Therapeutic Effects of Tumor Necrosis Factor Receptor Superfamily 25 (TNFRSF25)-Mediated Expansion of T-Regulatory Cells in Allergic Lung Inflammation and Airway Remodeling

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THERAPEUTIC EFFECTS OF TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY 25 (TNFRSF25)- MEDIATED EXPANSION OF T-REGULATORY CELLS IN ALLERGIC LUNG INFLAMMATION AND AIRWAY REMODELING

By

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Chronic allergic lung inflammation results in airway remodeling and permanent structural changes. Here we show that administration of agonists to TNF receptor superfamily 25 (TNFRSF25) in vivo causes expansion of natural and allergen-specific CD4+FoxP3+ regulatory T-cells (Tregs), which can prevent allergic lung immunopathology in the acute setting, and if administered during chronic allergic lung inflammation, inhibits inflammation and reverses airway remodeling. TNFRSF25-expanded Tregs reduced eosinophilia in the bronchoalveolar lavage fluid, reduced expression of T-helper type-2 (Th2) cytokines and blunted allergen-specific Th2 effector responses. Previously established chronic airway remodeling, as measured by goblet cell hyperplasia and subepithelial collagen deposition, was reversed in mice treated with TNFRSF25-agonists but not in controls. The results indicate that in vivo Treg expansion by TNFRSF25 agonists, during chronic lung inflammation leads to durable suppression of inflammation and reversal of airway remodeling, which may be a result of shifting the antigen-specific T-cell population toward a regulatory response.
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1.1 ALLERGIC ASTHMA

Allergic asthma constitutes a diseased state in which the immune system has inappropriately reacted to non-pathogenic antigens, also known as allergens (2, 3). Asthma has a significant impact on the health of patients due to its chronic nature which often will have direct and in-direct financial costs to the patient. This disease affects 235 million people worldwide, and is a number that is only growing, though the reason that allergic asthma is on the rise in the western world is still up to debate (4). The “Hygiene Hypothesis” has been the dominant theory for the past few decades, which postulates that the excessive use of anti-microbials and the lack of early exposure to certain parasitic or bacterial infections leads to the development of an immune response to commonly exposed allergens (reviewed in (5)). But recently, another idea has received stronger support based on cohort studies looking to identify risk factors for allergic asthma: genetic and environmental factors, combined with severe early childhood respiratory infection, are the strongest indicators associated with development of allergic asthma (reviewed in (6)). This theory focuses primarily on immunological pre-disposition towards dysfunction that is easily enhanced by severe viral infections, with subsequent sensitization to common allergens.

1.1.1 Cellular and molecular mechanisms in asthma

Allergic-type asthma is characterized by an overzealous inflammatory response to common allergens that contributes to airway hyperreactivity and
airway remodeling (Figure 1). In a large number of patients suffering from asthma, exposure to the offending allergen(s) leads to the development of airway inflammation characterized by eosinophilia in the bronchoalveolar lavage fluid (BALF), mononuclear cell infiltration into the lung parenchyma, and airway remodeling (as reviewed in (2)). It is apparent that the main driver of this inflammation is a T-helper type-2 (Th2) polarized CD4+ T-cell mediated response with significant cross-talk with the innate immune response during initiation, progression, and exacerbation of the disease.

Figure 1 Cellular mechanisms of allergic asthma (adapted from (1))

1.1.1.1 Adaptive immunity in asthma

The adaptive immune response in the setting of allergic asthma is a driving force for the initiation and "memory"-like responses to allergens. Natural
Killer T-cells (NKT) are essential to the initiation phase of the disease in animal models of allergic asthma (7). Though their frequency is relatively low in any immune compartment, it is believed that activation via the invariant-T-cell receptor, by glycolipid-based antigen via CD1d presentation, drives NKT-cell proliferation and the release of IL-4 and IL-13, both key cytokines in the skewing of CD4+ T-cell responses towards a Th2-bias and the development of several aspects of allergic asthma (8). CD1d knockout (KO) mice, which lack NKT-cells, were resistant to the development of allergen-induced airway hyperreactivity, a pathophysiological component allergic asthma, while Jα18 KO mice, which lack iNKT-cells, failed to develop ovalbumin-induced allergic asthma (7-10).

CD4+ T-helper cells have also been shown to be essential to the development of allergic asthma (11, 12). Antigen-presentation by dendritic cells (DC) activates CD4+ naïve T-helper cells toward different helper subsets, depending on the cytokines in the microenvironment. There exist certain DC subsets (DCs expressing FcεR1α) that inherently skew immune responses toward a Th2 phenotype without cytokine influence (13, 14), additionally, if naïve T-cell activation and differentiation occurs in the presence of IL-4, differentiation is pushed toward the Th2 lineage via STAT6, and these CD4+ T-cells will up regulate the transcription factor GATA3, the characteristic Th2 transcription factor (15).

CD4+ Th2 effector cells are major sources of IL-4, IL-5, and IL-13, all cytokines essential for the development and progression of allergic asthma. These cytokines lead to enhanced serum IgE, increased presence of
eosinophils in the airways, goblet cell hyperplasia, and airway remodeling. Co-stimulation and specific cytokines from CD4+ Th2 effector cells leads to class-switching within activated B-cells towards immunoglobulin isotypes that contribute to the immunopathology associated with asthma (reviewed in (16)). CD4+ Th2 memory responses, in conjunction with allergen-specific responses by innate responses via bound IgE, are responsible for the subsequent sensitivity to allergen.

Another T-helper subset that has recently been found to be associated with certain types of human asthma is the T-helper type 17 (Th17) cell. This association is based on elevated IL-17 levels in the blood and lungs of asthmatic patients (17-19). Th17 cells are characterized by their production of IL-17a, IL-17f, IL-21, and IL-22 during immune responses, and are important in the clearance of extracellular pathogens. Th17 cells are differentiated from naïve CD4+ T-cells by the presence of TGF-β, IL-1β, IL-23, and IL-6 during activation, resulting in STAT3 signaling with subsequent up-regulation of the characteristic Th17 transcription factor, RORγt (20, 21). Through the use of animal models and patient studies, Th17-associated allergic asthma was characterized as having a significant presence of neutrophils in the airways with a severe clinical course, often observed in patients that are poorly responsive to steroid treatment (22-24).

The role B-cells have in allergic asthma is as memory B-cells and plasma cells, active producers of allergen-specific IgE, the quintessential atopy-related immunoglobulin (reviewed in (25)). Class-switching of allergen specific immunoglobulin to IgE, after allergen has activated and matured the
B-cell, requires two molecules from Th2 effector cells, either IL-4 or IL-13 via IL-4Rα signaling, and CD40L via CD40 signaling (26-28). Subsequently, B-cells become IgE-producing plasma cell, increasing the levels of circulating IgE, which are picked up by mast cells and eosinophils via surface FcεR1. Mast cells and eosinophils are innate immune cells, but by collecting allergen-specific IgE on their surface, these cells have acquired an ability to respond in an antigen-specific manner via the variable region of IgE. Once IgE-FcεR1 is cross-linked by allergen, a number of “early-” and “late-” phase responses occur. In mast cells, degranulation results in the release of molecular mediators of the immediate hypersensitivity (early) response, as well as the release of cytokines and chemokines involved in the consequent recruitment and activation of the inflammatory cells into the site of allergen exposure (late). IgE cross-linking on APCs activates them to promote IgE production from B-cells and Th2 cytokine production by effector T-cells (reviewed in (29)).

B-cells can also become IgE memory B-cells that reside in secondary lymphoid organs. IgE memory B-cells are long-lasting and are ready to respond when allergen is re-introduced into the system. Subsequent allergen exposures results in either differentiation of IgE memory B-cells into IgE plasma cells or IgE memory B-cells expediting cellular immunity in the lymph nodes. The IgE memory B-cells comprise a unique subset of memory B-cells differentiated directly from IgE class-switched B-cells, and not IgG1 memory B-cells that had undergone secondary class switch (30).
The major effector molecules produced by the adaptive immune response are the Th2-associated cytokines and chemokines, though these molecules are known to be also produced by cells of the innate immune response. This group of cytokines includes IL-4, IL-5, IL-9, IL-13, IL-17 (a and f), and IL-25 (IL-17e) (Figure 2). Each of these cytokines have effects in multiple aspects of asthma pathogenesis: IgE production, mast cell growth, eosinophil accumulation, airway remodeling, and airway hyperreactivity (32-35).

In allergic asthma, IL-4 is essential in the early differentiation of the Th2 phenotype, but is dispensable in the effector phase of the disease. This is likely due to redundant signaling by amplified IL-13 signaling (36). The importance of IL-4 in T-cell responses can be observed during CD4+ T-helper
cell differentiation from naïve to T-helper type 2 by IL-4 signaling via IL-4Rα. The ligation of IL-4 to IL-4Rα results in STAT6 signaling with subsequent activation of the GATA3 transcription factor (37, 38). Signaling of IL-4Rα is also required during B-cell class switching of the immunoglobulin constant region to the IgE isotype (28). IL-4 signaling has also been implicated in a number of other aspects of allergic asthma, including modulating smooth muscle cell response to IL-13, up-regulating FcεR1 on mast cells, induction of the mucin gene, and increased expression of eotaxin, a chemoattractant for eosinophils (39-42).

