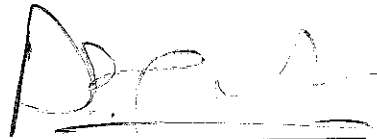


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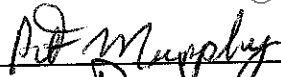
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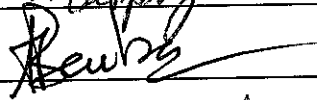
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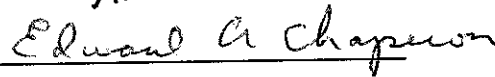
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**TH₁₇ CELLS AND SUPPRESSOR OF CYTOKINE SIGNALING IN
HOUSE DUST MITE MODEL OF ASTHMA: EFFECT OF BY FLT-3
LIGAND.**

By

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A THESIS

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Abstract:

Asthma is a chronic inflammatory disorder of the airways. In this disease an allergic reaction occurs that shifts T-helper (Th) cells towards a Th2 subtype response to innocuous antigen characterized by a predominant production of the cytokines IL-4, IL-5, IL-9, and IL-13. These cytokines together with mediators from mast cells, basophils and eosinophils lead to IgE class switching, chronic inflammation, massive mucus secretion, and the contraction of smooth muscle cells. However it is unclear how the inflammatory response in the lung is modulated during an allergic reaction. In this study the role of suppressor of cytokine signaling proteins (SOCS) and Th17 cells was examined in the regulation of cytokines, allergic and inflammatory response in the airways.

Most research have used an asthma model with OVA as an antigen and intraperitoneal injections as the route of sensitization, neither being typical of the clinical situation. This study set out to establish a mouse model of asthma closer to clinical situations by using a common antigen, House Dust Mite, and sensitizing the mice intranasally before determining the presence of inflammatory SOCS proteins and the novel helper T cell, Th17.

Mice were sensitized either by intranasal administration (IN) or by intraperitoneal injection (IP) with house dust mite antigen. Airway hyperresponsiveness (AHR) was determined by response to methacholine using both non-invasive and invasive methods. H & E staining and Tri-Chrome staining was used to compare morphology and collagen deposition, respectively. Periodic Acid Schiff (PAS) staining was performed to visualize goblet cell

hyperplasia and mucus deposition, and immunohistochemistry (IHC) was performed to visualize the expression of SOCS 1, 3, and 5.

Intranasal sensitization followed by aerosol challenge with HDM elicited a consistent and significant increase in AHR to methacholine in mice while the IP method was not effective with this antigen. The Th17 cell in asthma was examined and the hematopoietic growth factor FMS-like tyrosine kinase 3 ligand (Flt3L) was used to help determine if the Th17 cell is pro-inflammatory or a regulatory cell in the case of asthma. 5 µg IP injections of Flt3L were given to mice with established AHR and allergic airway inflammation on ten consecutive days. IHC was performed and the expression of SOCS 1, 3 and 5 decreased after treatment with Flt3L. Th17 cells, as indicated by their phenotype of CD4+IL-23R+ and mRNA expression of ROR-γ, were significantly present in the mice treated with HDM. When treated with Flt3L the number of these cells in the lungs and spleen decreased, suggesting that the Th17 cell is also a contributor to asthma symptoms.

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LIST OF ABBREVIATIONS

AHR-	Airway Hyperresponsiveness
APC-	Antigen Presenting Cell
BALF-	Bronchoalveolar Lavage Fluid
DC-	Dendritic Cell
FL-	FMS-like tyrosine kinase 3 ligand
Flt3L-	FMS-like tyrosine kinase 3 ligand
H&E-	Hemotoxylin and Eosin
HDM-	House Dust Mite
IHC-	Immunohistochemistry
IgE-	Immunoglobulin E
IL-	Interleukin
IFN- γ -	Interferon Gamma
IN-	Intranasal
IP-	Intraperitoneal
JAKs-	Janus Kinases
Mch-	Methacholine
MHC-	Major Histocompatibility Complex
OVA-	Ovalbumin
PBS-	Phosphate Based Saline
RBC-	Red Blood Cell
ROR- γ t	Retinoic acid-related Orphan Receptor Gamma Thymus
RT-PCR	Reverse Transcription Polymerase Chain Reaction

SEN-	Sensitized
SCF-	Stem Cell Factor
STAT-	Signal Transducer and Activator of Transcription
Th-	T-helper
TGF- β -	Transforming Growth Factor Beta
TNF- α -	Tumor Necrosis Factor Alpha
Tregs-	T-Regulatory Cells

I. INTRODUCTION

A. Bronchial Asthma:

Asthma and allergic disorders are becoming epidemic problems across the world and its increased incidence has been well established in the industrialized nations. Nearly half of the population of these countries has been sensitized to one or more allergens. Allergies are more than just nuisances but can be life threatening in the instances like anaphylaxis and asthma (Holgate, 1999).

Allergies pay a heavy cost to societal healthcare systems. Allergies are the sixth leading chronic disease in the US and cost the healthcare system \$18 billion annually. Asthma can be a burden on the health system as well as in productivity in work and school. In the U.S. in 2002, 12 million people had an asthma attack, 14.7 million school days were missed, and 11.8 million work days were missed. There were 13.9 million outpatient asthma visits, 1.9 million visits to the emergency department, 484,000 asthma hospitalizations, and 4,261 people died from asthma (2006a).

Asthma is a chronic inflammatory disorder of the airways. The chronic inflammation is associated with airway hyper-responsiveness (AHR) (2006b). There are several types of asthma such as atopic asthma, non-atopic asthma, exercise-induced asthma, cold-air induced asthma, night time asthma, and occupational asthma. All the subtypes are not well defined but all share some similarities. Some similarities include wheezing and chest tightness, but non atopics may show no history of allergy and have normal immunoglobulin E levels while atopics have these symptoms (Humbert, 2000; Lemiere, 2006; Walia et al., 2006). These studies focus on the mechanisms of atopic asthma.

Asthma is associated with chest tightness and shortness of breath caused by airway inflammation, obstruction, and airway hyper-responsiveness (Koh et al., 2003). These symptoms occur in two phases following exposure to an allergen, with an early phase occurring in the first 5-15 minutes of exposure and a late phase 2-9 hours later (Howarth et al., 1987). In the early phase mast cells are activated by being cross linked with IgE, which is specific to the antigen, then degranulates releasing histamine, chemokines, cytokines, and leukotrienes. This causes local responses such as constriction of the airways, increased mucus production, and edema. Due to the activities in the early phase the late phase is signaled by the migration of neutrophils, eosinophils, and monocytes to the area adding to the inflammation (Holgate and Kay, 1985).

Allergic reactions shift T-helper (Th) cells towards a Th2 subtype response to antigen. When exposed to allergen, antigen presenting cells (APCs), such as dendritic cells take up the antigen and then migrate to regional lymph nodes. In the lymph nodes the dendritic cells process the antigen into small peptide fragments and express it association with the Major histocompatibility complex (MHC) class II molecules. The antigen is then recognized by T cell receptor (TCR) of CD4+ T cells. These actions activate the CD4+T cells and can cause differentiation into Th2 cells (Upham and Stumbles, 2003; Vermaelen et al., 2001).

B. TH2 Cells

Th2 cells typically arise in helminthic infections but they also develop when a person has allergies. Th2 cells are characterized by a predominant production of the cytokines IL-4, IL-5, IL-9, and IL-13. Each of these cytokines aid in the airway obstruction in different ways: IL-4 induces Th cells towards a Th2 subtype and causes class switching in B-cells to IgE which aids in inflammation. IL-5 recruits and activates eosinophils. IL-9 works as a T cell and mast cell growth factor, as well as works synergically with IL-4 and IL-5 with IgE formation and eosinophil maturation respectively. IL-13 aids in IL-4 production, class switching and assists in airway hyperreactivity and glycoprotein hypersecretion (Coffman et al., 1993; de Vries, 1998; Devos et al., 2006; Hamid and Minshall, 1996; Repa et al., 2004). Studies have been performed to identify the genes and proteins that are responsible for the expression of these cytokines in allergy. Recently one set of molecules that has been looked at closely are the suppressor of cytokine signaling (SOCS) molecules.

C. Suppressor of Cytokine Signaling

SOCS are a family of molecules that work as a negative feedback of specific cytokine signals. Janus kinases (JAKs) are responsible for the signaling of a large number of cytokines. SOCS work by interfering with the binding of cytokine receptors and intracellular molecules like JAK (Arakawa et al., 2004; Krebs and Hilton, 2000). The SOCS family consists of SOCS-1 through SOCS-7 and the cytokine-inducible SH2-domain-containing protein (CIS) (Wormald and Hilton, 2004). SOCS-1, 3, and 5 have been implicated in its involvement in asthma due to their interaction with inflammatory cytokines.

SOCS-1 has been implicated in a wide range of cytokine signaling pathways due to its ability to bind to all JAK family tyrosine kinases through its SH2 domain and the blocking of their activity. Interferon-gamma (INF- γ) has been shown to cause expression of SOCS-1, which in turn blocks expression of STAT 6 and IL-4 (Heller et al., 2004). SOCS-1 also can block INF- γ production when induced by IL-4 through blocking of STAT 1. Thus SOCS-1 may be a mechanism for the mutual suppression of both Th1 cells and Th2 cells (Yoshimura et al., 2007). SOCS-3 is known to enhance airway hyper-responsiveness and skew cells towards a Th2 response. This is a consequence of IL-4 inducing SOCS-3 causing it to preferentially inhibit IL-12, which is necessary for a Th1 response (Seki et al., 2003). SOCS-5 is a mirror of SOCS-3 and has been shown to skew towards a Th1 response by inhibiting IL-4 signaling (Ohshima et al., 2007; Seki et al., 2002).

D. Mouse Model of Acute Asthma

Researchers have gathered information on asthma, such as the type of immune response, by utilizing animal models especially mouse models which are relatively inexpensive. Animal models are used because they offer opportunities to study disease pathogenesis and to develop new therapeutics. Mouse models of asthma have been developed to gain insight on the allergic immune response, modeling clinical behavior of allergic asthma, and finding insights into pulmonary pathophysiology (Epstein, 2004). Acute airway inflammation induced in mice is characterized by accumulation of antigen-specific CD4⁺ effector cells and eosinophils surrounding the airways and blood vessels (Fulkerson et al., 2005).

Most models sensitize the animal with ovalbumin (OVA), and many do not show the typical chronic inflammatory and lung structural changes seen in most patients with asthma. These models typically have 40-80% eosinophilia in their bronchoalveolar lavage fluid (BALF) compared to 1-3% typical of human asthma (Brightling et al., 2003; Fulkerson et al., 2005; Najafi et al., 2003; Ward et al., 2002). Researchers have also found that in humans IgE and mast cells mediate early-and late-phase allergic responses, which are unnecessary for the generation of allergic asthma in mice. This raises the question of the validity of mouse models since they are not exact replicas of human disease (Epstein, 2004).

It is important to develop a valid mouse model to help in the understanding of the progression and treatment of the disease. Prolonged allergen exposure likely triggers a distinct array of immunological responses that result in chronic inflammation and impact airway integrity and function (Fulkerson et al., 2005). Common allergens are Ragweed, Bermuda grass, rye grass, white oak, Russian thistle, *Alternaria* mold, cat dander, house dust mite, German cockroach, and peanut (2006a).

House dust mite (HDM) is an antigen that has been focused on in the sensitization of individuals with asthma. Thirty percent of children are allergic to house dust mites, a figure that has increased with the incidence of asthma. There are several groups of HDM allergens. The ones typically studied are of the species *Dermatophagoides farinae* and *Dermatophagoides pteronyssines* (Thomas et al., 1998).

