolerance was transferable to naïve rodents via regulatory T cells (Tregs) that produced TGF-β (83). The induction of TGF-β was somewhat expected since oral tolerance, utilized in the lung transplant studies, was mediated typically by this cytokine (78). IL-10 has been shown to suppress AHR (202). However, neither IL-10 nor TGF-β was induced strongly in the lung or spleens of tolerized animals in the current study.

Recent studies report that IL-35, may have potent suppressive effects on AHR and allergen-specific IgE (203). Though not examined in the current study, it is interesting to speculate that col(V)-induced tolerance and suppressed IgE responses may have been mediated by IL-35.

CONCLUSIONS

In our study of AAD and the effect of col(V)-induced tolerance, we discovered that asthmatics have circulating anti-col(V) antibodies and that in severe cases there is an increased col(V) in the lungs. In this thesis, we propose the mechanism by which complement, IL-17 and the auto-antigen col(V) are linked to allergic airway disease. Airway epithelial cells normally express the CRPs: CD55 and Crry on their cellular surfaces. During the induction of allergic airway disease, inflammatory cytokines including IL-17 are up regulated. At the same time, airways are undergoing goblet cell metaplasia under the control of IL-13. We show after goblet cell metaplasia has occurred, CRPs are no longer expressed or are expressed at decreased levels on airway epithelial cells. Cells in the surrounding lung parenchyma still express both CRPs similar to unsensitized animals. At the same time, IL-17 is increasing the amount of C3a, which has also been shown to increase IL-17 levels (192). As IL-17 and C3a continue in a feed-forward loop, C3a also acts on smooth muscle cells to cause contraction of these cells and increase AHR in these animals (Figure 27).

We also demonstrated that using col(V)-induced tolerance altered AAD in a mouse model. We found that after the induction of tolerance, mice had decreased AHR when challenged with methacholine; there was also a decrease in localized responses to OVA following tolerance. In contrast, other known markers of the AAD phenotype such as lung infiltration, goblet cell metaplasia, and the induction of Th2 cytokines remained unchanged in the animals. Additionally, IL-17 levels were decreased after tolerance induction along with C3a levels. A rise in C5a levels along with the modulation of

smooth muscle contraction was also observed in the treated animals. We conclude that col(V) causes a decrease of IL-17 which can directly affect the levels of C3a leading to decreases in this protein. These finding along with the increase in C5a lead to a decrease in airway hyper-responsiveness (Figure 28). These results support the hypothesis that col(V)-induced tolerance modulates the asthmatic response.