The cytokine IL-5 acts on IL-5R, which signals through JAK-STAT (STAT1 and STAT5) and Ras-MAPK pathways. IL-5 is primarily produced by CD4+ T-helper type 2 cells, and is important in the recruitment, retention and activation of eosinophils in the airway mucosa. The importance of IL-5 in the development of eosinophilia within the airways and lung parenchyma has led to much work focusing on IL-5 blocking therapies, though early clinical trials have not shown much physiological effect by anti-IL-5 treatments though systemic eosinophilia is well controlled (43). Issues with sample size, cohorts chosen, and methodologies in these trials suggest that refinement of the studies would have yield promising results. Recent work has indicated that anti-IL-5 treatment in the right setting could yield clinically relevant results (44-46).

IL-9 was first characterized as mediating Th2 immune responses in a mouse model of Leishmaniasis (47, 48). It is primarily expressed in CD4+ T-cells, though there is evidence that a unique subset of T-helper cells, Th9, can
be defined by their dedicated production of IL-9. Signaling of IL-9 occurs via a heterodimer of IL9Rα and the common γ-chain, leading to the activation of STAT1, STAT3, and STAT5. IL-9 signaling occurs on effector T-cells, with highest expression on Th2 and Th17 subsets, as well as mast cells, airway epithelium, and smooth muscle cells. IL-9 has a number of effects on both the adaptive and innate immune system. In the context of immune responses in asthma, IL-9 induces T-cell growth, increases IgE production in B-cells, helps in mast cell growth and survival, enhances chemokine production from epithelial cells, and up-regulates IL-13 production from airway smooth muscle cells (reviewed in (49)).

The cytokine IL-13 has been found to be an essential molecule in mediating eosinophilic lung inflammation, characteristic for allergic asthma, as well as airway hyperreactivity and airway remodeling (33, 34, 50, 51). IL-13 ligates to either IL-13Rα1, as a heterodimer with IL-4Rα, or IL-13Rα2, which is believed to act as a decoy receptor to sequester IL-13. Signaling via IL-13Rα1/IL-4Rα, activates the JAK/STAT pathway, specifically through STAT6 (52). IL-13 is produced by a number of cell types including CD4+ Th2 and T-helper type 1 (Th1) T-cells, CD8+ T-cell, NKT-cells, mast cells, basophils, eosinophils and airway smooth muscle cells (53, 54). The receptor pair of IL-13Rα1 with IL-4Rα is expressed on B-cells, monocytes, macrophages, dendritic cells, eosinophils, basophils, fibroblasts, endothelium, and airway epithelium and smooth muscle cells. IL-13 signaling on these cell types, are responsible for much of the effector phase of allergic asthma (reviewed in (55)). The effects of IL-13 include augmenting airway smooth muscle cell
contractility, enhancing mucus production from bronchial epithelium, increasing Th2 cytokine and chemokine production, up-regulation of metalloproteinases, proliferation of myofibroblasts, and increasing deposition of collagen in the setting of subepithelial fibrosis (reviewed in (53)).

The cytokines IL-17a and IL-17f, of the IL-17 family, are able to signal via IL-17RA and IL-17RC, with downstream signaling through Act1 and the ERK pathway, with subsequent NFκB and TAK1 activation. IL-17a and IL-17f are produced by Th17 cells, iNKT-cells, γδ T-cells, and certain lymphoid tissue-inducer cells in response to extracellular pathogen infections; all subsets express the characteristic Th17 transcription factor RORγt. The receptors for IL-17 (a and f) are expressed on epithelial cells and fibroblasts, and IL-17 signaling causes these cells types to produce a number of cytokines and chemokines, such as KC, MIP-2, MCP1, IL-6, G-CSF, and IP-10. All of these cytokines and chemokines enhance innate immune responses to extracellular pathogens (reviewed in (56)). The specific role of IL-17 in asthma seems to be quite complicated, seeming to be dependent on the timing and source of the IL-17. Using asthma animal models, it has been determined that IL-17a is essential to the disease pathogenesis and the development of the characteristic airway infiltration by eosinophils and neutrophils, as well as airway hyperreactivity (57). In support of these studies in genetically modified mice, antibody-mediated neutralization of IL-17a prior to allergen challenge resulted in blunted airway inflammation (58). Complicating the role of IL-17 in asthma is the finding that production of IL-17 from γδ T-cells, the major source
of IL-17 in allergic asthma, is a negative regulator of allergic inflammation and airway hyperreactivity in mouse models of asthma (59, 60).

1.1.1.2 Innate immunity in asthma

The innate immune response is also highly implicated with the development and immunopathology in allergic asthma. Eosinophils, macrophages, dendritic cells (DC), mast cells, and the newly discovered sub-class of innate-like cells have shown to have a role in disease pathogenesis, often times using the same cytokines of the adaptive response as well as having direct interactions with the adaptive arm.

Allergic lung inflammation, a key component of allergic asthma, is characterized as excess leukocytosis into the airways. Eosinophils are the primary cell type that many identify as a key marker for allergic lung inflammation, and are observed at significant levels in bronchoalveolar lavage fluid (BALF) collected from asthmatic patients. The actual percentage of eosinophils that is observed in BALF from patients with eosinophilia is approximately 4.8% in mild-to-moderate asthmatics (61). The primary function of eosinophils during “normal” immune responses is during parasitic infection, acting as effector cells for the expulsion and control of the parasites. In the setting of allergic asthma, eosinophils play a key role in the disease pathogenesis because of their release of IL-13, TGF-β, and cationic proteins: major basic protein (MBP), eosinophilic peroxidase (EPO), and eosinophilic cationic protein (ECP), which are toxic to airway epithelial cells (62) and enhance airway hyperreactivity in primates (63). Interestingly, eosinophilia
does not consistently correlate with airway hyperreactivity in some mouse models of allergic asthma, suggesting the role of eosinophils in the airways differ to some degree between species, mouse strains, and experimental models (64, 65). Additionally, the importance of eosinophils in airway remodeling is contentious (66, 67). It is likely that both eosinophil-dependent and eosinophil-independent mechanisms are involved in airway remodeling, with both pathways involving IL-13 and TGF-β, two cytokines produced by eosinophils in the lung that contribute to airway remodeling.

In the lung, most macrophages reside within the airway and during homeostatic conditions act in an “immunosuppressive” manner. This is described as active phagocytosis and killing of any microbes in the respiratory tree which in turn does not result in the release of “danger signals” to alert other cells of pathogen invasion (68, 69). Through interactions of CD200R on macrophages and CD200 on airway epithelium, immune responses to antigen is controlled in the lungs (70). The likely reason airway macrophages act in an “immunosuppressive” action is to protect the lungs from excess immunopathology. A strong immune response often results in collateral damage to the surrounding tissues, but in the airways, excess inflammation and repair results in the development of an epithelial lining that is in a poor state of gas exchange (71). To preserve the physiological characteristics of the airway epithelium, mucosal immune responses are locally controlled, with minimal bystander inflammation elicited. During pathological immune responses in the lung, such as in the setting of allergic asthma, alveolar macrophages can be “alternatively-activated”, acquiring an M2 phenotype,
which produce chemokines CCL17 and CCL22, potent chemoattractants for CCR4+CD4+ Th2-cells to the lungs (72).

Dendritic cells, the professional antigen-presenting cells, represent a key member of the innate response that bridges the innate and adaptive immune responses. A number of signaling molecules activate dendritic cells to enhance MHC Class-II presentation of antigen, up-regulate co-stimulatory molecules, and produce several cytokines with either inflammatory or tolerogenic properties. In the setting of allergic asthma, DC activating molecules include TLR ligands (LPS and dsRNA), cytokines (TSLP, IL-25, IL-33, IL-10), and various members of the TNF super family (TL1A and CD40) (reviewed in (73)). Work from a number of groups has shown the importance of DCs in the initiating events that lead to allergic asthma during childhood, as well as how phenotypic changes to DCs after severe respiratory infection early in life leads to the progression of allergic asthma into adulthood (reviewed in (74)). The model suggests that early life events, such as environmental exposures, infections, and nutrition, compiled upon genetic predisposition to atopy, lead to the recruitment of DCs with enhanced expression of FcεR, to the airways. This leads to the lung being primed toward a Th2 immune response with subsequent exposure to allergen resulting in the release of chemokines for Th2 memory cells and enhanced presentation of allergen, leading towards the activation of a robust Th2 immune response (13, 75). In addition to the importance of antigen processing and presentation, DCs present and secrete a number of important co-stimulatory molecules. In allergic asthma, molecules of the
Immunoglobulin family and TNF superfamily have been found to be quite important both in disease pathogenesis and progression. Within the Immunoglobulin family, CD80 and CD86, the quintessential co-stimulatory molecules, are needed as “signal 2” during T-cell activation, while ICOS-L, when ligated to its cognate receptor ICOS, enhances T-cell production of cytokines. OX40L, a ligand within the TNF superfamily, is highly up-regulated on DCs after TSLP signaling, and can co-stimulate CD4+ Th2 effector responses, enhancing Th2 cytokine production and markers of activation (reviewed in (76)).