It appears that HDM has a prominent role in asthmatic individuals and thus an appropriate animal model should be developed to help determine the

immunological effects of this antigen. This could help in the search for developing a human model of asthma specifying antigens that are involved in the disease. Since allergies vary from people and mice due to genetic differences the strain of mice used may affect the results. Also the route of immunization may make a difference because the antigen is in areas with different types of cells. It is important to determine suitable routes of immunization and strains of mice that could maximize the model of asthma.

Studies have examined the effect of local application of antigen to the lungs and its response. Enander et al. found that mice do develop acute asthma when sensitized by an intranasal or subcutaneous application (Enander et al., 1985). Repa et al. demonstrated that a subcutaneous (SC) injection route is more effective in displaying a typical Th2 response than an intraperitoneal (IP) injection or aerosol sensitization even though that did sustain equal allergic airway inflammation (Repa et al., 2004). Nelde et al found that intranasal sensitization produced greater asthma characteristics compared to the intraperitoneal route which is similar to the epicutaneous route (Nelde et al., 2001).

Studies have also been done to observe the strain of mice that would be most effective in becoming a mouse model of asthma. The BALB/c mouse is a strain known for developing Th2 responses (Tournoy et al., 2000; Zhang et al., 1997) and is commonly used to develop models. The first study looked to develop a mouse model of asthma using the BALB/c mouse, house dust mite as an antigen, and comparing two routes of sensitization: intranasal versus intraperitoneal.

After establishing that the intranasal route of sensitization with HDM leads to acute asthma symptoms I wanted to examine the mechanisms of reversal of asthma symptoms and the cells involved. It has been established that CD4+ T cells are involved with allergic asthma which appears to be the work of the Th2 subtype however, recently there has been a discovery of a novel subtype of CD4 helper cells the Th17 cell.

E. Th17 Cell

There are some discrepancies in different studies: Th1 dominant disease led to the discovery of the Th17 cell. In studies of experimental autoimmune encephalitis (EAE) and collagen-induced arthritis (CIA) researchers found that blocking of the p40 subunit of IL-12, a Th1 cytokine, lead to the ablation of the diseases but if just the Th1 specific cytokine IFN- γ was blocked the disease would still progress. More studies were done on the IL-12 family and a cytokine that shared the p40 subunit but had a unique p19 subunit, IL-23, was found and it has been linked to the inflammatory disease and production of cells that produce IL-17 (Constantinescu et al., 1998; Harrington et al., 2006; Leonard et al., 1995; Oppmann et al., 2000; Segal et al., 1998).

The Th17 cell is developed from naïve CD4+ cells that are activated in the presence of TGF- β 1 and IL-6. ROR- γ t has been shown to be the transcription factor for Th17 cells (Weaver et al., 2006). Even though TGF- β 1 is known to cause the development of Foxp3+ Tregs, in combination with IL-6 as a critical cofactor the cells form the transcription factor ROR- γ t developing Th17 cells (Harrington et al., 2005; Park et al., 2005; Weaver et al., 2006). TGF- β 1 causes the upregulation of ROR- γ t while IL-6 inhibits the production of Foxp3, which

can block the production of ROR- γ t (Ichiyama et al., 2008; Zhou et al., 2007) . TGF- β 1 also causes the cell to upregulate the IL-23R and the IL-23 cytokine is necessary for a full protective TH17 response (Figure 1) (Weaver et al., 2006)

Th17 cells are known for producing IL-17, IL-17F, IL-6, TNF- α , and IL-22. These cytokines suggest a proinflammatory nature for this subset of effector cells (Chung et al., 2006; Harrington et al., 2005; Mangan et al., 2006; Park et al., 2005; Schmidt-Weber et al., 2007; Zheng et al., 2007). IL-17 and IL-17F have been shown to induce recruitment of neutrophils depending on the target cell population activities of these cytokines which include inducing expression of GM-CSF, G-CSF, CXC chemokines, metalloproteinases, and IL-6 (Kolls and Linden, 2004; Weaver et al., 2007)

Studies have shown that IL-17 cytokines may play a role in asthma. Suzuki et al. showed that there is increased levels of IL-17F in OVA sensitized mice (Suzuki et al., 2007). Even though it has been shown that IL-17 is expressed in asthmatics it is unsure if it present as a pro-inflammatory or as a negative regulator of asthmatic features. Schnyder-Candrian et al. found a dual effect for IL-17 showing that it is necessary for induction of asthma but negatively regulates the disease (Schnyder-Candrian et al., 2006).

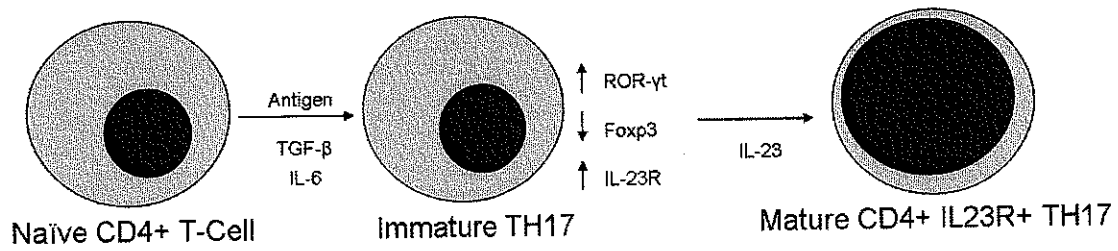


Figure 1: Maturation of TH17 Cells: When a naïve CD4+ T-cell is presented an antigen in the presences of TGF- β and IL-6 it leads to the differentiation of the cell into a Th17 cell. TGF- β and IL-6 are cofactors and both are necessary to form a Th17 cell with IL-23 needed to complete maturation and maintenance of the cell; TGF- β with other cofactors can form a Treg since IL-6 is not present to suppress Foxp3 activity.

Stockinger's article showed another factor that is being looked at in the Th17-Treg dichotomy. IL-2 discourages the development of Th17 by activating STAT 5 and inhibiting Th17 cell production but is needed in the development of Tregs. Tregs lowers the development of Th1 or Th2 pathology but may promote Th17 development since the cells scavenge its negative regulator IL-2 (Laurence et al., 2007; Stockinger, 2007a; Villarino et al., 2007).

This dual role of IL-17 may be explained by examining Th17s link with regulatory T cells, which are known to reverse asthmatic features if adoptively transferred to an asthmatic mouse (McGee, 2006). TGF- β and IL-6 are the crucial differentiation factors for Th17 cells (Stockinger, 2007b; Veldhoen et al., 2006) and TGF- β is also required for T regulatory cell development in the periphery but is suppressed when IL-6 is present (Weaver et al., 2006). To examine this discrepancy in the role of TH17 in inducing or negatively regulating asthma FMS-like tyrosine kinase 3 ligand (Flt3L) was examined since it is a drug that has been shown to reverse AHR (Bharadwaj et al., 2007).

F. FMS-like Tyrosine Kinase 3 Ligand

Flt3L is a hematopoietic growth factor shown to stimulate the proliferation of hematopoietic stem and progenitor cells (Lyman, 1995; Lyman et al., 1993; Maraskovsky et al., 1996). The Flt3 receptor is preferentially expressed on hematopoietic stem cells and is a member of the type III tyrosine kinase receptor family (Kiyoi and Naoe, 2002; Reber et al., 2004; Rosnet et al., 1996; Rosnet et al., 1993). Flt3L has low toxicity in comparison to some other growth factors such as IL-3 and Stem Cell Factor (SCF); both known to have asthma-like toxicity

profiles (Broudy, 1997; Gebhardt et al., 2002; Itakura et al., 2001; Reber et al., 2004; Volc-Platzter et al., 1991).

This makes Flt3L a good drug for treatment of asthma since its ability to mature hematopoietic stem cells have been able to reverse asthma symptoms. The exact mechanisms of how Flt3L reverses asthmatic features are still under investigation. Findings by Maraskovsky et al. show that Flt3L affects dendritic cells maturation level and causes expansion of hematopoietic cells to become dendritic cells. This shows how the drug may affect APCs and what type of effector cell response arises when an antigen is presented (Maraskovsky et al., 1996). Most Flt3L expanded DCs are immature, which implies that they have the potential to induce tolerance (Mosca et al., 2002; Reber et al., 2004).

Studies have shown that immature DCs in vitro as well as in vivo induce the development of T-regulatory cells (T-regs) which are known to reverse asthmatic features (Bharadwaj et al., 2007). Immature DCs circulate and take up antigen which migrate to the regional lymph nodes and may encounter naïve T cells and differentiate these cells into T-regs instead of effector cells. The effect of Flt3L in the HDM model on the SOCS proteins and the Th17 cell is not known. Therefore, in this study mice where intranasally sensitized and aerosol challenged to HDM antigen to examine the effect Flt3L would have on SOCS proteins and the novel Th17 cell.

II. Materials & Methods

Mice

Four to five weeks old BALB/c mice purchased from Harlan Laboratories (Indianapolis, IN) and were housed in separate cages according to treatment protocol. Food and water were provided ad libitum. According to National Institutes of Health guidelines, the research protocol of this study was approved by the Institutional Animal Care and Use Committee (IACUC) of Creighton University.

House Dust Mite Antigen

Allergenic extract was the whole bodies of mites, *Dermatophagoides farinae* and *Dermatophagoides pteronyssines* purchased from Hollister-Stier Laboratories LLC (Spokane, WA).

Immunization Protocol

One group of mice received intraperitoneal injections of 10 μ g concentration of house dust mite extract emulsified in alum (Pierce, Rockford, IL) in a volume of 100 μ l on day 0 and day 14. Another group of mice received a 10 μ g concentration of house dust mite via the intranasal route on day 0 and day 14. On days 28-30 all of the mice received an aerosol challenge of a 1% concentration of house dust mite antigen and a 5% concentration was given on day 32. AHR was measured on day 33 and all of the animals were euthanized on day 34 (figure 2). To test the effect of Flt3L on day 34 mice were randomly selected into groups with a control group and one sensitized group receiving IP injections of 100 μ l of PBS on days