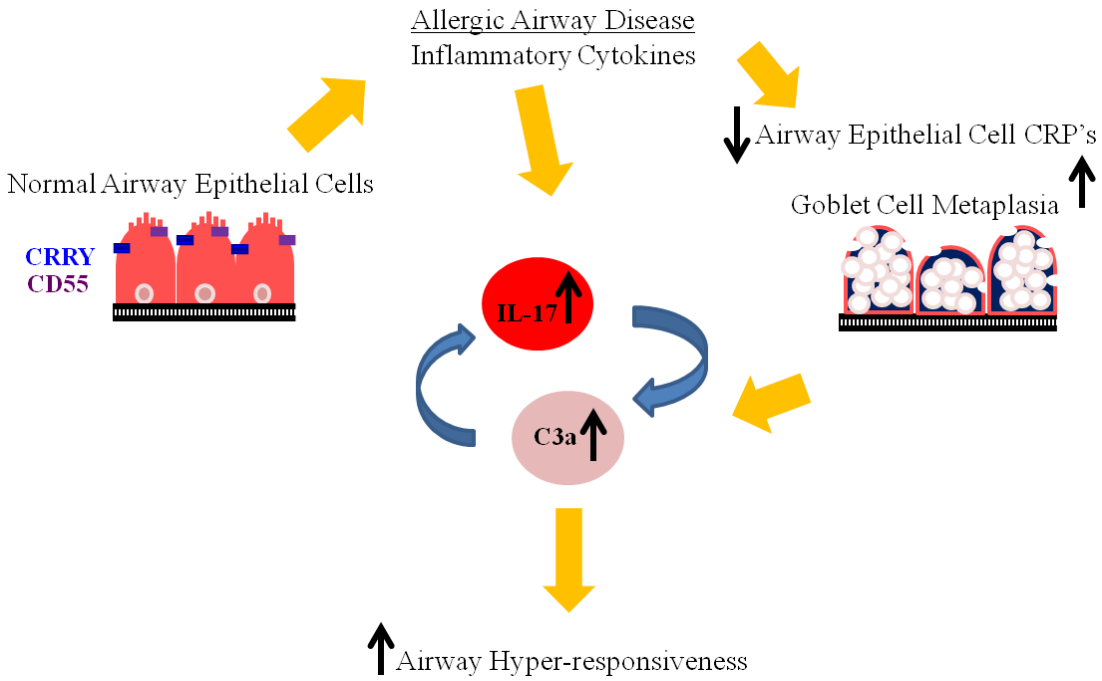

Figure 27. Complement involvement in AAD.

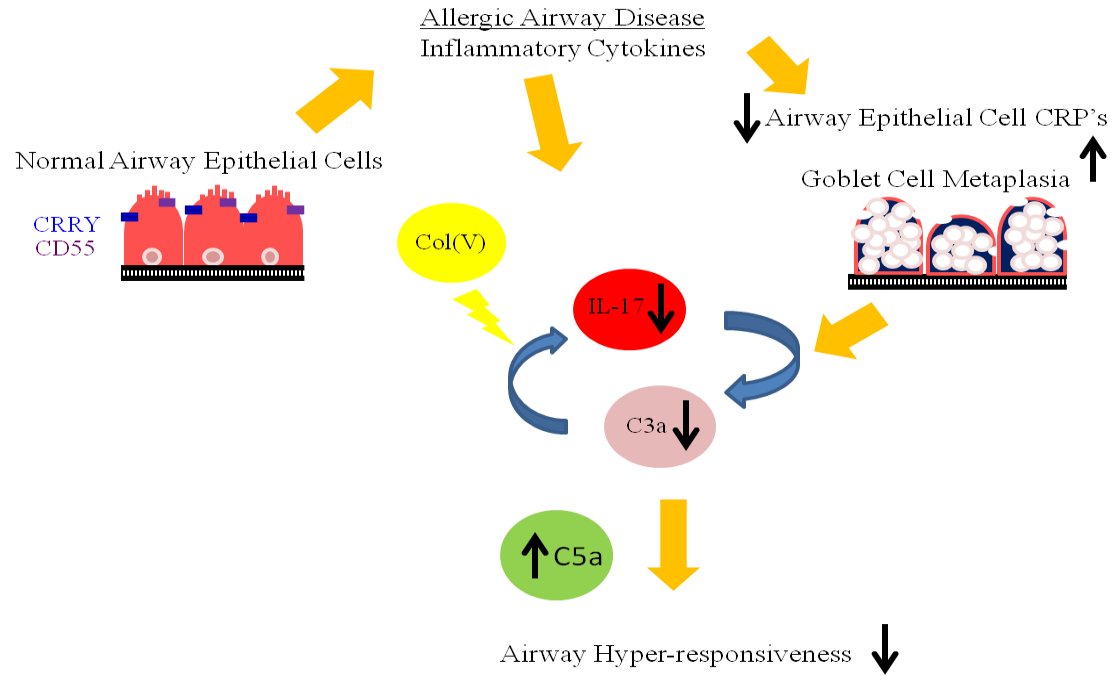

Figure 28. Col(V)-induced tolerance modulation of AAD.

FUTURE DIRECTIONS

Modulation of AAD by col(V)-induced tolerance

The discovery that col(V)-induced tolerance regulates AAD is a novel finding. There are however, a number of future studies that are needed to further elucidate the exact mechanism by which this regulation is occurring. One of the studies that would further elucidate the mechanism would be to define the role of IL-17 in our model. To confirm the role of IL-17, specifically in regulating C3a levels, using anti-IL-17 to bind IL-17 and prevent the binding of IL-17 to its receptors would be useful to study. This would be done in the absence of col(V)-induced tolerance, to fully understand the role of IL-17 following the induction of tolerance. One possible complication to studying the role of IL-17 in the regulation of C3a is that C3a is not only induced by IL-17. In the normal course of AAD, C3a is produced; because of this fact determining the true effect of IL-17 would be difficult.