Tissue resident mast cells in the lung mucosa are important early mediators of the acute asthmatic response. The surface receptor FcεR1 on mast cells efficiently binds antigen-specific IgE and is primed for action upon aeroallergen exposure. Once antigen enters the system and cross-links IgE, an immediate hypersensitivity response is elicited from the mast cells, mediated by the release of preformed granule-associated effector molecules such as histamines, leukotrienes, proteases and cytokines. These mediators trigger smooth muscle contractions as well as increase microvascular permeability. Th2 effector cytokines IL-4, IL-5, IL-9, IL-13, and IL-25, as well as the chemokine CCL2, are all produced by mast cells upon degranulation, and help mediate the late-phase response observed by enhanced Th2 adaptive immune responses in the lungs. It has also been found that mast cells are significant sources of MMP-3 and -9, both molecules heavily implicated in the airway remodeling process that occurs in chronic airway inflammation (reviewed in (29)).
Recently, a variety of new cell-types have been discovered to have significant roles in immune response, specifically at mucosal sites such as the lung. These innate-like cells (ILCs) include a number of different types of cells, including nuocytes, lymphoid tissue-inducer cells (LTi), and natural helper cells (NHC) (reviewed in (77)). Nuocytes are especially of significance in the setting of allergic asthma. Nuocytes are expanded by IL-25 and IL-33, two epithelial-derived cytokines with significant roles in innate signaling for Th2 effector responses. These cells are early and abundant source of IL-5 and IL-13 in the lung, representing a major source of non-T-cell-derived Th2 cytokines (78).

As it has been recognized that leukocytes do not act without stromal influences and crosstalk, epithelium-derived cytokines are being investigated to define their roles in the initiation and progression of asthma and the associated pathological sequelae (Figure 2).

The work of Dr. Ziegler and others has shown that thymic stromal lymphopoietin (TSLP), of the IL-7 family, is necessary and sufficient for the development of allergic asthma in mice using both knockout mice and transgenic expression of TSLP (79, 80). TSLP is constitutively produced by lung epithelium, and signals via the TSLPR/IL-7Rα heterodimer. Signaling by TSLP occurs on a number of different cells, including CD4+ T-cells, eosinophils, mast cells, NKT-cells, and importantly, DCs. TSLPR/IL-7Rα ligation by TSLP results in STAT5 activation. In the setting of allergic asthma, it has been found that TSLP can induce proliferation in Th2 CD4+ T-cells and enhance Th2 cytokine production by NKT-cells, CD4+ T-cells and epithelial
cells. TSLP up-regulates a number co-stimulatory molecules on DCs, including, CD40, CD80, and OX40L, while also enhancing survival of eosinophils (reviewed in (31)).

IL-25 (IL-17e), of the IL-17 family of cytokines, has shown to be sufficient to initiate an asthma-like phenotype in animal models with transgenic expression of the cytokine (81). IL-25 acts on the heterodimer IL-17RB and IL-17RA, activating the STAT6 and ERK intracellular signaling pathways. IL-25 is produced by the epithelium, T-cells, and mast cells, while acting on T-cells, NKT-cells, and the newly discovered nuocytes and natural helper cells (78, 82). The cytokine has been shown to be important in the initiation and enhanced production of Th2 cytokines IL-4, IL-5, and IL-13, and plays a key role in linking the innate and adaptive immune responses (82, 83). Interestingly, it has been found that IL-25 can block the differentiation of CD4+ naïve T-cells to the Th17 phenotype, further complicating the role of the IL-17 family of cytokines in the allergic asthma (84).

IL-33, of the IL-1 family, is another recently discovered epithelial-derived cytokine, and is quite central to allergic asthma. Airway epithelium and smooth muscle cells produce IL-33, which signals through the heterodimeric receptor of IL-33Rα (ST2L) and IL-1R accessory protein, with intracellular signaling by the MAPK pathway leading to NFκB activation (85, 86). IL-33, like IL-25, acts on a number of cell types to enhance Th2 immune responses. IL-33 enhances the “alternative activation” of macrophages toward the M2 phenotype as well as stimulates eosinophil differentiation in the bone marrow. IL-33 can also expand nuocytes, which subsequently are early sources of IL-5.
and IL-13, as well as up-regulate markers of maturation and activation on DCs: CD80, CD40, and OX40L (87).

1.1.1.3 Immunoregulatory mechanisms in asthma

In allergic asthma, there are a number of immunoregulatory mechanisms that have either failed, or have become unable to suppress the inflammatory response to allergen. The process of sensitization in patients and animal models is a result of broken “tolerance” to the antigen. Tolerance to a specific antigen starts at the antigen-presenting cell (APC), primarily the dendritic cell, but can include macrophages and B-cells in certain situations. In a naïve patient or animal, naïve T-cells with T-cell receptors specific for the allergen are presented the allergen by APCs, which results in either tolerogenic or inflammatory phenotypes in the T-cells. Often MHC Class-II presentation of allergen with no co-stimulation (low CD80/86) or increased indoleamine 2,3-dioxygenase, will result in CD4+ T-cell anergy, which is considered a state in which the antigen-experienced T-cell is unable to respond to the antigen upon re-stimulation, unless a large amount of IL-2 is present (88-90). Another mechanism for tolerance induction is the differentiation of CD4+ naïve T-cells to CD4+FoxP3+ T-regulatory cells (Treg), via the presence of TGF-β (enhanced with retinoic acid) during antigen-presentation (91, 92). The differentiation of naïve CD4+ T-cells to iTregs is considered a major component to peripheral tolerance (extrathymic regulation), and often times occurs at sites of tissue inflammation and mucosal lymphoid structures (93-96). DCs with surface expression of the integrin CD103 (itgax) have been shown to have increased activity of aldehyde dehydrogenase and
retinaldehyde dehydrogenase 2 (two essential enzymes for the conversion of retinol to retinoic acid), and are mucosal residing DCs that are efficient at the induction of FoxP3 transcription factor, the key immunoregulatory transcription factor in T-cells (97). In allergic asthma, a number of mechanisms have been shown to break this tolerance to antigen, including defective Treg function, reduced number of Tregs, and dendritic cells with Th2-bias ((13), reviewed in (32)).

CD4+FoxP3+ Regulatory T-cells (Tregs) serve an important role in the control of lung inflammation in asthma (Figure 3), as evidenced by the reduction of airway inflammation by therapeutic treatment with Tregs in allergic asthma models or depletion in allergen-induced asthma (98-105). Additionally, most mice strains are inherently tolerant to ovalbumin aeroallergen, but this tolerance can be broken by disrupting the induction of iTregs at the site of mucosal interaction with the allergen (106). Many types of Tregs (Th1-like, Th2-like, Foxp3/TGF-β) can potently block the development of airway hyperreactivity and allergic lung inflammation in murine models (reviewed in (32, 107)). In humans, a strong presence of antigen-specific Tr1 regulatory cells, which are FoxP3+ or FoxP3- but produce IL-10 proficiently, is associated with healthy, non-atopic patients, as well as patients clinically responding to specific-antigen immunotherapy (108, 109).
The suppressive effects Tregs have in allergic asthma include direct suppression of innate mediators of allergic asthma (mast cells and eosinophils), controlling CD4+ Th2 immune responses either directly with the T-cell or via modulation of APCs, and regulation of IgE-producing B-cells (reviewed in (111)). Molecular mechanisms for this suppression include the presence of IL-10, ICOS, TGF-β, IL-35, and the induction of indoleamine 2,3 dioxygenase in DCs (99, 112-116). Currently, therapeutic approaches to enhance Treg numbers for treatment of allergic asthma encompass a variety of strategies: specific-allergen immunotherapy, polysaccharides from
Streptococcus pneumoniae, probiotic cocktails, and IL-2/Anti-IL-2 monoclonal antibody complex (117-120).

Deviation of Th2 immunity towards Th1 type responses has shown promise in mouse models of asthma. A number of vaccine models using modified infectious agents, peptides, or primed DCs, have shown to be efficacious in reducing parameters of allergic asthma, primarily through the induction of a prominent CD8+ Tc-1 response and enhancing CD4+ Th1 type responses, at the expense of CD4+ Th2 immunity (121-124). This strategy may be an ideal approach for reducing immunopathology associated with allergic asthma, but carries a risk for enhancing bystander inflammation that occurs with the clearance of viral infections, such as influenza (125).

1.1.2 Pathology associated with allergic asthma

Allergic asthma is characterized by a dysregulated immune response with subsequent pathological sequelae, which includes severe lung inflammation, airway hyperreactivity and airway remodeling.

1.1.2.1 Allergic lung inflammation and airway hyperreactivity

The pathology observed in allergic asthma falls into two categories: immunological and physiological. The allergic responses in the lung stem from sensitization of the host to allergens: a result of genetic predisposition, diet, environmental exposures, and a history of severe viral infections in the lung. When allergen exposure occurs, an inflammatory process begins, starting with the immediate degranulation of mast cells and eosinophils (Figure 4), and the eventual release of Th2 cytokines, IgE production, and
recruitment of inflammatory cells to the lung mucosa (Figure 5) and airway lumen (Figure 4) (2). In parallel, airway smooth muscle cells, which have proliferated and have increased sensitivity to histamine by IL-13, significantly contract the bronchial airways in response to allergen-induced degranulation of mast cells and eosinophils. The physiological measurements for mice and humans involve airway challenge with methacholine and subsequent measurement of airway resistance and lung compliance. Airway hyperreactivity is observed as enhanced airway resistance and reduced lung compliance. Both allergic lung inflammation and airway hyperreactivity are pathological responses that are responsible for much of the clinical symptoms suffered by the patients during acute asthmatic exacerbations (2).