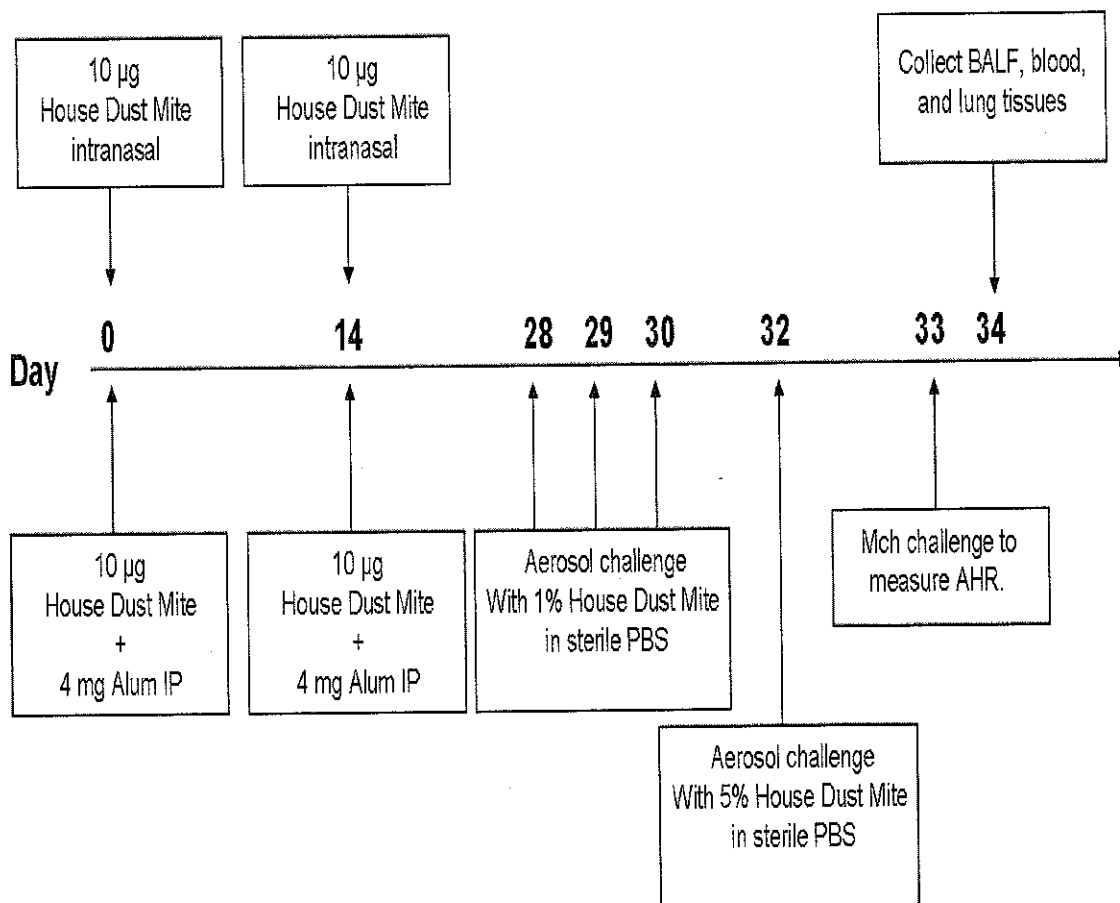


Figure 2: Immunization protocol to establish asthma: A. Sensitization phase:

Day 0 and day 14 a group of mice received an IP injection of house dust mite antigen while another group received an intranasal injection of HDM and another of PBS. B. Challenge phase: Day 28 through 30, and day 32 aerosol challenge with house dust mite antigen. Day 33, methalcholine challenge.

34-43 and one sensitized group receiving IP injections of a 5 µg concentration of Flt-3 ligand in 100 µl solution of PBS. On day 45 the control group received an aerosol challenge of PBS while the two sensitized groups received a 5% aerosol challenge of HDM. On day 46 AHR was measured and mice were sacrificed (Figure 3).

Airway Responsiveness using non-invasive and invasive method

On day 33 AHR was established using a single-chamber, whole-body plethysmograph (Buxco Electronics, Troy, NY) and aerosolized acetyl β-methacholine (Sigma) in a dose-dependent manner (.031 g, .062 g, .125 g, .25 g, .50 g, and 1.0 g in 10ml PBS). To test the effect of Flt3L the same non-invasive method was used on day 33 and day 46. Randomly selected mice AHR were also confirmed on day 46 with a more rigorous method of invasive tracheostomy. Tracheostomized mice were placed in PLY4111-R/C plethysmograph single chamber (Buxco Electronics, Troy, NY) and were mechanically ventilated (140 breaths/min and a tidal volume of 0.15 ml) using Harvard rodent ventilator (model 683, Harvard Apparatus, South Natick, MA). Anesthesia was given by intraperitoneal injection of sodium pentobarbital (1.6mg/20g body weight). Lung specific airway resistance (RL) was measured. In both methods, the mice were challenged with increasing doses of nebulized methacholine (up to 100 mg/ml) to measure AHR.

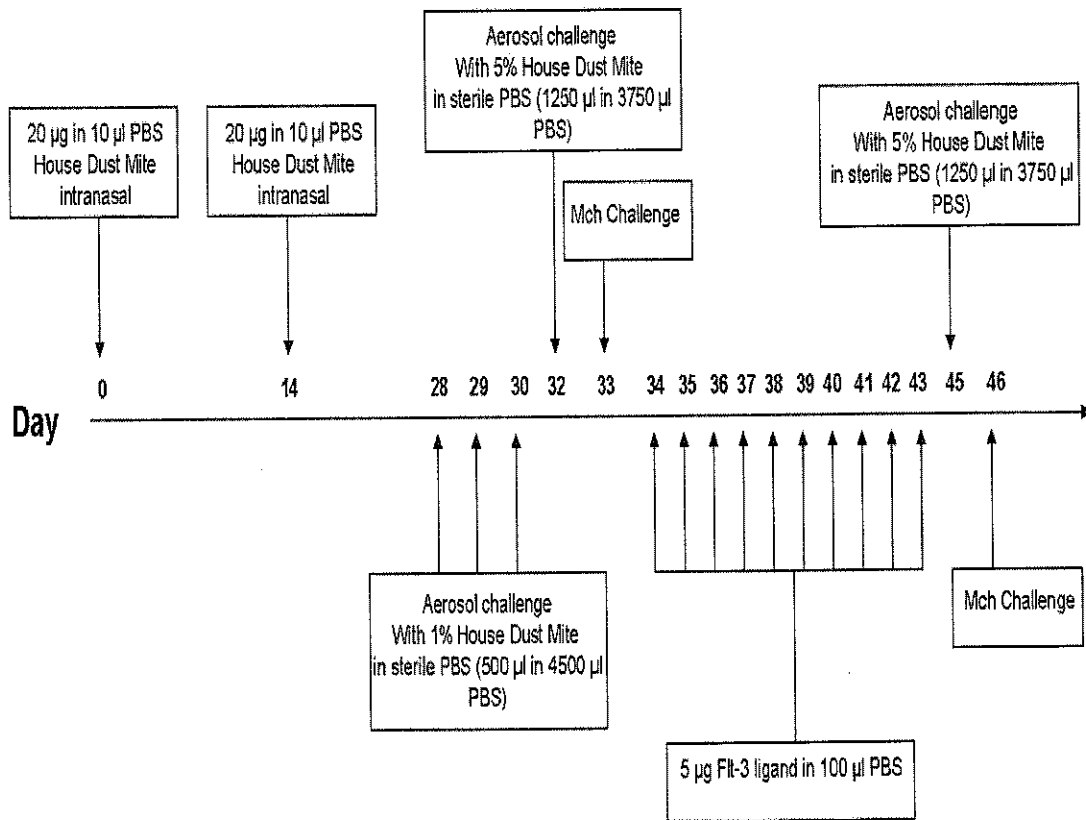


Figure 3: *Immunization protocol and administration of Flt3:* Sensitization phase: day 0 and day 14 groups receive 20 µg of house dust mite antigen intranasally and the control group receives PBS. Challenge phase: Day 28 through 30, and day 32 aerosol challenge with house dust mite antigen. Day 33, methacholine challenge. On days 34 through 43 one group of mice receive 5 µg injections of Flt3L each day. Day 45 both groups receive an aerosol challenge and on day 46 they receive Mch Challenge and are sacrificed.

Immunohistochemistry

Immunohistochemistry (IHC) was done using a standard Avidin-Biotin Complex (ABC) protocol as recommended by Vector Labs. Endogenous peroxidase was blocked using 3% H₂O₂ in methanol for 20 minutes. The substrate used was 2,2-diaminobenzene (DAB) in which positive staining was indicated by the presence of brown precipitate. The sections were counterstained with Gills number 2 hematoxylin for 10 seconds. Negative controls lacking the primary antibody were run. The results were examined via light microscopy.

Histology

The lungs of the mice were removed and were placed in paraffin then sectioned into 5 micron sections. Hematoxylin and Eosin (H&E) staining was performed to observe morphology. Trichrome staining for collagen deposition was performed via the Masson's Trichrome Staining Kit by IMEB inc. using their company's recommended protocol. Periodic Acid-Schiff (PAS) staining was performed to observe and compare mucus production in the lungs of the mice using Sigma-Aldrich staining kit and protocol.

RT-PCR

To analyze expression of ROR- γ mRNA was prepared from isolated CD4+IL-23R+ cells using Trizol (Sigma-Aldrich) reagent protocol. Gene Amp PCR System 2400 (Perkin Elmer) was used at 36 cycles for each product. ROR- γ : Forward 5'- TGA GGA AAC CAG GCA TCC TGA ACT – 3', Reverse 5' – TGT GTG GTT GTT GGC ATT GTA GGC– 3' T_m: 55° C.

Tissue preparation and Isolation of Th17 cells

To isolate Th17 cells, lungs and spleens were harvested from Balb/c mice. The tissues were cut into fragments, followed by digestion using collagenase D (Roche laboratories) (1 mg/ml) and 5 ml of RPMI 1640 (Cambrex). The samples were incubated at 37°C in a CO₂ incubator for 90 minutes. Tissue was disrupted with 1 ml syringe and the cell suspension was poured over 40 µm filter (BD bioscience) and collected into 15 ml tube. RBCs were lysed using Tris-buffered ammonium chloride solution and suspension was neutralized with a solution containing 4% FBS in sterile PBS. The suspension was centrifuged at 350g for 15 minutes. The supernatant was discarded and the pellets washed in 10 ml Hanks balanced salt solution and centrifuged. CD4⁺ T cells were pre-enriched by depleting unwanted cells by using a cocktail of antibodies. After the addition of microbeads the suspension was incubated at 4°C for 30 minutes. The suspension was processed in the AutoMACS to obtain CD4⁺ cells, which were then washed in PBS and 100 µl was placed in a 96 well plate. After titrations it was determined to use a 1:200 ratio for CD4 (Percp) and 1:500 for IL-23R (FITC). The procedure called for staining of CD4 with 5.0 µl dilutions to the cell suspensions. The suspension was incubated for 30 minutes. After incubation, the cells were washed 3 times by centrifugation at 350g for 5 min then resuspend in PBS 4 for flow cytometry. To stain for IL-23R, a donkey anti-goat secondary conjugated with FITC was used and that involved adding the dilution for another 30 minute incubation period. Cells are washed and then resuspend in Flow cytometry staining buffer.

Data analysis

Data was analyzed using GraphPad Prism statistical analysis and graphing software and Microsoft Excel. Multiple group comparison was made using ANOVA. A p value of less than 0.05 (<0.05) was considered significant.

III. Results

Effect of HDM sensitization and challenge on pulmonary function, histology and SOCS expression

Pulmonary Function.

Airway responsiveness was determined by non-invasive whole body plethysmograph. Mice of the intranasal group showed significant airway hyper-responsiveness compared to the IP and PBS group in the 100 mg/ml and 50 mg/ml dosage of methacholine. Representative mice of the intranasal group had a mean enhanced pause (Penh) of 4.38. IP had 1.83 and PBS had a value of 0.99 with the 100 mg/ml dose of methacholine (Figure 4).

Lung Histology.

The mice were sacrificed and lungs harvested for H&E staining, Masson Trichrome staining, and PAS staining to demonstrate histological hallmarks of asthmatic airways. PBS animal group displayed normal airway morphology, while the IP group showed some signs of acute asthma with the intranasal group showing the most airway remodeling. The intranasal group displayed the hallmarks of airway remodeling with collagen deposition, epithelial cell hypertrophy and smooth muscle cell hyperplasia as well as showing more mucus production compared to the other groups (Figure 5).