In trying to identify the mechanism of alteration of AAD by col(V)-induced tolerance, another factor to study is the contribution of C5a. Studies have shown the reciprocal role of C3a and C5a as both activators and inhibitors of AHR and AAD. Previous studies have demonstrated that the absence of C3a leads to decreased AHR (142). Similar results have also been observed in animals studies using both guinea pigs and mice that have blocked anaphylotoxin receptors, specifically C3a receptors (201, 204, 205). Our studies show that C5a is up regulated after col(V)-induced tolerance, which we hypothesize, is a result of col(V)-induced tolerance. Using our model of col(V)-induced tolerance, treating mice with a C5a inhibitor after the induction of

tolerance could answer the possible role of C5a modulation. The established protocol of AAD induction would be performed with the addition that before each intranasal challenge with OVA, the animals would be given the C5a inhibitor by nebulization. Another use for the inhibitor would be to give the inhibitor by i.p. injection following each OVA intranasal challenge. Blocking the activity of C5a would allow the study of the contribution of C5a during the changes in AAD brought about by col(V)-induced tolerance.

The transfer of tolerance is another area of study that could greatly benefit this experimental model. Previous studies using the rat lung transplant model by the Wilkes lab have utilized the transfer of col(V) reactive cell. Using these studies and others that have been used for the transfer of allergic airways responses in rats, one of the studies that would further elucidate the potential of this mechanism would be the transfer of tolerized cells into animals before the induction of allergic airway disease. Using the model of allergic airway disease, animals would be injected with col(V) tolerized lymph node cells by the tail vein approximately 24 hours before OVA challenge. The time point before OVA challenge was selected because lung damage occurs during the challenge phase of our model. Using these transferred cells would allow the study of the contribution of the cells and the potential for further study.

Another aspect in studying the mechanism of col(V)-induced tolerance regulation of AAD is determining the cell that is responsible for the generation of tolerance in the model. Previous studies have shown the involvement of plasmacytoid DCs (pDC) in the development of tolerance, specifically as it pertains to transplantation and in the animal model of asthma (206, 207). These cells have also been linked to AHR in various

models, including limiting AHR in both ADD and in respiratory syncytial virus infection (RSV) infection (157, 208). The regulation of IL-17 has also been linked to pDCs, specifically Kang et al. demonstrated in a model of lupus that peptide tolerance generated pDCs that suppressed Th17 cells (209). Using our model to demonstrate the involvement of pDCs would require the treatment of mice before the induction of tolerance. Previous studies have depleted pDCs by i.p. injection using an anti-pDCA antibody (210). The timing of the depletion will be important, and studies have used various time frames for the depletion. Studies involving the measurement of pDC depletion will be necessary to find the optimum depletion and timing. The depletion of pDCs will provide insight into the mechanism by which col(V)-induced tolerance is regulated in AAD.

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CURRICULUM VITAE

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Education

2012	Doctor of Philosophy, Microbiology and Immunology Indiana University, Indianapolis, IN
2005	Master of Science, Biology Jackson State University, Jackson, MS
2003	Bachelor of Science, Biology Jackson State University, Jackson, MS

Academic Experience

2007	Teaching Assistant –Department of Microbiology and Immunology, Indiana University, Course J210 Microbiology for students enrolled in health related disciplines including nursing, allied health, dental hygiene, and pre-medicine.
2002	Summer Research Assistantship. Minority Medical Educational Program (MMEP). Fisk University. Nashville, TN.
2001	Summer Research Assistantship. Health Careers Opportunities Program (HCOP). Talladega College. Talladega, AL.