Figure 4: Bronchoalveolar lavage fluid (BALF) cells were fixed and stained with Wright-Giemsa. BALF from naïve (Left) and asthmatic (Right) mice are shown.
1.1.2.2 Airway remodeling in allergic asthma

Airway remodeling is a significant contributor to chronic airway dysfunction and clinical symptoms experienced by patients (2), and is a result of excess T-helper 2 type cytokines (IL-5 and IL-13) as well as eosinophils (a major source of TGF-β) (34, 126-128). Remodeling is characterized by a number of parameters, including goblet cell hyperplasia/dysplasia (Figure 6), airway fibrosis (Figure 7), airway smooth muscle hyperplasia/hypertrophy, and increased airway vascularization. Airway fibrosis is a result of significant deposition of collagen types I, III, V into the subepithelial space, primarily from fibroblasts that have migrated to the area. Another characteristic of airway
remodeling is the conversion of fibroblasts to myofibroblasts (129, 130), which leads to enhanced bronchial constriction to allergen challenge.

Figure 6: Periodic Acid-Schiff staining for mucus in the bronchiole epithelium of lungs from a mouse model of allergic asthma (200x).

Figure 7: Sirius Red-Picric acid staining of collagen deposited in the subepithelial space in the bronchiole of a lung from a mouse model of chronic airway remodeling (320x).

Research into airway remodeling in the setting allergic asthma indicates that TGF-β and IL-13 are key cytokines in the remodeling process due to their
actions on fibroblasts and epithelial cells. It is believed that factors such as MMP-2, -3 and -9 as well as EGFR and VEGF, are also important in the process of collagen deposition and other aspects of remodeling in the airways, possibly as downstream mediators of IL-13 and TGF-β signaling (reviewed in (131)). Recent work by Doherty et al. has found the TNF superfamily member LIGHT is necessary and sufficient for remodeling in two different chronic models of allergic asthma, indicating the number of molecules involved in airway remodeling are quite diverse and likely create a network of redundancies (132). Any intervention to reverse this process likely requires an ability to affect a number of up-stream promoters of the remodeling process.

1.1.3 Models of allergic asthma

To study both disease pathogenesis and the efficacy of potential therapies, a number of animal models of allergic airway disease have been developed. Currently, most research uses mouse, rat, sheep, or non-human primate models of the disease. The animal models can be broken down into two types: models for acute asthma, usually involving short-term exposure to allergen, and models for chronic asthma, which sustain aeroallergen exposure for a number of weeks to months.

1.1.3.1 Acute mouse models

In the development of mouse models of allergic asthma, there have been a number of protocols and allergen exposure systems used to induce a reliable and representative model of asthma. The majority of models can be
categorized as either: allergen sensitization then aerosol exposure (Figure 8) or nasal-only introduction of allergen. These models have been used to understand cellular and molecular mechanisms important in disease initiation, progression, and treatment.

![Antigen Sensitization and Exposure Protocol](image)

**Figure 8: Example protocol for an acute model of allergic asthma**

A number of other models involve cytokine-instillation, transgenic over-expression of molecules, or adenoviral delivery of genes, but they are primarily used only to study the mechanism of disease pathogenesis for the specific cytokine or gene-product. The allergens of interest used in models of acute asthma include chicken egg ovalbumin, house dust mite allergen (HDM), and cockroach extract antigen (CEA). Using ovalbumin requires priming with adjuvant, such as potassium aluminum sulfate dodecahydrate (ALUM), since most mouse strains are not inherently sensitized to the chicken product (except in the A/J strain) (133, 134). The HDM and CEA models do not require any sensitization, but allergen exposure is primarily via intranasal instillation, versus allergen aerosolization used in ovalbumin-sensitized models. All three models develop symptoms and pathology similar to allergic asthma, including severe lung inflammation, airway infiltration by eosinophils
and other leukocytes, goblet cell hyperplasia, and airway hyperreactivity. Interestingly, there seems to be a varied response in regards to airway inflammation and airway hyperreactivity, depending on the mouse strain. BALB/C mice were found to consistently display airway hyperreactivity after allergen exposure, while C57BL/6 mice were reliable models for allergic lung inflammation in response to allergen (135, 136). A significant benefit when using the ovalbumin-sensitization/aerosolization model is that both BALB/C and C57BL/6 strains have a number of tools that allow for monitoring of ovalbumin-specific responses: transgenic mice (OT-II and DO11.10) and immunological reagents (OVA:I-A\textsuperscript{b} and KJ-126 tetramer antibodies).

1.1.3.2 Chronic mouse models

Early animal models for allergic asthma began with acute models with short periods of allergen challenge, usually a single exposure or multiple exposures over a 1-week period. Though these models reflect much of the immunopathology associated with allergic asthma in humans, one key component was not always present: airway remodeling.

![Figure 9: Sample protocol for a chronic model of allergic asthma](image-url)
To further develop the mouse model allergic asthma, a number of groups have modified the acute models to include an increased number of allergen exposures over a longer period of time (Figure 9). For example, McMillan and Lloyd et al. extended airway exposures to approximately 40 days in sensitized mice (137), while Wegmann and Renz et al. gave ovalbumin aerosolization twice a week for 12-weeks in sensitized-mice (138). A third model by Doherty and Croft et al. gave intranasal instillation of house dust mite allergen or ovalbumin once a week for approximately 6 weeks (132). All of these models show varying degrees of airway remodeling, allergic lung inflammation and airway hyperreactivity: three important clinical parameters relevant to the human disease. Additionally, these chronic models of allergic asthma represent the ideal setting to test the efficacy of any therapeutic intervention in established asthma and airway remodeling because of the similarity to patients in the clinical course and pathology.

1.1.3.3 Pre-clinical models

In the development of therapies for asthma, larger animal models have been utilized to evaluate efficacy and possible adverse effects. The models developed have used sheep, Ovis aries, or non-human primates, Macaca fascicularis (cynomolgus macaque) and Macaca mulatta (rhesus macaque).

In sheep, two different allergen challenge models have been employed. First discovered and developed was a model using the parasite Ascaris suum, a natural allergen for sheep, which replicated much of the pathology observed in human patients (reviewed in (139)). Recently, a model using the known
human allergen, house dust mites (HDM), has been developed in sheep. The HDM model begins with sensitization of sheep to HDM, then airway challenge with intranasal allergen administration. The immunopathology observed in both models comprise of increased IgE production, early- and late-phase bronchoconstriction, airway hyperreactivity, and airway inflammation and remodeling (140, 141). A number of current asthma medications were found to be efficacious in the sheep model of asthma, and the model is believed to be one of the best predictors for clinical success in humans (139). The limiting factors for expanded use of the sheep models of allergic asthma is the availability of reagents available for proper immunological monitoring and the cost of developing a sheep-specific version of biological therapies.

Pre-clinical animal models that share more anatomical and physiological similarities to humans use two species of macaques: rhesus and cynomolgus macaques. The earliest model of allergic asthma model in non-human primates used rhesus macaques, which could be sensitized to Ascaris suum by intestinal infection of the monkey. This model was developed based on observations that wild-caught rhesus macaques were found to be naturally sensitive to soluble extracts of Ascaris by skin test. The hypothesis was that these animals were infected by Ascaris suum or a related parasite and became sensitized to the allergen, displaying a Th2-type immune response upon challenge. This early model in rhesus macaques displayed many of the typical “asthmatic” features including early- and late-phase bronchoconstriction in response to Ascaris aerosol challenge, migration of eosinophils, lymphocytes, and dendritic cells to the airway, and Th2 cytokine
and chemokine induction (142). A similar model was developed in cynomolgus macaques that were selected for atopic responses to Ascaris extract, similar to the rhesus macaque model. The cynomolgus model is currently used by a number of groups and displays similar “asthmatic” features as discussed with the rhesus macaque (143).

Chronic models of allergic asthma in non-human primates have also been developed to fully evaluate the effects of potential therapeutics on established asthma with features of airway remodeling. A rhesus macaque model of chronic allergic asthma has been established at the National Primate Research Center at the University of California at Davis, and begins with sensitization of rhesus macaques with subcutaneous injections of HDM, followed by 13 weeks of HDM aeroallergen exposure. This chronic model of asthma uses a known human allergen and successfully displays similar airway remodeling observed in patients with long-standing asthma, in addition to the pathological immune and physiological responses to aeroallergen challenge (144). The development of a reliable chronic asthma model in rhesus macaques, combined with the number of immunological tools available for use in that species, suggests that the HDM-induced allergic asthma model in rhesus macaques is the ideal model for pre-clinical studies in asthma therapeutics.
1.2 TNF SUPERFAMILY

1.2.1 TNF superfamily members

Figure 10: TNF superfamily of receptors and ligands (adapted from (145))

The TNF superfamily consists of a number of receptors and ligands, all of which have significant but varying effects on the immune system. There are approximately 29 receptors and 20 ligands within the superfamily (146)
(Figure 10), with cross-ligation with certain receptors and ligands. The receptors are characterized by the presence of a cysteine-rich domain (CRD) in the extracellular domain, and are primarily membrane-bound, with a few exceptions, including Decoy Receptor 3 (DcR3) which lacks a transmembrane domain (147). As a superfamily, they affect multiple aspects of the immune system including: homeostatic maintenance, activation, and suppression. They often work in both redundant and non-redundant roles in complex immune networks, and have started to garner attention for their position as key intersections of the immune response and potential as effective immune-modulating therapeutics. The TNF superfamily is involved in proliferation, morphogenesis, differentiation, and apoptosis, and often times can have varying effects depending on the immune and spatial-temporal context (106, 146, 148-150) (Table 1).