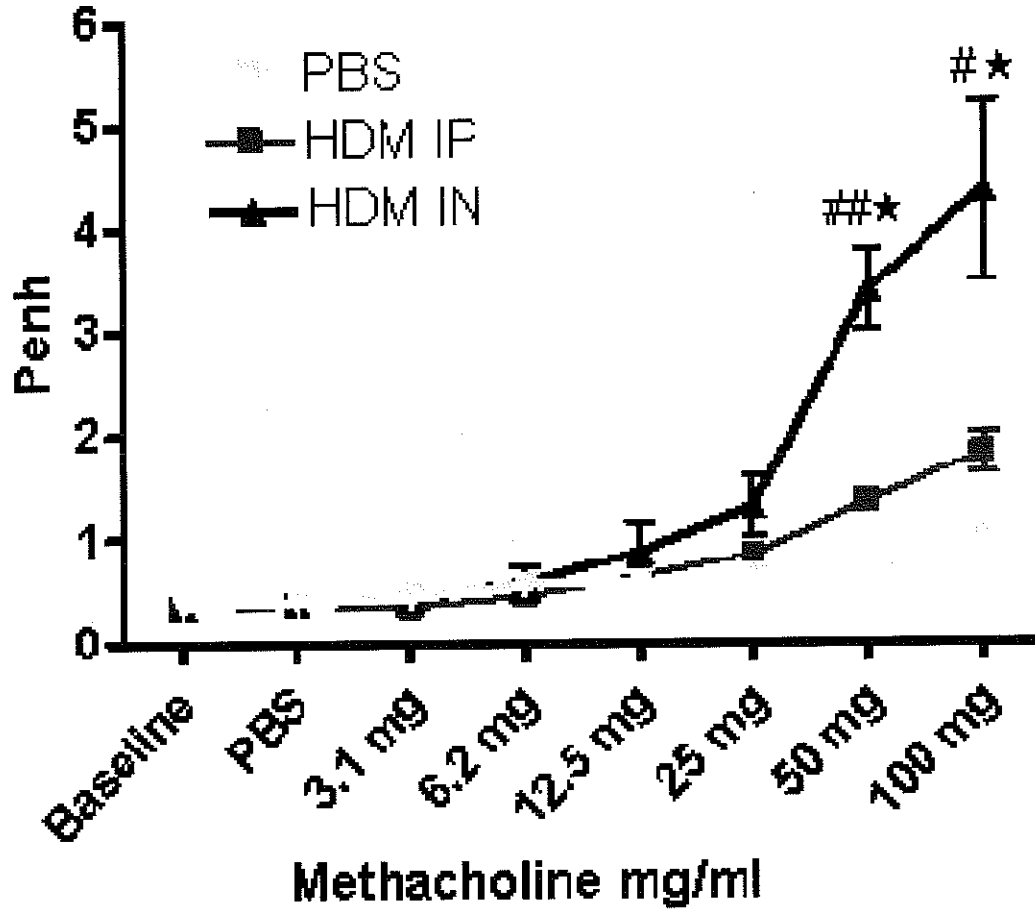


Figure 4: Establishment of airway hyperresponsiveness to methacholine: Pulmonary function was evaluated by non-invasive technique in unrestrained animals using single chamber whole body plethysmograph. The results are presented as mean \pm SE of 3 mice per group. *HDM IN vs. HDM IP $p < 0.05$ #HDM IN vs. HDM IP $p < 0.01$, #HDM IN vs. PBS $p < 0.01$ ##HDM IN vs. PBS $p < 0.001$

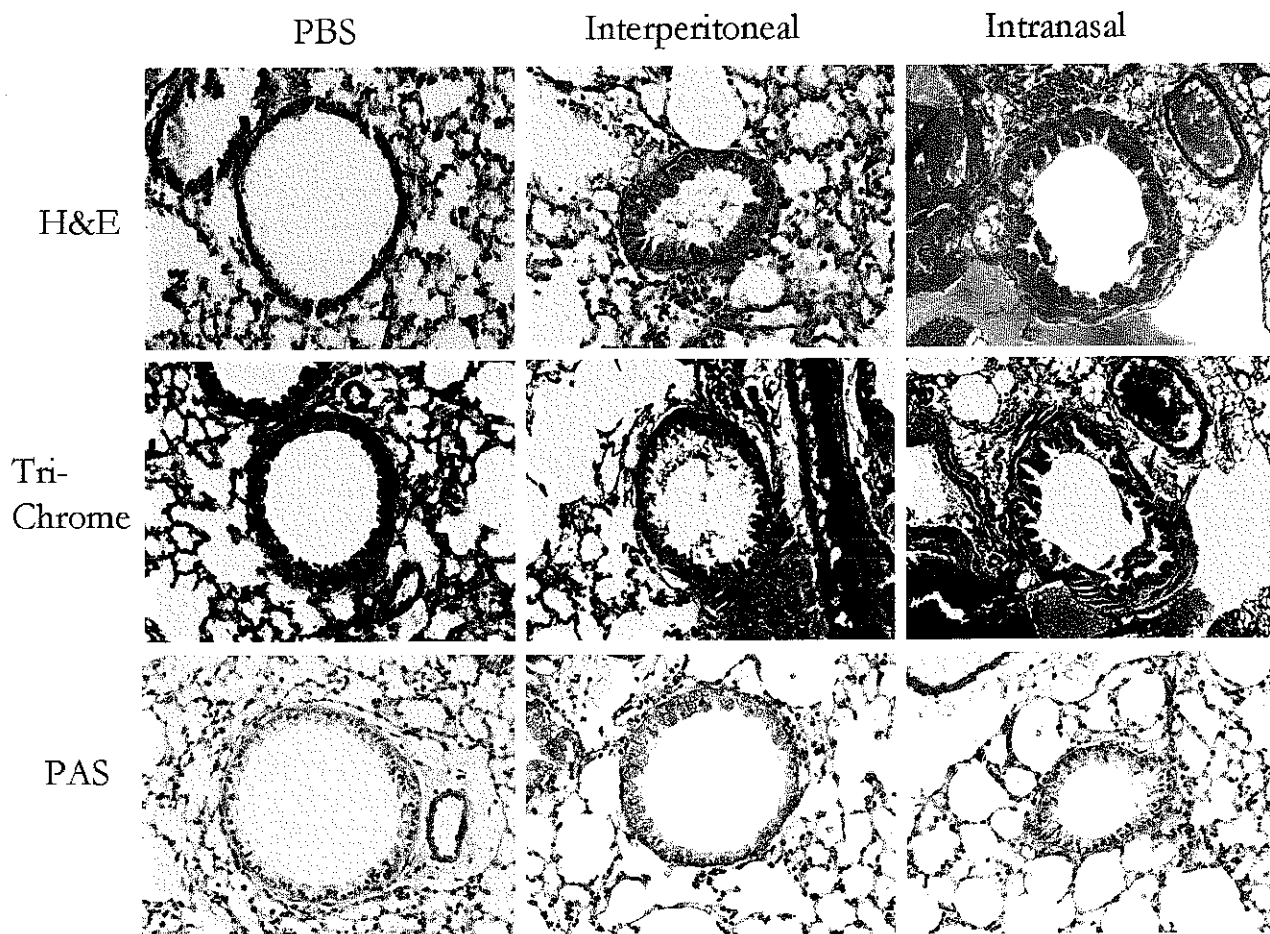


Figure 5: Lung histology in HDM-sensitized and challenged mice: Lung sections (5 μ m) were stained with H&E to show histological changes, Tri-chrome to show collagen deposition and PAS to show mucus cells. Results are representative of 5 mice per group.

SOCS expression in the lung.

SOCS-1, 3, and 5 were expressed strongly in the IN group compared to the other two groups. Expression is most visible in the epithelial lining with light expression in the inflammatory cells in the IN group (Figure 6, 7, 8).

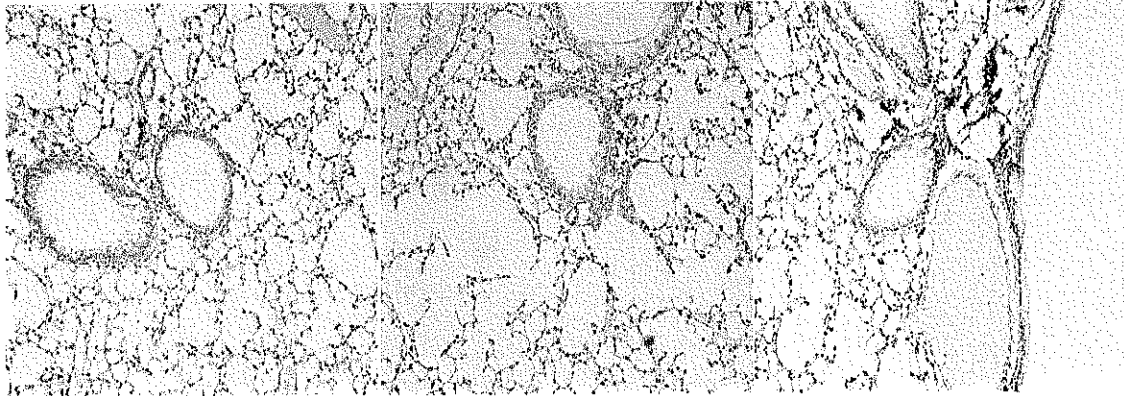
IHC SOCS 1

Negative Controls

PBS

Interperitoneal

Intranasal



SOCS 1

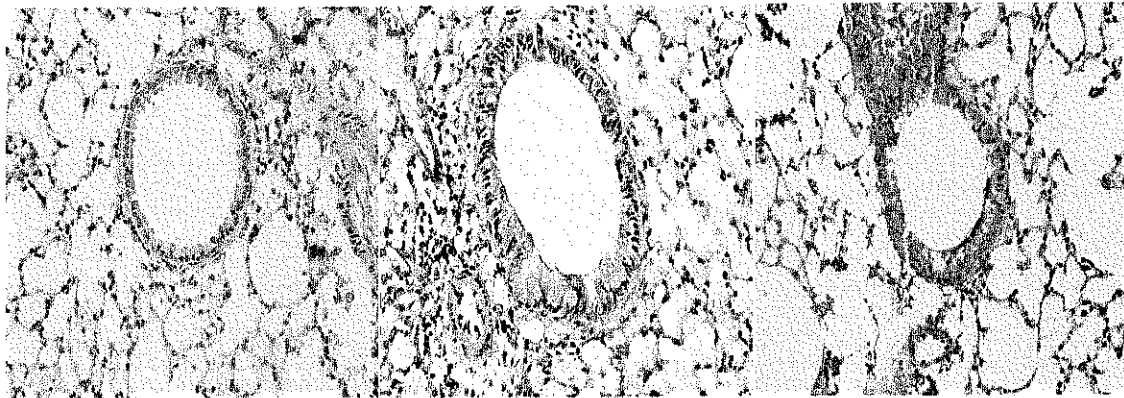


Figure 6: *Immunohistochemical localization of SOCS 1 in the lung:* Expression of SOCS was confirmed in the lungs of sensitized and challenged mice where HDM was administered. Results are representative of 5 mice per group.