Society/Professional Memberships

2010-	Trainee Member, American Thoracic Society
2004-	Trainee Member, American Association of Immunologists

Academic and Professional Honors

2011	Minority Trainee Travel Award Recipient, American Thoracic Society
2009	John Wallace Fellowship Recipient, Autumn Immunology Conference
2009	National Institutes of Health Harper Scholar Minority Fellowship Award
2004	Minority Travel Award, Sponsored by the American Association of Immunologists and National Institutes of Health MARC
2003	National Institutes of Health Bridges to the Doctorate Fellowship Award

Peer Reviewed Publications

Benson, H.L., H. Suzuki, **J. Lott**, A.J. Fisher, P. Pandiya, C. Walline, K.M. Heidler, R. Brutkiewicz, J.S. Blum, D.S. Wilkes. 2012. Donor Lung Derived Myeloid and Plasmacytoid Dendritic Cells Differentially Regulate T Cell Proliferation and Cytokine Production. Respiratory Research. 13(1):25.

Zhou, D., P. Li, Y. Lin**, J.M. Lott**, A.D. Hislop, D.H. Canaday, R.R. Brutkiewicz, J.S. Blum. 2005. Lamp-2a facilitates MHC class II presentation of cytoplasmic antigens. Immunity. 22(5): 571-581.

Lott, J.M., S. Sehra, P. Mehrotra, E.A. Mickler, A. J. Fisher, R.G. Presson, I. Petrache, M.F. Busk, S. Goenka, S.E. Wenzel, M.H. Kaplan, D.S. Wilkes. Type V Collagen-Induced Tolerance Prevents Airway Hyper-responsiveness, IL-17 and C3a Production. American Journal of Respiratory and Critical Care Medicine. **Under Revision**.

Vittal, R., E.A. Mickler, K. Roththar, C. Zhang, **J.M. Lott**, A. Emtiazdjoo, S. Frye , K.M. Brown, A.J. Fisher, G.N. Smith, O.W. Cummings, D.S. Wilkes. Overexpression of Type V Collagen in Patients with IPF and Tolerance Induction by Nebulizing Type V Collagen in Bleomycin-Injured Mice. **Manuscript In Preparation.**

Abstracts and Invited Seminars

A. Abstracts

Lott, J.M., S. Serha, M. Busk, M.H. Kaplan, S. Balzar, S.E.Wenzel, D.S.Wilkes. Type V Collagen Induced Tolerance Prevents Allergic Airway Disease. American Thoracic Society International Conference May 2011.

Lott, J., S. Serha, M. Busk, M. Kaplan, D.Wilkes. Tolerance to collagen V limits inflammatory and airway reactivity in an asthma model. 39th Annual Autumn Immunology Conference Nov. 2010.

Lott, J., M. Vasko, D. Wilkes. The Effects of $S1P_1$ on $CD19^+$ B cell Function. 38th Annual Autumn Immunology Conference Nov. 2009

Lott, J., M. Vasko, D. Wilkes. The Effects of $S1P_1$ on $CD19^+$ B cell Function. Annual Biomedical Research Conference for Minority Students Nov. 2009.

Lott, J.M., J.A. Cameron, J.S. Blum. MHC class II-restricted processing and presentation of a cytoplasmic antigen. Experimental Biology May 2005.

Lott, J.M., D. Zhou, J.A. Cameron, J.S. Blum. Immunological responses to cytoplasmic antigens: role of MHC class II antigens. Experimental Biology May 2004.

B. Invited Seminars

2012	Department of Medicine, Vanderbilt University School of Medicine, Nashville, TN
2012	Department of Surgery, University of Pittsburgh School of Medicine, Pittsburgh, PA
2012	Department of Medicine, University of Colorado-Denver School of Medicine, Aurora, CO

Research Support

NIH NIGMS R25 GM079657-04. Indiana University Initiative for Maximizing Graduate Student Diversity. Hal E. Broxmeyer, PI. August 2009-February 2012.

NIH NHLBI R01 HL081350. David S. Wilkes, PI. MMPs in Alloimmune and Autoimmune Lung Disease. June 2006-May 2009. (Minority Supplement)

NIH NIGMS R25 GM67592. Bridges to the Doctorate Program. Hal E. Broxmeyer, PI. June 2003-August 2005. (JSU Mentor-Joseph Cameron) (IU Mentor-Janice S. Blum). Academic Year Support.

NIH NIAID R01 AI049589. Janice S. Blum, PI. MHC Class II Restricted Cytoplasmic Antigen Presentation. (Minority Summer Supplement)