In the setting of immune cell activation and proliferation, TNF superfamily signaling activates various mitogen-activated protein kinases through the initial recruitment of a TNF Receptor Associated Factor (TRAF), sometimes through a TNF Receptor Associated Death Domain (TRADD) molecule. During the activation of the apoptotic pathway, TNF superfamily signaling recruits both a TNF Receptor Associated Death Domain (TRADD) molecule and a Fas-associated Death Domain (FADD) molecule (145, 151). TNF superfamily members have been implicated in T-cell memory responses (CD30), T-cell activation and co-stimulation (4-1BB) (152), Ig class-switching (CD40, APRIL) (27, 153), NKT-cell co-stimulation (TL1A) (149, 154), and immune suppression (OX40, FAS) (155, 156). The molecules of the TNF
superfamily also have non-immune influences, such as homeostatic maintenance of bones (RANK, OPG) (157), though the most notable role of the TNF superfamily is in the modulation of immune responses.

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Receptors</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>TNFR1/TNFR2</td>
<td>Programmed cell death/cellular proliferation</td>
</tr>
<tr>
<td>LTβ</td>
<td>LTβR</td>
<td>Lymphoid organogenesis</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas/DcR3</td>
<td>Apoptosis induction</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40</td>
<td>B-cell co-stimulation, Ig class-switching</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TRAILR/OPG</td>
<td>Apoptosis induction in dendritic cells</td>
</tr>
<tr>
<td>APRIL</td>
<td>TACI/BCMA</td>
<td>T-cell and B-cell proliferation, Ig class-switching</td>
</tr>
<tr>
<td>OX40L</td>
<td>OX40</td>
<td>T-cell co-stimulation, development of T-cell memory, Treg expansion</td>
</tr>
<tr>
<td>BAFF</td>
<td>BAFFR/TACI/BCMA</td>
<td>B-cell development and plasma cell differentiation</td>
</tr>
<tr>
<td>RANKL</td>
<td>RANK</td>
<td>Dendritic cell proliferation/bone development</td>
</tr>
<tr>
<td>TL1A</td>
<td>DR3</td>
<td>T-cell and NKT-cell co-stimulation, Treg expansion</td>
</tr>
<tr>
<td>4-1BBL</td>
<td>4-1BB</td>
<td>T-cell co-stimulation, T-cell memory</td>
</tr>
<tr>
<td>CD30L</td>
<td>CD30</td>
<td>T-cell differentiation, development of T-cell memory</td>
</tr>
<tr>
<td>GITRL</td>
<td>GITR</td>
<td>Regulation of Tregs</td>
</tr>
<tr>
<td>LIGHT</td>
<td>HVEM/LTβR</td>
<td>T-cell memory</td>
</tr>
</tbody>
</table>

Table 1: TNF superfamily members in immune functions (adapted from (158))
1.2.2 TNF superfamily in health and immunity

Figure 11: TNF superfamily members in disease (adapted from (159))

The TNF superfamily of ligands and receptors have multiple roles in immune modulation; they can affect either the effector or regulatory immune responses, and often times both arms. The importance of these molecules in health is reflected by the number of diseases that have been found to be highly associated with changes in expression or function of members of the TNF superfamily, or unique SNPs within their loci (Figure 11). In patients with inflammatory bowel disease, both Crohn’s disease and ulcerative colitis, there are reports of elevated levels of TL1A in the blood stream and inflamed mucosa of patients with active disease, with animal models replicating similar disease parameters with enhanced gut TL1A expression (160-162). For patient cohorts with systemic lupus erythematosus (SLE), there have been GWAS and association studies showing increased risk for disease with certain SNPs in and around the CD40 loci, with mouse models confirming the
importance of CD40/CD40L to disease pathogenesis (163, 164). And in allergic asthma, bronchial biopsies from mild-to-moderate asthmatics show elevated numbers of OX40+ cells (165) with another study showing elevated levels of soluble CD30 in the serum of atopic asthmatics (166). A third study found enhanced levels of BAFF in the sputum of asthmatic children (167). In addition to genetic and immunological studies of patient cohorts, there have been a number of animal models of allergy and auto-immunity showing the significant role TNF superfamily members play in disease pathogenesis as well as their potential as therapeutic targets. TNFRSF25/TL1A, CD30/CD30L, OX40/OX40L, CD40/CD40L, LIGHT/HVEM/LT-β receptor are ligand/receptor pairs implicated in various aspects of the development and progression of allergic asthma, and some have already been studied as targets for immunotherapy (106, 132, 149, 168, 169).

1.2.3  TNF receptor superfamily 25/TNF-like ligand 1A

1.2.3.1  TNF receptor superfamily 25

TNFRSF25 is a tumor necrosis factor (TNF) family receptor that contains a death-domain in its intracellular domain, and has therefore been categorized as Death Receptor 3 (DR3). It was discovered 16 years ago, and was identified by a number names including “WSL-1,” “Apo-3,” “Lymphocyte-associated receptor of death (LARD),” “TNF receptor-related apoptosis-mediated protein (TRAMP),” and “TR-3” (170-175). A similar receptor to TNFRSF25 is TNFRSF6B, also known as Decoy Receptor 3 (DcR3, TR6); the main difference between the two molecules a lack of intracellular signaling
domain in DcR3 (147). TNFRSF25 is a type 1-membrane protein made up of 417 amino acids (aa) with a molecular weight of approximately 45 kDa.

Figure 12: Diagram of TNFRSF25. (SS= N-terminal signal sequence, ECD= Extracellular domain, TM= Transmembrane domain, ICD= Intracellular domain, DD= Death domain)

As diagrammed in Figure 12, the molecule has an N-terminal signal sequence (aa 1-24) followed by an extracellular domain (ECD) (aa 25-198), a transmembrane domain (aa 199-224), and an intracellular cellular domain (ICD) (aa 225-417). The ECD is comprised of four homologous cysteine-rich domains (CRD) while the ICD contains a death domain (DD), an essential component for intracellular signaling. Currently the crystal structure is not available for TNFRSF25, but by aligning structural elements of the primary sequences of TNFRSF25 with other TNF superfamily members that were previously determined by crystallography, specifically TNFR1 and DR5, comparative modeling was done. TNFR1 is the closest related TNF superfamily to TNFRSF25, with 30% sequence similarity: 44% in their ECD, 32% in their ICD, and 48% in their DD (170-174, 176, 177). The gene was mapped in humans to the short arm of chromosome 1, position 1p36.3, and in mice to chromosome 4, region E1. At the protein level, mouse and human TNFRSF25 are 63% homologous (94% in the DD and 52% in the ECD) (176).

Expression of the TNFRSF25 is primarily in lymphocyte-rich tissues like the spleen, thymus and peripheral blood lymphocytes (PBL), and less
abundantly in the small intestine and colon (170-173, 175, 176). A number of cell-types within the adaptive and innate immune systems express TNFRSF25 on their surface. Naïve and activated CD4+ T-cells, naïve and activated CD8+ T-cells, and CD4+ T-regulatory cells, all have surface expression of the receptor, though at varying levels (173, 175, 178, 179). NKT-cells, a subpopulation of CD11c+ cells, and NK-cells also express TNFRSF25 on their surface, while B-cells do not (149).

The Death Domain is the intracellular signaling domain for TNFRSF25 that can induce downstream activation of NFκB or apoptosis. Upon ligation with TL1A, the cognate ligand for TNFRSF25, the adaptor molecule TNF receptor-associated death domain (TRADD) is recruited to the DD, followed by TNF receptor-associated factor 2 (TRAF2) and receptor interacting protein 1 (RIP1), resulting in the formation of complex 1 (Figure 13) (151, 170, 171, 174, 180, 181).

This complex initiates a signaling cascade resulting in activation of NFκB and MAPK (ERK1/2, JNK, and p38), and the induction of cIAP-2, an anti-apoptotic factor (180, 182). The alternative signaling pathway is mediated through the formation of complex 2 with the recruitment of Fas-associated death domain (FADD) and caspase 8 to TRADD. Caspase 8 then initiates the caspase 3-mediated cell death pathway (Figure 13) (151). Recent work by Pobezinskaya et al. showed that in primary lymphocytes obtained from mice, cell death could not be induced after exposure to TL1A, even with pretreatment with cycloheximide or a SMAC mimetic (which blocks cIAP-2). These results, combined with the fact that previous observations of
TNFRSF25-mediated induction of cell death pathways were done in cell lines, suggests that induction of the cell death pathway by TNFRSF25 ligands is not a physiological response but rather a by-product of using cell lines.