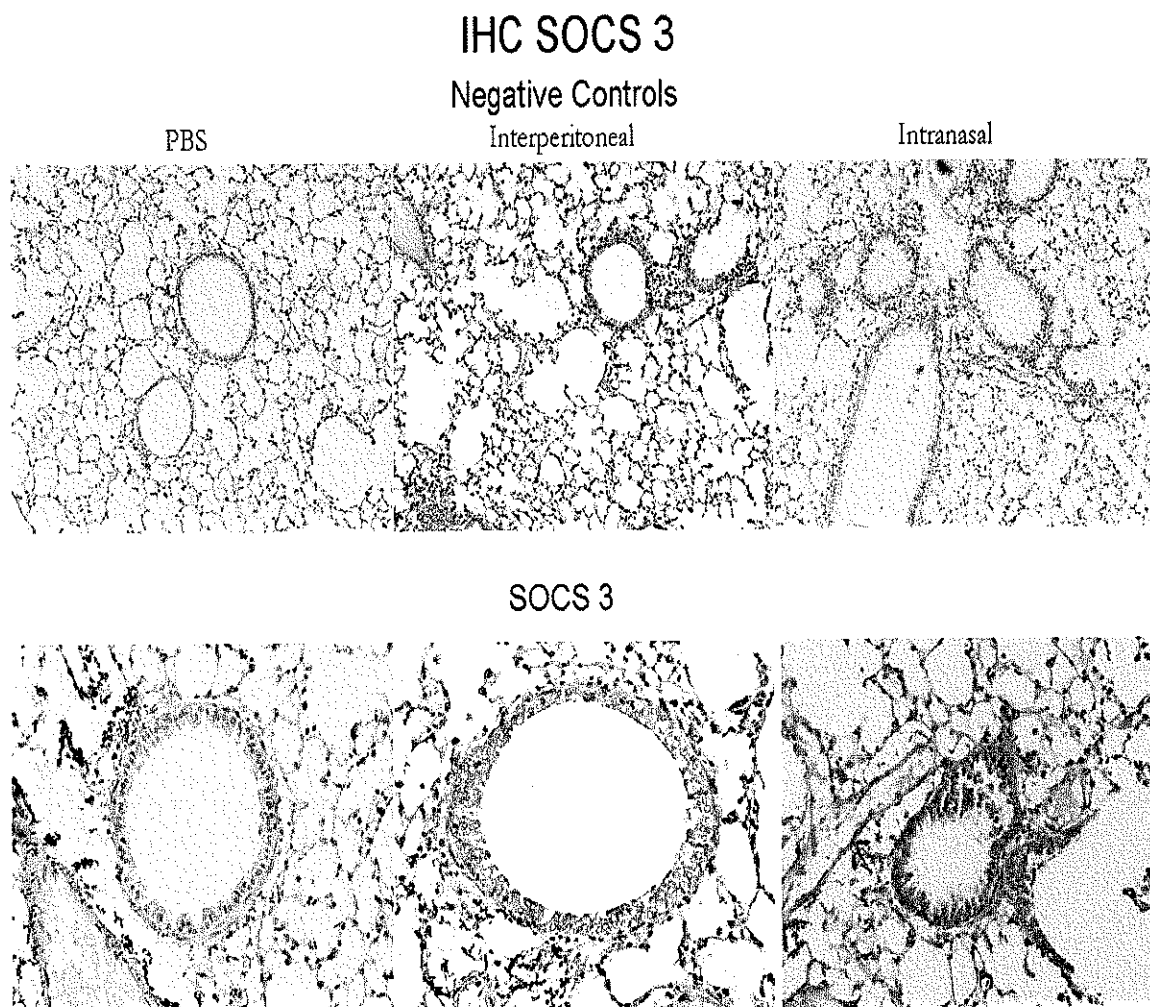


Figure 7: *Immunohistochemical localization of SOCS 3 in the lung:* Expression of SOCS was confirmed in the lungs of sensitized and challenged mice where HDM was administered. Results are representative of 5 mice per group.

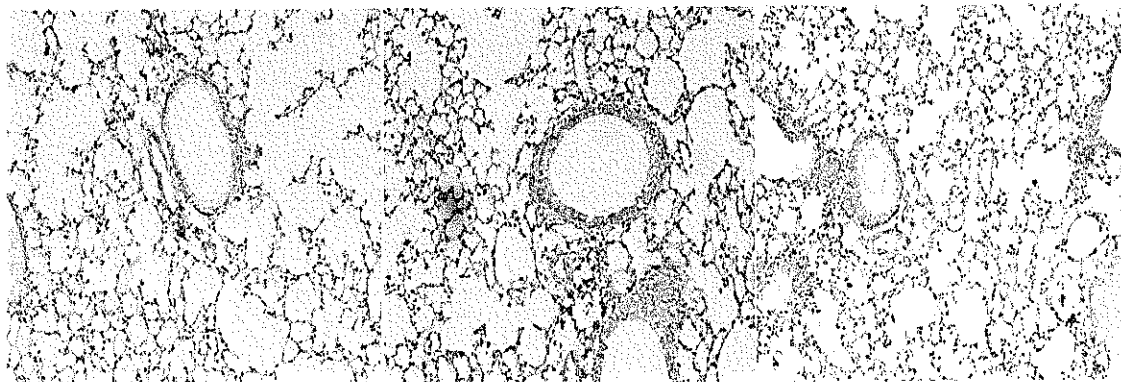
IHC SOCS 5

Negative Controls

PBS

Interperitoneal

Intranasal



SOCS 5

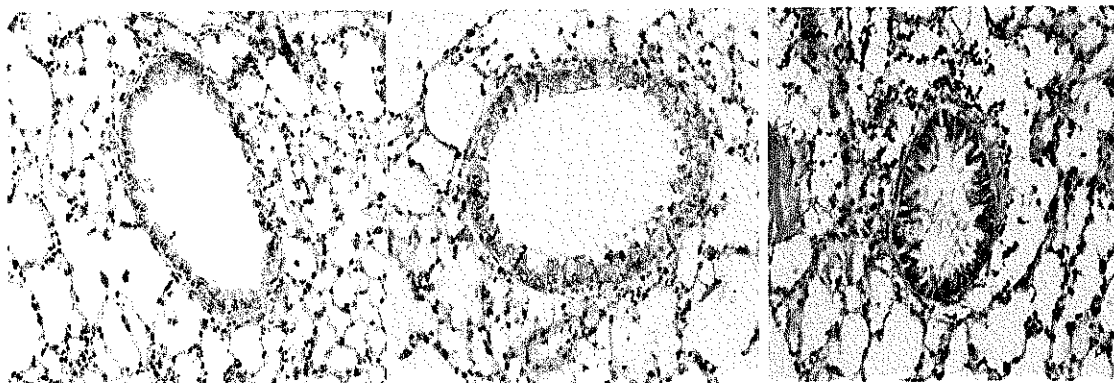


Figure 8: *Immunohistochemical localization of SOCS 5 in the lung:* Expression of SOCS was confirmed in the lungs of sensitized and challenged mice where HDM was administered. Results are representative of 5 mice per group.

Effect of Flt3L on AHR, SOCS expression and Th17 development

Effect of Flt3L on AHR in HDM-sensitized and challenged mice.

HDM-sensitized and challenged mice exhibited AHR on days 33 and 46 according to the protocol followed in figure 3. The AHR in response to methalcholine was examined and established with non-invasive whole body plethysmograph. Administration of 100 mg/ml methalcholine exhibited Penh values of 4.25 ± 0.43 (n = 5) on day 33 and 3.86 ± 1.01 (n = 4) on day 46 (Figure 9 and 10). On day 46 tracheostomy was performed on the mice with RL (cm H₂O.s/ml) of 3.25 for PBS, 7.17 for the HDM group and 1.59 for the HDM+FL group at 100mg of methalcholine (n=3) (Figure 11).

Lung Histology.

The mice were sacrificed and their lungs harvested for H&E staining histological hallmarks of asthmatic airways. The PBS animals displayed normal airway morphology, while the sensitized group showed typical airway remodeling. The sensitized group displayed the hallmarks of airway remodeling with collagen deposition, epithelial cell hypertrophy and smooth muscle hyperplasia. The Flt3L treated group showed a reversal of these hallmark signs (Figure 12).

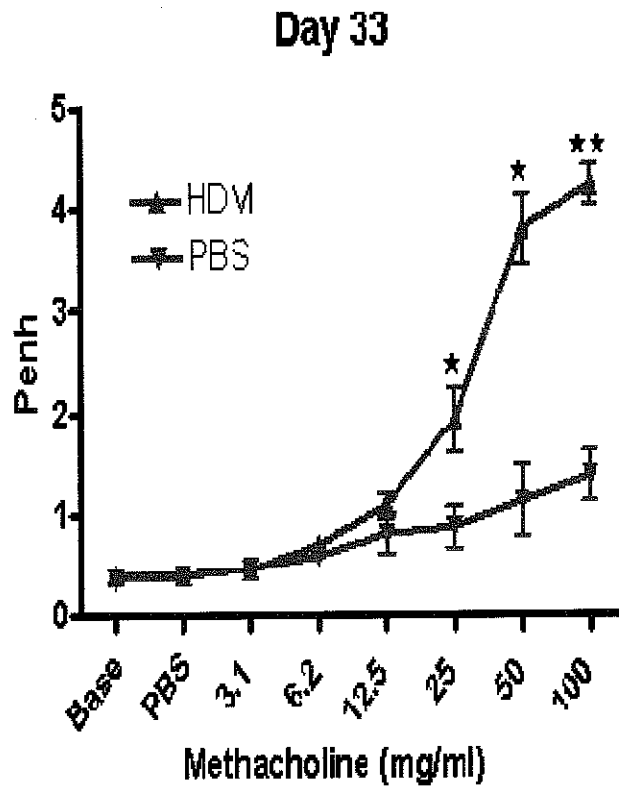


Figure 9: *Establishment of Airway hyperresponsiveness to methacholine:* Pulmonary function was evaluated by non-invasive technique in unrestrained animals using single chamber whole body plethysmograph. The results are presented as mean \pm SE of 5 mice per group. ***HDM vs. PBS $p \leq 0.0001$, **HDM vs. PBS $p \leq 0.0005$, *HDM vs. PBS $p \leq 0.05$

Day 44

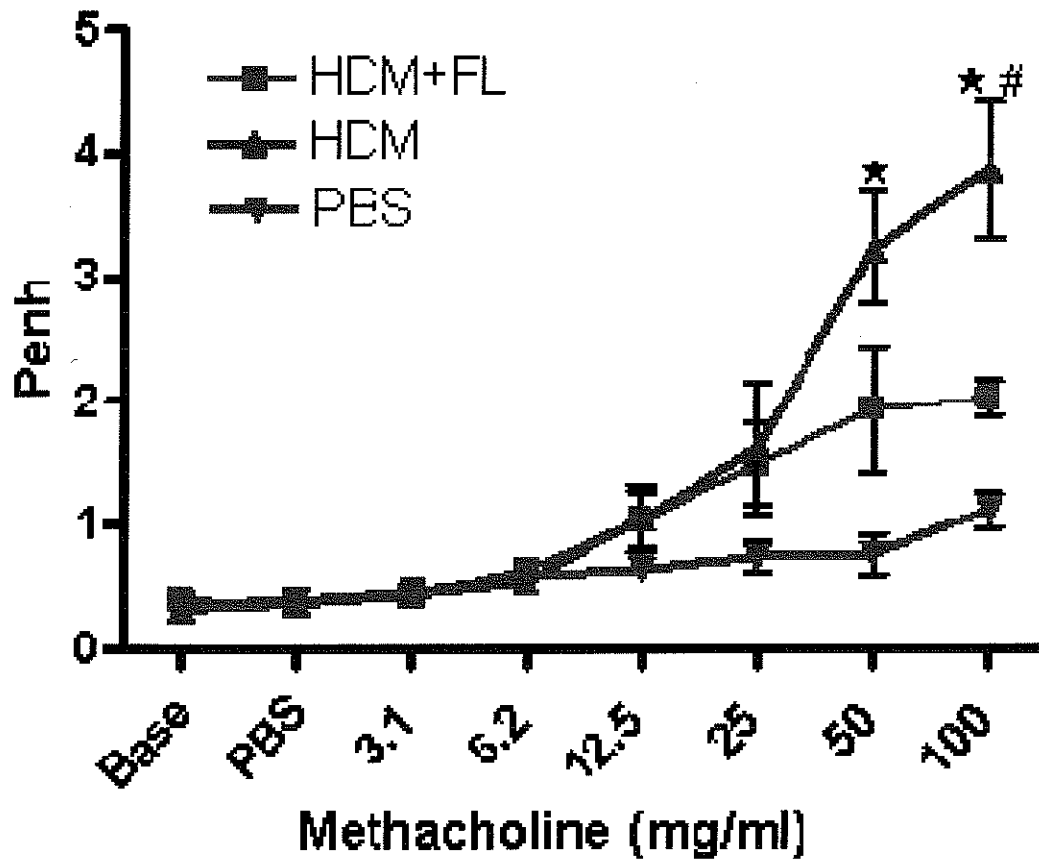


Figure 10: *Effect of FL on Airway hyperresponsiveness to methacholine:* Pulmonary function was evaluated by non-invasive technique in unrestrained animals using single chamber whole body plethysmograph. The results are presented as mean \pm SE of 4 mice per group. **HDM vs. PBS $p \leq 0.001$, *HDM vs. PBS $p \leq 0.01$, #HDM vs. HDM+FL $p \leq 0.05$

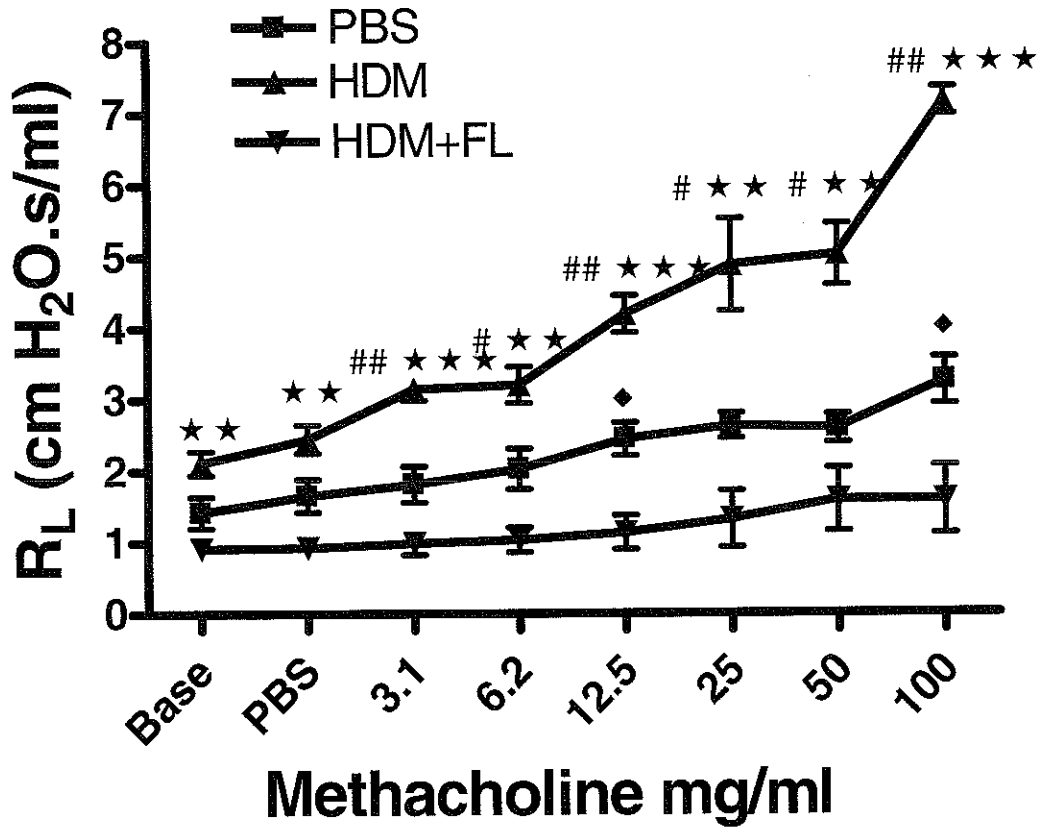


Figure 11: Confirmation of AHR using invasive method in tracheostomized mice: On day 46 pulmonary function was measured as specific airway resistance (R_L) and reported with units of $\text{cm H}_2\text{O.s/ml}$. The results are presented as mean \pm SE of 3 mice per group. ***HDM vs. HDM+FL $p \leq 0.001$, **HDM vs. HDM+FL $p \leq 0.01$, ##HDM vs. PBS $p \leq 0.01$, #HDM vs. PBS $p \leq 0.05$, •PBS vs. HDM+FL $p \leq 0.05$

PBS

HDM

HDM+FL

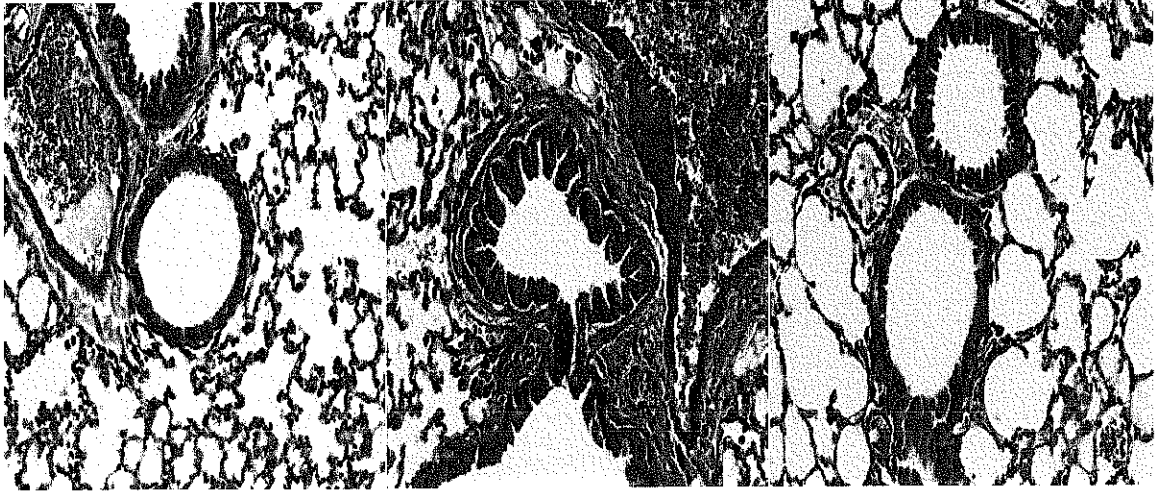


Figure 12: *Effect of FL on histological changes in the lung:* The Flt3L treated group shows a reversal of the signs of asthma in the airway. Results are representative of 5 mice per group.

Effect of Flt3L on SOCS expression

It was previously established that SOCS 1, 3, and 5 are expressed in the House Dust Mite model of asthma. When the mice were treated with Flt3L there was a reversal of the expression of the three SOCS molecules (Figure 13, 14, 15)

Flow cytometry analysis of CD4 and IL-23R on lung and spleen CD4⁺T-cells.

Isolated spleen CD4⁺ cells were selected for expression of IL-23R to compare between the PBS, sensitive, and Flt-3 treated groups (Figure 16A and Figure 17A). The sensitized group had a significantly larger amount of cells expressing CD4⁺IL-23R⁺ cells compared to the other groups and had a significantly higher percentage of these cells compared to the total amount of cells (Figure 16B and C). Isolated lung CD4⁺ cells were also gated for expression of IL-23R and once again the sensitized group showed a significantly higher amount and percentage of cells that expressed IL23R (Figures 17B and 17C)

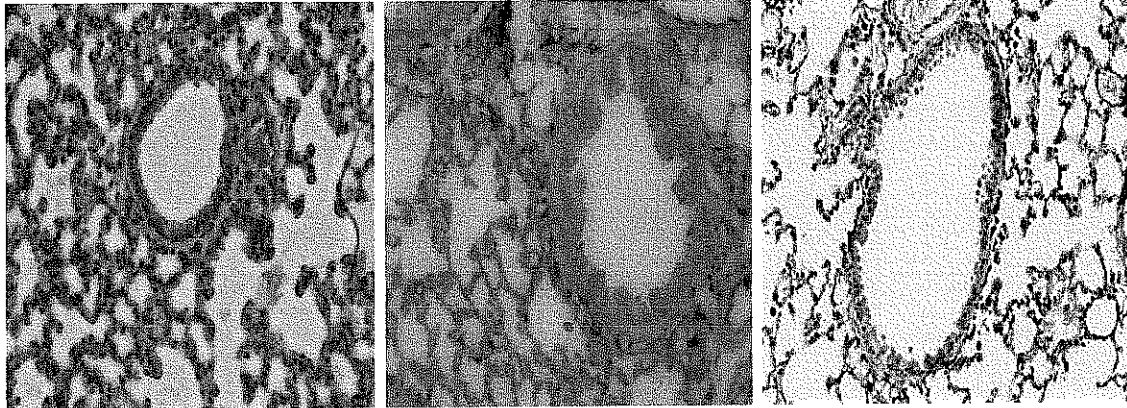
IHC SOCS 1

Negative Controls

PBS

HDM

HMD+FL



SOCS 1

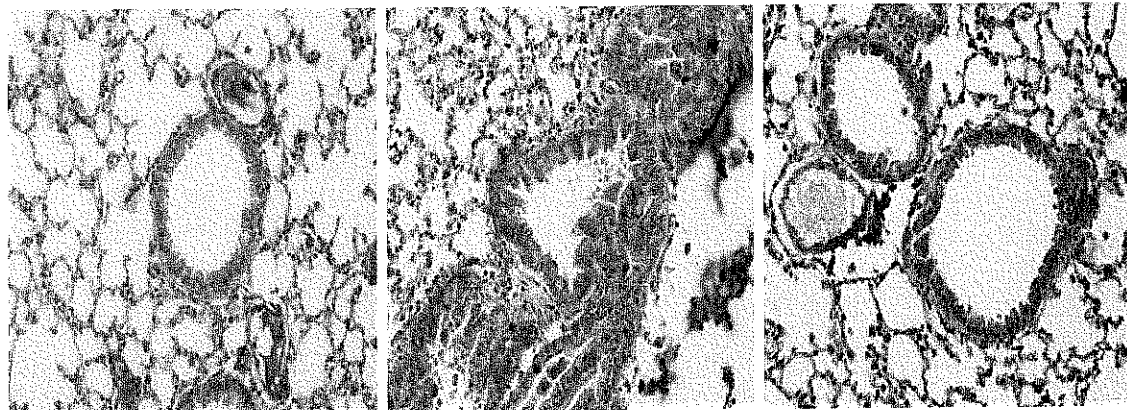


Figure 13: *Effect of FL on immunohistochemical localization of SOCS 1 in the lung: Staining shows effect of Flt3L on SOCS staining. Results are representative of 5 mice per group.*