1.2.3.2 TNF (ligand) superfamily 15/TNF-like ligand 1A

TNF-like ligand 1A (TL1A) (TNFSF15) is the cognate ligand for TNFRSF25 and TNFRSF6B (184). It was originally identified as vascular endothelial growth inhibitor (VEGI) for its ability to block the proliferation of vascular endothelium (185), with subsequent work done by Migone et al. showing its ligation with and activation of TNFRSF25 (184). TL1A is a type-II membrane
bound protein that can be cleaved at the surface to make a soluble form, though the protease involved is not currently known. The mouse homolog of TL1A consists of 252 aa, while the human TL1A has two splice variants, one of 192 aa, the other 251 aa. The mouse and human sequences share 63.7% homology, and are mapped to chromosome 4, region C1, and the short arm of chromosome 9, position 9q32, respectively. The crystal structure for human TL1A has been resolved and found to exist as a homotrimer (186). TL1A is constitutively expressed in endothelial cells (at significant levels in the vascular endothelium of the kidney and prostate), and can also be induced by IL-1β or TNFα (184, 187). In macrophages and dendritic cells, TL1A expression is highly up-regulated upon cross-linking of FcγR or ligand-mediated activation of TLR4 or TLR11 (149, 154, 188). LPS stimulation or TCR engagement on CD4+ or CD8+ T-cells can induce TL1A expression (149, 189); though at rest both cell types do not express any TL1A. Additionally, it has been found that human monocytes, upon stimulation with immune complexes, up-regulate surface expression of TL1A and release soluble TL1A (190).

1.2.3.3 Multiple roles of TNFRSF25 signaling in immune responses

TNFRSF25 signaling by TL1A has a number of important functions in the immune response. TNFRSF25 stimulation on activated T-cells enhances both proliferation and cytokine production, while blocking its activity suppresses T-cell memory cytokine production in response to restimulation (149, 184). Interestingly, TNFRSF25 knockout mice, like TL1A knockout mice, do not show any gross developmental or phenotypic abnormalities in
lymphoid or non-lymphoid tissues (178). Importantly, the significance of TNFRSF25 signaling by TL1A is quite apparent when studying these knockout mice or TNFRSF25 dominant-negative transgenic mice in various disease models, such as experimental autoimmune encephalitis (a model for multiple sclerosis) (154, 178), allergic asthma (149, 154), and autoimmune arthritis (191). In all of the models, the lack of TNFRSF25 signaling resulted in a reduction of a number of markers related to disease severity. With the use of blocking antibodies for TNFRSF25, a number of different colitis models have also been shown to be dependent on the TNFRSF25:TL1A pathway (162, 192).

TNFRSF25 signaling has been studied in the context of a number of immune pathways. In the setting of Th1 immunity, TNFRSF25 has been found to enhance immune responses in a number of cell types. T-cells, NK cells, and NKT-cells, in the presence of IL-12/IL-18 signaling, increase IFN-γ production in a dose-dependent fashion to TL1A (193). Generally, TL1A enhances Th1 activity by reducing the threshold for TCR-induced activation (189, 194). Additionally, CD8+ T-cells proliferate upon TNFRSF25 signaling and have enhanced differentiation towards becoming cytotoxic T-cells, resulting in enhanced killing of myeloma tumor cells (195).

In Th2 immune responses, TNFRSF25 signaling has been shown to enhance IL-4 and IL-13 production from NKT-cells, IL-4 from activated CD4+ T-cells and is essential for the development and progression of allergic lung inflammation and airway hyperreactivity (149, 154). Additionally, transgenic
expression of TL1A in T-cells resulted in enhanced IL-13 production in the gut, leading to an IBD-like disease in mice (192).

TNFRSF25 signaling in Th17 results in a similar enhancement of immune responses. In vitro work has shown that Th17 differentiation is blocked by TL1A, but enhances the proliferation of previously differentiated Th17 effector cells, as well as increases IL-17 production in those cells. In the setting of experimental autoimmune encephalitis, a Th17-mediated disease model, both TL1A and TNFRSF25 knockouts had reduced cellular infiltration into the spinal cord and better clinical scores (162, 178, 192). In mice with transgenic expression of TL1A on CD11c+ cells, an increased number of IL-17+ cells and up-regulated IL-17 production was found in the gut, which displayed spontaneous goblet cell and paneth cell hyperplasia (196).

The regulatory arm of the adaptive immune response is also affected by TNFRSF25 signaling. TNFRSF25 is constitutively expressed on the surface of CD4+FoxP3+ Treg and stimulation by TL1A or other agonists can expand Tregs both in vivo and in vitro (150, 196)(Khan SQ, Podack ER, unpublished). TNFRSF25 agonists can also reduce the ability of CD4+FoxP3+ Tregs to suppress CD4+ effector T-cell proliferation when present in an in vitro suppression assay (192). TL1A signaling affects peripheral tolerance mechanisms by blocking the induction of FoxP3 during in vitro differentiation of CD4+ naïve cells with APCs and TGF-β. Interestingly, previously expanded Tregs by TL1A or TNFRSF25 agonist, were better suppressors of CD4+ T-cells proliferation in vitro when TL1A was not in the suppression
culture, compared to naïve Treg controls (150)(Khan SQ, Podack ER, unpublished).

1.3 T-REGULATORY CELLS

1.3.1 T-regulatory cells in health

The importance of FoxP3+ T-regulatory cells is easily observed in the clinical pathology associated with the human disease Immune dysregulation, Polyendocrinopathy, Enteropathy, X linked syndrome (IPEX) which is a result of a germ-line mutation in the FoxP3 loci. It is characterized by Treg developmental deficiency or dysfunction that results in a severe, multi-organ, autoimmune response with clinical manifestations that include enteropathy, chronic dermatitis, endocrinopathy, hepatitis, nephritis, and thrombocytopenia (197). Genetically modified mice with mutations within the FoxP3 loci show a similar phenotype and are identified as “scurfy” mice: X-linked recessive inheritance of scaly skin, runting, progressive anemia, thrombocytopenia, leukocytosis and lymphadenopathy, all of which lead to death by three to four weeks of age (198, 199). Beyond IPEX and scurfy mice, other diseases and their animal models have been identified as having deficient numbers or defective Tregs. Tregs are at reduced levels in autoimmune diseases such as juvenile idiopathic arthritis, psoriatic arthritis, hepatitis C-associated mixed cryoglobulinemia, autoimmune liver disease, SLE, and Kawasaki’s disease (as reviewed in (200)). Additionally, deficient Treg function was found in patients with Type-1 diabetes or multiple sclerosis (201, 202). In mouse models of autoimmune diseases and tolerance, Tregs have been found to
play important roles in controlling pathological immune responses (106, 203-205).

1.3.2 FoxP3: Master regulatory transcription factor

Forkhead/winged helix transcription factor 3 (FoxP3) is a nuclear transcription factor that was identified by Fontenot et al. as the key molecule essential for the development of CD4+ Tregs, and has become known as the “master regulatory factor” due to its importance in initiating suppressive mechanisms within the adaptive immune response (206). The induction and stabilization of FoxP3 requires IL-2 signaling via IL-2Rβ and subsequent STAT5 activation (207). The ability for FoxP3 to induce regulatory functions within a cell involves modulating a number of factors, including up-regulation of key suppressive molecules, down-regulation of effector functions, and epigenetic changes to sustain the phenotype (208). The full array of binding partners and sites for FoxP3 has not been fully described, but a number of gene loci are directly affected by FoxP3 activity. Upon FoxP3 induction, expression in the loci for Il2ra, Irf4, Ctla4, and Icos was up-regulated while expression in the loci for Zap70, Il2, and Jak2 were down-regulated (208, 209). Further studies identified specific factors with direct or indirect interactions with FoxP3 which were required for proper development of regulatory function. Irf4 is an essential FoxP3-controlled transcription factor for Tregs in controlling Th2 immune responses (210). AML1/Runx1 has direct contact with FoxP3, and this interaction results in the suppression of IL-2 and IFNγ, and the increased expression of regulatory-associated molecules CTLA-4, GITR, and IL2Rα in Tregs (211).
1.3.3 Natural and induced T-regulatory cells

T-regulatory cells are categorized as either thymically-derived “natural” Tregs (nTreg) with TCR-specificities for self, or peripherally-induced “adaptive or induced” Treg (iTreg) with TCR-specificities for non-self. Thymically-derived nTregs have survived both positive and negative selection in the thymus, with moderate affinity to self-antigen, and have induced FoxP3 transcripts at the late-double positive stage (CD4+CD8+) as a thymocyte (212). These nTregs contribute to self-tolerance in the periphery by controlling any T- and B-cells that survive negative-selection with TCR or BCR specificities for self. Additionally, it has been reported that nTregs play a role in antigen non-specific suppression of immune responses, better known as “bystander suppression” (213). Peripherally-induced iTregs have survived both positive- and negative-selection in the thymus due to TCR-specificities for non-self, and are circulating in the periphery. iTregs are derived from CD4+ naïve T-cells that were induced in secondary lymphoid organs or peripheral tissue sites to stably express the FoxP3 transcription factor. FoxP3 induction and stabilization in CD4+ T-cells requires TCR-engagement, IL-2 and TGF-β, with the presence of retinoic acid enhancing this process. Gut-residing CD103+ dendritic cells express significant amounts of aldehyde dehydrogenase and retinaldehyde dehydrogenase, the two enzymes required for the conversion of Vitamin A to retinoic acid, and have been found to be efficient inducers of iTregs, contributing to the tolerizing environment of the gut mucosa to food and commensal bacteria (97). Currently, there are not any reliable markers delineating between nTregs and iTregs, though it has been suggested that
Helios, an Ikaros transcription family member, potentially could be a marker for nTregs (214). Opposing evidence has recently been reported: Helios could be induced in iTregs, in vitro and in vivo (215, 216).