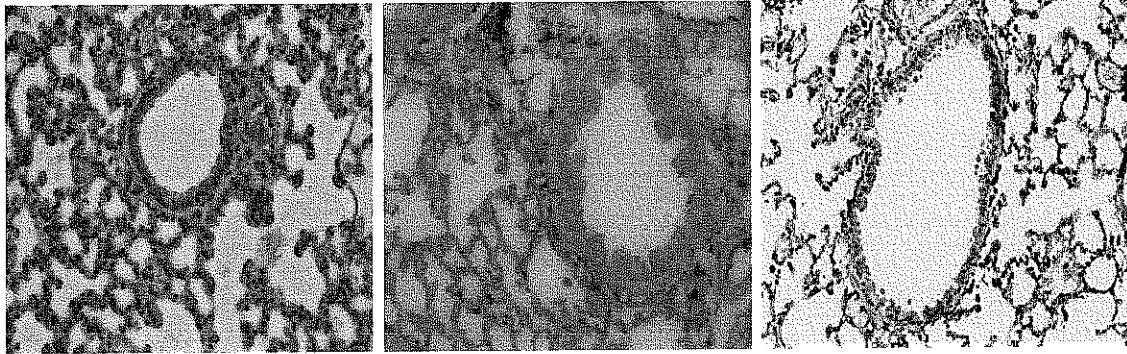
IHC SOCS 3

Negative Controls

PBS

HDM

HMD+FL



SOCS 3

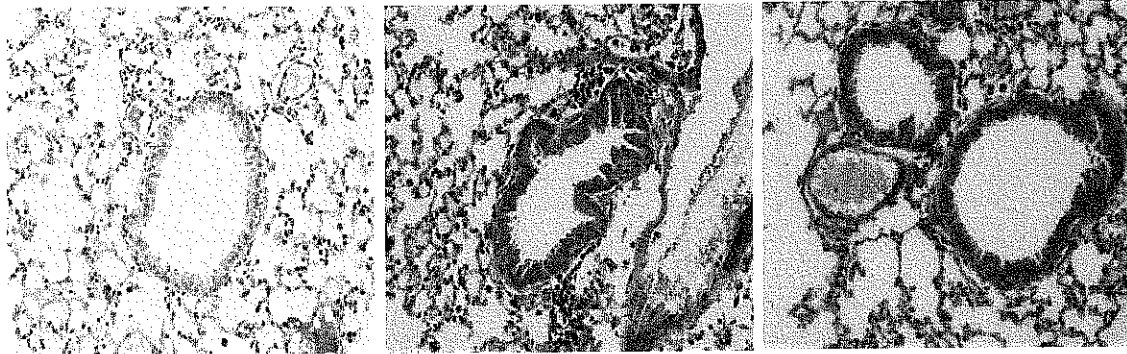


Figure 14: *Effect of FL on immunohistochemical localization of SOCS 3 in the lung: Staining shows effect of Flt3L on SOCS staining. Results are representative of 5 mice per group.*

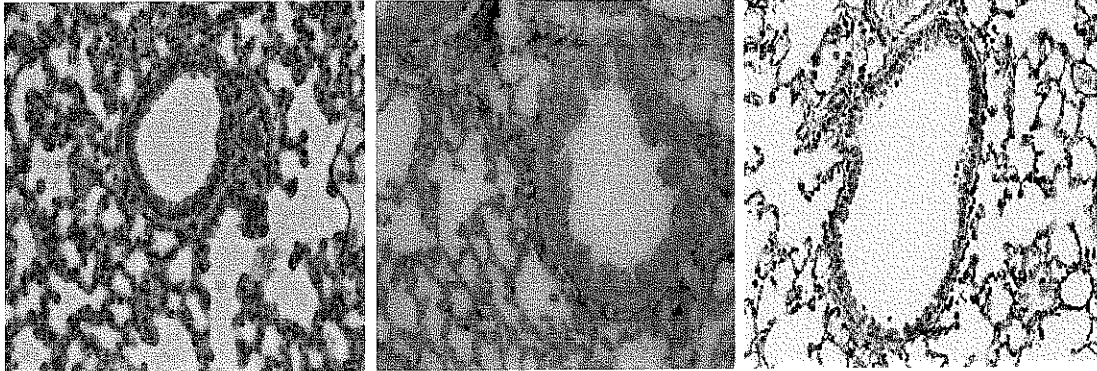
IHC SOCS 5

Negative Controls

PBS

HDM

HMD+FL



SOCS 5

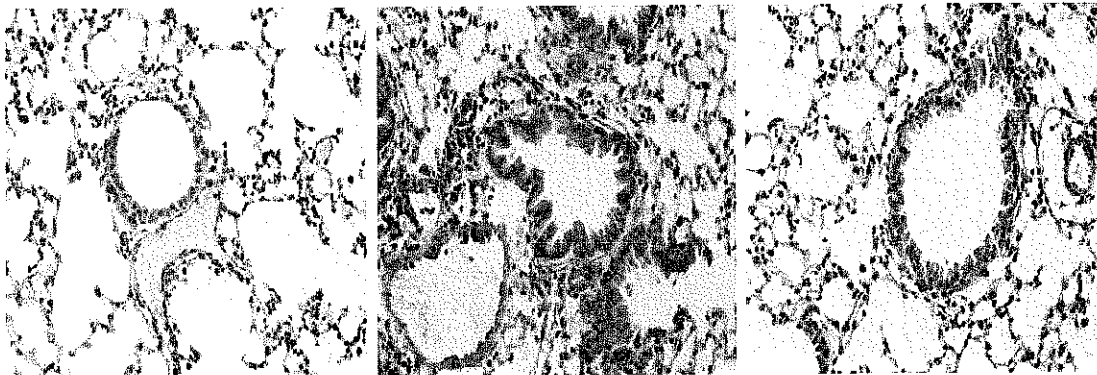


Figure 15: *Effect of FL on immunohistochemical localization of SOCS 5 in the lung:* Staining shows effect of Flt3L on SOCS staining. Results are representative of 5 mice per group.

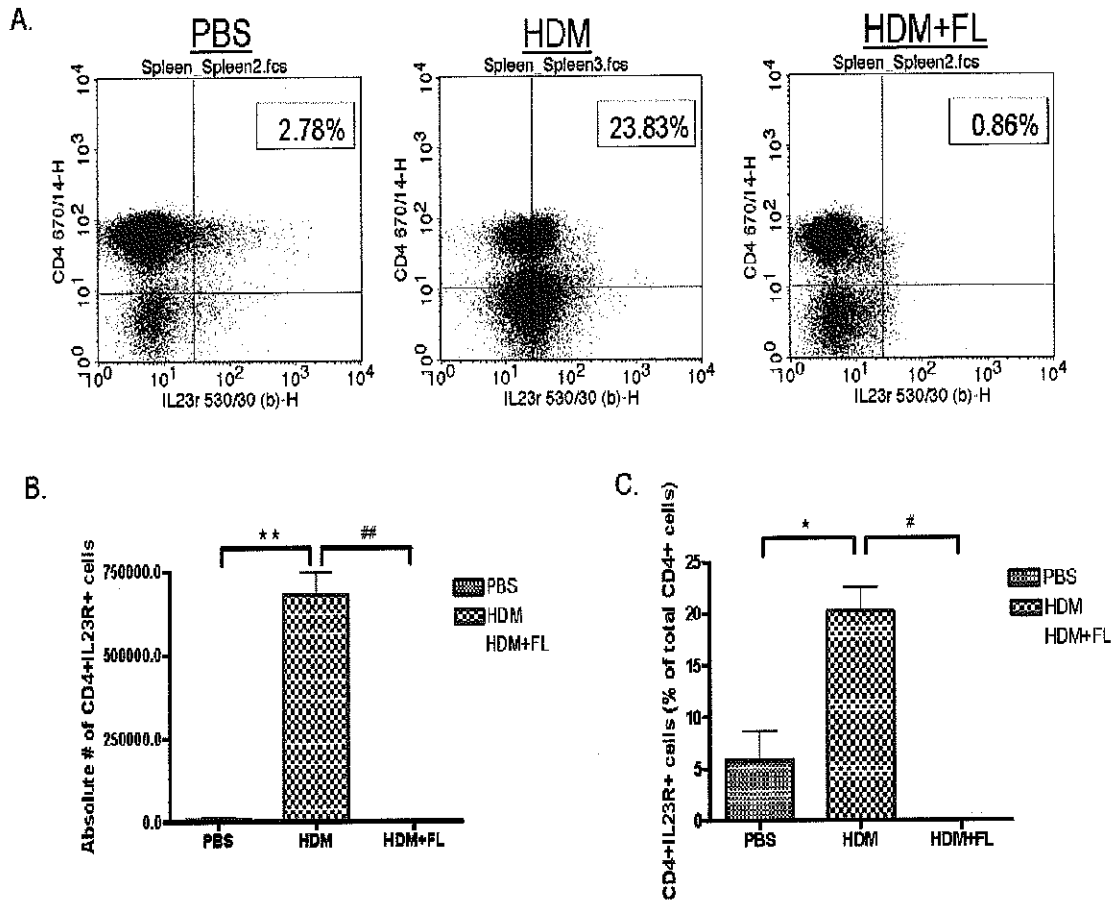


Figure 16: Effect of FL on TH17 cells in the spleen: **A.** These graphs display the shift of cells that express CD4 and IL23R in the spleen. The percentage of CD4+IL-23R+ cells of the total amount are in the upper right quadrant (Representative of four mice for each group) **B.** Displays the number of cells from the spleen that expressed the markers CD4 and IL-23R **C.** The percentage of CD4+ cells in the spleen with the CD4 and IL-23R was also determined with flow cytometry. The results are presented as mean \pm SE of 3 mice per group. **HDM vs. PBS $p \leq 0.001$, ##HDM vs. HDM-FL $p \leq 0.001$, *HDM vs. PBS $p \leq 0.01$, #HDM vs. HDM-FL $p \leq 0.01$.