1.3.4 Suppressive mechanisms

Figure 14: Regulatory T-cell mechanisms of suppression (adapted from (217))

In the context of Treg-mediated suppression of immune responses, a number of molecular mechanisms are involved in this function (Figure 14). The immunosuppressive cytokine IL-10 is known to be quite essential in the control of immune responses and sustaining tolerance to allergens. IL-10 affects a number of cell-types including, DCs, T-cells, and B-cells. On DCs, IL-10 can prevent maturation and reduce co-stimulatory molecules CD80 and CD86 as well as MHC Class-II presentation of antigen (218, 219). IL-10 can
also induce a long-lasting anergic state in CD4+ T-cells, as well as inhibit ongoing T-cell activity (220, 221).

Another potent suppressor of immune responses is TGF-β. TGF-β has a multiplicity of roles in systemic immunoregulation including being essential for the differentiation of peripherally induced Tregs from naïve CD4+ T-cells (93), suppressing Th2 differentiation by blocking GATA3 (222), regulating Th1 differentiation through the inhibition of T-bet (223), and blocking the activation of macrophages (224). It has also been shown that defective TGF-β signaling on effector T-cells blunts the ability of Tregs to suppress autoimmune responses (225).

A common surface marker for CD4+FoxP3+ Tregs, CTLA-4 of the Ig super family, is also a molecule used by Tregs to control immune responses. In genetically modified mice with Tregs lacking CTLA-4, the spontaneous development of a lymphoproliferative disease occurs with their Tregs unable to function in an in vitro suppression assay (226, 227). CTLA-4 ligation of CD80/86 on the surface of DCs results in the up-regulation of indoleamine 2,3 dioxygenase, a potent enzyme that converts tryptophan to kynurenine. The degradation of an essential amino acid creates a nutrient deficient environment that suppresses proliferation of T-cells (228, 229). Additionally, CTLA-4 blocks CD28 engagement of CD80/86 due to CTLA-4’s higher avidity for the surface molecules on DCs (230). This action essentially blocks co-stimulation of TCR-engaged T-cells, likely resulting in anergy induction (231, 232).
IL-2Rα (CD25) was previously the main identifier for CD4+ Tregs, prior to the discovery of FoxP3 as a reliable marker. Though IL-2Rα is considered the low-affinity receptor, it is recognized that Tregs need IL-2 for proliferation and survival (reviewed in (233)). This “need” results in Tregs being a potential “IL-2 sink” in the immune microenvironment, a mechanism of suppression that deprives CD4+ effector T-cells (Teff) of IL-2 resulting in the loss of survival signal and apoptosis of Teff (234).

Recently a new suppressive cytokine produced by Tregs was discovered: IL-35, of the IL-12 family. It is a heterodimeric protein comprised of IL-12 p35 and Ebi-3, and is regulated by the master regulatory transcription factor FoxP3 (235). IL-35 signals on CD4+ naïve and Teff cells via heterodimers or homodimers of IL-12Rβ2 and gp130, and results in the induction of iTregs or suppression of Teff responses, respectively (236, 237). Tregs unable to produce either IL-12p35 or Ebi3 (i.e. IL-35) are less suppressive in vitro, and are unable to control inflammation in a cell-transfer model of IBD (235).

Treg-mediated suppressive mechanisms can be categorized as antigen-specific or antigen-non-specific. This delineation is not necessarily absolute for each mechanism, but a description of how Tregs target their suppression. Antigen-specific suppression is mediated through an APC presenting MHC Class II-restricted antigen to both Tregs and Teff. Treg recognition of its cognate antigen allows targeted suppression through the release of IL-10 or TGF-β into the microenvironment, CTLA-4 engagement of CD80/86 on the APC presenting the specific antigen, or physically blocking proper immune synapse formation between antigen-specific Teff and APCs (reviewed in
All of these mechanisms similarly occur with antigen-non-specific suppression, but does not require antigen-specific engagement by the Treg. Antigen-specific suppression by Tregs is quantitatively better than antigen-non-specific suppression, in a dose dependent manner, likely due to efficient and direct delivery of immunosuppression to activated Teff (239).

Allergic asthma has been shown to be loss of tolerance to allergen, often times due to reduced numbers of antigen-specific Tregs, or reduced functionality of Tregs. The lack of immune control by Tregs leads to the development of the hallmarks of allergic asthma: allergic lung inflammation, airway hyperreactivity, and airway remodeling. Therapies that increase the numbers of antigen-specific Tregs, specifically at sites of allergen exposure, represent an immunotherapy with promise to be quite successful in the clinic.
CHAPTER 2: RESULTS

Allergic asthma is a chronic disease that affects a significant part of our population, and is primarily treated with therapies targeted to reduce symptoms and progression of the disease. Tolerance induction, employing specific-allergen immunotherapy, has shown to be effective as a long-term solution to atopic responses to allergen and is associated with an increase in circulating CD4+FoxP3+ T-regulatory cells (Tregs). These results from clinical studies, combined with the work in mouse models of allergic asthma, suggests that any treatment modality that increases the frequency of circulating Tregs are likely able to affect a number of immunopathological parameters associated with allergic asthma. The discovery that in vivo signaling of the TNFRSF25 pathway with monoclonal agonistic antibody can robustly expand Tregs has presented a novel tool to deliver Tregs without ex vivo isolation and delivery, and is an ideal candidate for expanding circulating and tissue-resident Tregs in models of allergic lung inflammation, a hallmark of allergic asthma. We hypothesize that this expansion of Tregs by TNFRSF25 agonists, in the setting of allergic lung inflammation, can suppress markers of immunopathology in both the acute and chronic setting.

2.1 TNFRSF25 SIGNALING IN AN ACUTE MODEL OF ALLERGIC LUNG INFLAMMATION

The work by Dr. Schreiber and Dr. Wolf presented a novel technique for the expansion of CD4+ FoxP3+ T-regulatory (Treg) cells by in vivo signaling with an anti-TNFRSF25 agonistic antibody (clone 4C12) (240). To further
characterize the expanded Tregs, in vivo suppression was evaluated in a disease model. Based on previous work by a number of groups using ex vivo Treg harvesting, collection, and re-infusion as a therapy to prevent allergic asthma (105, 241, 242), and our own labs familiarity with the model, a treatment protocol using in vivo TNFRSF25 signaling to pre-expand Tregs for suppressing allergic lung inflammation was developed and tested in the lab (Figure 15).

Figure 15: Hypothetical model for TNFRSF25-mediated expansion of T-regulatory cells in the setting of acute allergic asthma

2.1.1 In vivo TNFRSF25 signaling expands T-regulatory cells in ovalbumin-sensitized mice

Figure 16: Acute model of allergic lung inflammation.
To properly evaluate the in vivo suppressive capacity of TNFRSF25-expanded Tregs, the acute model of allergic lung inflammation that had previously been used in the lab by both Fang et al., and Xiao et al. was employed (149, 243). Expansion of Tregs prior to sensitization would likely result in tolerance to ovalbumin rather than normal sensitization, therefore anti-TNFRSF25 agonistic antibody (clone 4C12) was injected i.p. after sensitization to ovalbumin with ALUM adjuvant, and peripheral blood was collected and analyzed to determine if Tregs would specifically be expanded after allergen sensitization. Due to the known co-stimulatory effects of TNFRSF25 signaling on activated CD4+ T-effector cells (Teff) (149, 184), an allergen-free period prior to TNFRSF25 signaling was created in the protocol (Figure 16), with the expectation that signaling in a non-inflammatory setting would allow for specific expansion of Tregs. These expectations are based on work by Dr. Schreiber using TNFRSF25 signaling in naïve mice and work by Ruby et al. in a murine model of multiple sclerosis using an agonist for OX40, also a TNF-receptor superfamily member (148, 150). Anti-TNFRSF25 agonistic antibody (clone 4C12), or isotype control IgG from Armenian hamster, was injected on day 12, seven days after the second ovalbumin/ALUM injection. Using FoxP3-RFP reporter mice (courtesy of Dr. Flavell (244)), the peripheral blood percentage of CD4+FoxP3+ Tregs was monitored. Similar to what was observed in naïve mice, ovalbumin-sensitized mice given 4C12 on day 12 showed a significant expansion in the percentage of CD4+FoxP3+ Tregs within the CD4+ gate, compared to control IgG-treated
mice, peaking four days after the injection of 4C12, at approximately 50% of the total CD4+ gate (Figure 17).