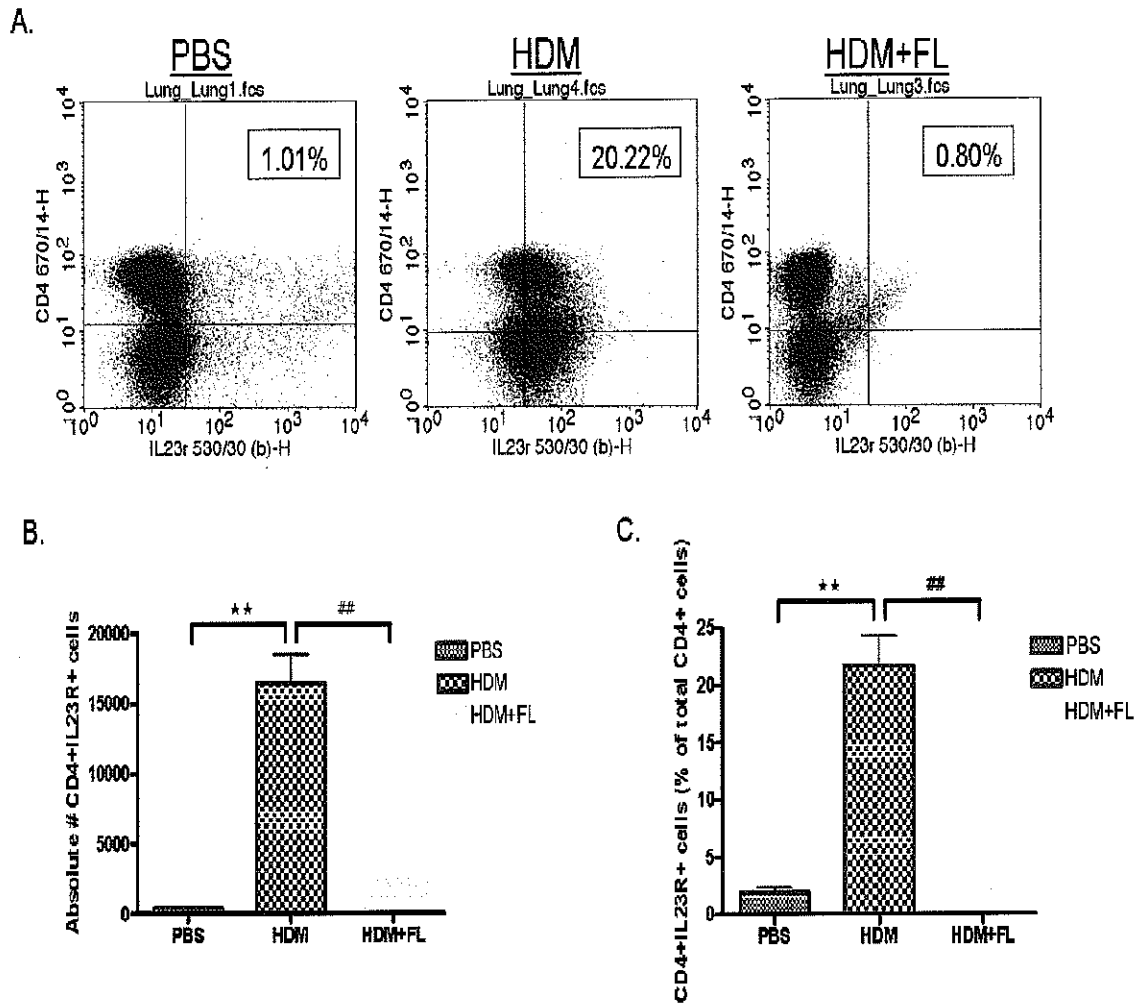


Figure 17: Effect of FL on TH17 cells in the lung: A. These graphs display the shift of cells that express CD4 and IL23R in the lung. The percentage of CD4+IL-23R+ cells of the total amount are in the upper right quadrant (Representative of four mice for each group) **B.** Displays the number of cells from the spleen that expressed the markers CD4 and IL-23R **C.** The percentage of CD4+ cells in the lung with the CD4 and IL-23R was also determined with flow cytometry. The results are presented as mean \pm SE of 3 mice per group. **HDM vs. PBS $p \leq 0.001$, ##HDM vs. HDM-FL $p \leq 0.001$.

Effect of Flt-3 ligand treatment on AHR.

The HDM-sensitized and challenged mice with established AHR were randomized into a group to receive PBS and a group to receive 5 µg of Flt-3 for 10 consecutive days. The Flt-3 treated group had a significant decrease in AHR compared to the sensitized group with a Penh of 2.03 ± 0.28 (n = 4) at 100 mg/ml of methacholine (Figure 10). Flt-3 treated group morphology revealed reduction in airway inflammation, airway epithelial hypertrophy, mucus hypersecretion, and collagen deposition compared to the sensitized group.

RT-PCR on ROR-γ in CD4+IL23R+ cells

There was expression of ROR-γ in the CD4+IL23R+ cells. This shows that these CD4+IL23R+ cells are Th17 cells because they are the only effector T helper cell that expresses ROR-γ (Figure 18).

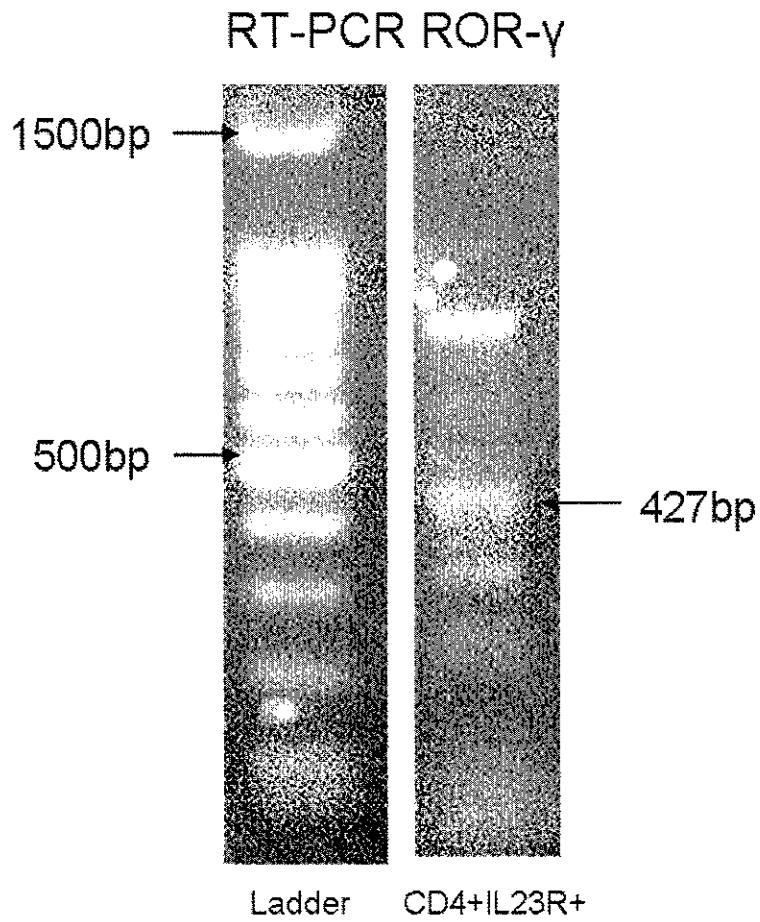


Figure 18: *mRNA of ROR- γ in TH17 cells:* RT-PCR analysis showed that the CD4+IL23R+ cells had RNA with a band at 427bp indicating it has ROR- γ mRNA.

IV. Discussion

Asthma is a disease that affects millions of people and is growing across the world. Accurate animal models need to be developed in order to fully understand the mechanisms of this disease. To help reach this goal our study looked to develop a mouse model of asthma that reflects the human condition as well as determine the role of SOCS proteins and the Th17 cell in asthma.

This experiment was done to determine if a common allergen that causes asthma (House Dust Mite) could sensitize the mice and if the route of sensitization could affect the severity of the condition. It was found that the house dust mite does cause an immune response in the BALB/c mouse and developed AHR and the histological features of asthma. It was also found that when the antigen is given intranasally it exacerbates the symptoms of this acute asthma compared to the IP and control group.

Our results show expression of the SOCS molecules 1, 3, and 5 in the intranasal group. SOCS-1 was expected due to its wide range effect on differing signaling pathways (Heller et al., 2004). SOCS-1 is known to be expressed to regulate IL-4 signaling when IL-4 is produced (Knisz and Rothman, 2007). In the presences of IL-4 SOCS-1 would be upregulated and bind to JAK1 and JAK2 blocking its ability to bind to STAT1 to activate the Th1 transcriptional factor Tbet, which leads to skewing towards the Th2 cell type (Inoue et al., 2007; Kubo et al., 2003). SOCS-3 is also expected since it has been noted that it causes a skew towards the Th2 subtype of helper cells in the presences of cytokines IL-4, IL-6, and IL-10 (Seki et al., 2003). This is done by binding to the IL-12R β 2 inhibiting STAT4 activation which is induced by IL-12 thus causing the cell to be unable to

become a Th1 cell(Inoue et al., 2007; Seki et al., 2003; Yamamoto et al., 2003). Since SOCS-5 appears to be responsible for cells to skew towards the Th1 type it would not be expected to be expressed in asthmatic mice. Activated by IL-12, SOCS5 disrupts the interaction of JAK1 and IL-4R by binding to the IL-4R α blocking IL-4 and shifting towards the Th1 subtype(Huang and Paul, 1998; Inoue et al., 2007; Kubo et al., 1997). Even though it was not expected there was SOCS5 expression. Ohshima et al. found that overexpression of SOCS-5 augmented eosinophilic airway inflammation in mice. They found that it did induce Th1 skewing but Th2 skewing still occurred in the mouse. The study suggested that a Th1 dominant state may augment the allergic phenotype in the asthma model (Ohshima et al., 2007).

It was also determined that the route of immunization had a greater amount of eosinophils in the BAL fluid, but overall house dust mite as an antigen did not evoke much of an eosinophil response as well as the Th2 cytokines (data not shown). Even though there was significant AHR in the intranasal group of animals there was not the expected increase of eosniophils. This points to the possibility that airway eosinophilia is not a requirement for allergen-induced AHR. Tournoy et al. found a production of IgE when sensitizing with Der p 1 but it was not further enhanced by repeated exposure of the extract. It was also found in other studies that the BALB/c strain of mice do not respond well to house dust mite (Lee et al., 1999; Tournoy et al., 2000; Zhang et al., 1997).

Different routes of immunization and strains of mice can affect the outcome of the sensitizations also. Other studies have found an increase in eosinophils with intratracheal instillation of the allergen but in their study the

author sensitized the mice with HDM and diesel exhaust particles (Ichinose et al., 2004). In a Human-Mouse Chimera model, where the researchers used SCID mice that were reconstituted intratracheally with human PBMC from allergic individuals, they found that Der p 1 resulted in AHR but did not cause an infiltration of eosinophils (Tournoy et al., 2001). These results are similar to ours in that our mice displayed AHR but did not have significant eosinophil infiltration that is typically displayed in the current models of acute asthma.

These studies did, however, show a difference in lymphocyte level in the BALF. The sensitized group had a higher level of lymphocytes compared to the control group and this trend was reversed with Flt3L treatment.

Flt3L reversed AHR in allergic mice and mice treated with the drug showed a decreased amount of CD4+IL23R+ cells and reversal of SOCS 1, 3, and 5 expression. The CD4+IL23R+ cells showed a high expression of ROR- γ suggesting that the cells are of Th17 lineage. These findings suggest that Th17 is present in the development of acute asthma in mice and may be a key contributor since its levels are decreased after Flt3L treatment, which occur with the reversal of asthma features. This could be due to Flt3L increasing the number of immature DCs in vivo. Immature DCs have shown the ability to activate T-regulatory cells that regulate activity of inflammatory and effector T cells by secreting such cytokines as IL-10 (McGee, 2006). These T-regs could have caused the down regulation of the Th17 cells and the reversal of asthmatic features. The upregulation of T-regs and the subsequent cytokines may also explain why SOCS 1, 3, and 5 were reversed in the mice treated with Flt3L.

Based off of studies done on Flt3L the cytokine looks like it would be effective in reversing symptoms of asthma. TGF- β was once considered for treatment because it is known to be needed to upregulate T-regs and mice carrying a Tgfb 1 null mutation develop a severe inflammatory disease leading to early death (Bettelli et al., 2006; Ivanov et al., 2006; Li et al., 2007; McGeachy and Cua, 2007). Studies have shown though that in the presence of proinflammatories like IL-6 TGF- β could cause the development of Th17 cells which can exacerbate asthmatic symptoms (Harrington et al., 2005; Park et al., 2005; Weaver et al., 2006). Flt3L has shown low toxicity in vivo unlike some other growth factors. A limitation of Flt3L is that to get desired results in studies daily injection of the drug had to be given (Reber et al., 2004). That is why there have been studies using Flt3L as a plasmid. Edwan et al. showed that the use of a mammalian expression vector pUMVC3-hFLex reversed AHR and airway remodeling and maintained airway protection in a chronic experimental asthma model (Edwan and Agrawal, 2007).

More research needs to be done on the mechanisms of Flt3L and the different functions of DCs and T-regs so they could potentially be used clinically to treat asthma. A better understanding of asthma itself and its mechanisms would help in the ultimate goal of having better treatments for the disease. Research is showing that allergic asthma is not just a Th2 driven disease and that Th1 as well as Th17 cells play a role in the disease. The concert of these effector cells need to be more closely examined in the disease to make further advancements in treatment.

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