Figure 17: Analysis of the peripheral blood mononuclear cells of control IgG- and 4C12-treated mice to determine the percentage of FoxP3(+) cells within the CD4(+) gate. Blood was drawn on day 12 (prior to i.p. injection), 13, 14, 15, 16 (prior to aerosolization) and 19. (Black arrow indicates when control IgG or 4C12 was injected; Red arrow indicates day of aerosolization). (* = p<.05, student’s T-test) (n= 3 – 4)

2.1.2 After pre-expansion of Tregs by in vivo TNFRSF25 signaling in sensitized-mice, subsequent airway eosinophilia is reduced after aeroallergen exposure

At the peak of Treg expansion, day 12 (four days after 4C12 administration), 0.5% w/v ovalbumin in saline was aerosolized using the BANG nebulizer (CH Technologies) into a Jaeger-NYU Nose-Only Directed-Flow Inhalation Exposure System (CH Technologies). Mice were aerosolized for one-hour, and then three days later at the peak of eosinophilia, mice were sacrificed and analyzed for a number of immunopathological parameters. Mice were lavaged with PBS, and the cells collected in the fluid were counted and analyzed for the percentage of eosinophils in the total cells collected. Subsequently, the total number of eosinophils in the bronchoalveolar lavage fluid (BALF) was calculated from these measurements. 4C12-treated mice showed significantly less eosinophilia within the BALF, as measured by
percent eosinophils in total BALF and number of eosinophils recovered, compared to control IgG-treated mice (Figure 18).

Figure 18: Bronchoalveolar lavage fluid (BALF) was collected on day 19, three days after ovalbumin aerosolization. (Left) The percentage of eosinophils in total cells recovered and (Right) the absolute number of eosinophils recovered is shown (* = p<.05, 1-way ANOVA, Tukey post-test, with respect to groups indicated) (n= 3 – 10)

2.1.3 Histopathology associated with allergic lung inflammation is reduced in mice administered TNFRSF25 agonistic antibody prior to aerosolization

Immune-mediated histopathology associated with allergic lung inflammation includes perivascular and peribronchial leukocytosis in addition to goblet cell hyperplasia and mucus hypersecretion. To evaluate these disease parameters in our treatment model, histological slides were made from formalin-fixed, paraffin-embedded lung samples procured at day 19. Control IgG-treated mice, which were sensitized and aerosol exposed to ovalbumin, showed significant mononuclear cell accumulation in the perivascular and peribronchial areas of the lung (Figure 19). Conversely, 4C12-treated mice showed significant reductions in mononuclear cell accumulation in the perivascular and peribronchial areas. Mucus hypersecretion in goblet cells is histologically identified using Periodic Acid-Schiff (PAS) staining for polysaccharides in bronchiolar epithelium. Histology slides from control IgG-
treated mice sacrificed on day 19 were stained with PAS and showed a significant number of PAS-positive nuclei in the bronchioles. Subjective evaluation of PAS stained slides of lungs from 4C12-treated mice on day 19 showed a minimal presence of PAS-positive nuclei, similar to what was observed in naïve airways (Figure 19).

Figure 19: Images from formalin-fixed, paraffin-embedded lung samples from PBS-aerosolized mice and ovalbumin-sensitized/aerosolized mice treated with control IgG or 4C12 analyzed on day 19. Representative images of (Top Row) Hematoxylin and Eosin stained or (Bottom Row) Periodic Acid-Schiff stained slides are shown (200x).

Quantitative analysis for the degree PAS staining in the bronchiolar epithelium was accomplished using ImageJ software. In the lungs of ovalbumin-sensitized and aerosolized mice treated with control IgG, a significant increase in the number of PAS-positive cells in the airways was observed, compared to naïve controls. Lungs procured from ovalbumin-sensitized and aerosolized mice treated with 4C12, showed a significant reduction in the number of PAS-positive cells in the bronchioles (Figure 20), with no difference compared to naïve controls. These data indicates that TNFRSF25-mediated
pre-expansion of Tregs was able to prevent the histopathology associated with allergic lung inflammation.

Figure 20: Images from PAS-stained slides were quantified using ImageJ software as described in Methods. The number of PAS positive cells per bronchiole in PBS-aerosolized mice, OVA-aerosolized and control IgG-treated, and OVA-aerosolized and 4C12-treated mice is shown. (*** = p<.05, 1-way ANOVA, Tukey post-test, with respect to groups indicated) (n= 3 – 4)

2.1.4 Th2 cytokine mRNA expression levels are reduced after TNFRSF25-mediated expansion of Tregs

To further investigate if TNFRSF25-mediated expansion of Tregs has any effect on the production of Th2 cytokines in the lung, mRNA expression of Th2 cytokines was quantified using real-time RT-PCR from the lungs of mice analyzed on day 19. The Th2 cytokines, IL-4, IL-5, and IL-13, all had an increase in the number of transcripts in the lungs of ovalbumin-sensitized and aerosolized mice treated with control IgG, when compared to non-aerosolized controls. Conversely, all three cytokines were significantly reduced in the lungs of ovalbumin-sensitized and aerosolized mice treated with 4C12, when compared to their control IgG-treated counterpart (Figure 21). FoxP3 mRNA expression levels were significantly increased in 4C12-treated mice, compared to control IgG-treated mice, reflecting a similar fold increase observed in flow cytometry analysis of the lungs (Figure 21 and Figure 22).
2.1.5 TNFRSF25 signaling increases Tregs at sites of allergen exposure

In the analysis of the lung, spleen, and mesenteric lymphnode, significant increases in the percentage of CD4+FoxP3+ Tregs at each site were observed in 4C12-treated mice, compared to control IgG-treated mice (Figure 22). Interestingly, the highest percentages occurred in the lung, followed by the spleen, and then the mesenteric lymphnode. This would suggest that Tregs were either proliferating at a higher level at the major site of allergen exposure (lungs), or were homing and entering at a higher level to the sites of inflammation and allergen entry; though neither is mutually exclusive, and a combination likely a possibility.
Figure 22: Total frequencies of FoxP3(+) cells within the CD4(+) gate for the (Left) lungs, (Middle) spleens, and (Right) mesenteric lymphnodes from mice analyzed on day 19. (* = p<.05, 1-way ANOVA, Tukey post-test, with respect to group indicated) (n= 3 – 10) (**) = p<.05, student’s T-test) (n= 3 – 4)

To determine if Tregs were proliferating in the tissue sites, intracellular Ki67 staining, a marker for cellular proliferation, was analyzed in the CD4+FoxP3+ Treg populations in various tissues procured on day 19. Lungs showed the highest percentage of Ki67 staining, in both control IgG- and 4C12-treated mice, compared to the spleen and mesenteric lymphnode, indicating allergen aerosolization leads to enhanced proliferation of Tregs at the site of allergen introduction (Figure 23). In all of the tissue sites, 4C12-treated mice showed a significant increase in the percentage of proliferating Tregs, compared to control IgG-treated mice (Figure 23).

Increased homing and migration of CD4+FoxP3+ Tregs could also contribute to the enhanced presence of Tregs by TNFRSF25 signaling. Messenger RNA expression of CCL17, CCL22, and CCR4 in RNA extracted from lungs on day 19 showed no significant differences between control IgG- and 4C12-treated mice (data not shown). These chemokines are important in the attraction and migration of CD4+FoxP3+ Tregs and CD4+FoxP3- Teff to the lung (245), and surface staining for CCR4 and CCR8 on circulating and
lung-residing T-cell subsets would likely be more informative than mRNA expression levels in the whole lung.

Figure 23: Total frequency of Ki67(+) cells within the CD4(+) FoxP3(+) Treg population from the (Left) lungs, (Middle) spleens, and (Right) mesenteric lymphnodes of mice analyzed on day 19. (* p<.05, student’s T-test) (n= 3 – 4)

2.1.6 TNFRSF25 signaling with simultaneous aeroallergen exposure results in enhanced allergic lung inflammation

Figure 24: Modified acute model of allergic lung inflammation with TNFRSF25 stimulation on the day of allergen aerosolization. Similar to the aforementioned acute model of allergic lung inflammation, in the modified protocol, either 20 µg control IgG or 20 µg 4C12 was injected i.p. on day 16, five minutes prior to aerosolization with 0.25% w/v ovalbumin in PBS for one-hour. Mice were sacrificed and bronchoalveolar lavage fluid cells analyzed on day 19.

Previous work by Fang et al., as well as work by other labs, showed that TNFRSF25 signaling enhances immune responses with TL1A acting primarily
as a co-stimulator of inflammation (149, 154, 162, 189, 193, 246). Interestingly, in our studies in the acute model of allergic lung inflammation, in vivo TNFRSF25 signaling during a non-inflammatory setting specifically expands CD4+FoxP3+ T-regulatory cells. Our hypothesis is that TNFRSF25 signaling, similar to OX40 signaling, can have dual effects on the immune system depending on the inflammatory setting (148). To confirm that TNFRSF25 signaling during the inflammatory setting enhances inflammation as hypothesized, a modified protocol to the acute model of allergic lung inflammation was developed. Instead of administering anti-TNFRSF25 agonistic antibody (4C12) on day 12, ovalbumin-sensitized mice were injected i.p. on day 16 with 4C12 or control IgG, 5 minutes prior to aerosolization with 0.25% w/v ovalbumin in PBS (Figure 24). On day 19, mice were analyzed for eosinophilia in the bronchoalveolar lavage fluid. As expected, mice given 4C12 on the day of aerosolization had an increased percentage and number of eosinophils in the BALF, when compared to control IgG-treated mice (Figure 25). Similar enhancement of inflammation was observed in a model of corneal infection with HSV when 4C12 was given 6 days post-infection, while 4C12 given two days prior to infection prevented immunopathology in the eye (Reddy P, Podack ER, unpublished).

Figure 25: Bronchoalveolar lavage fluid (BALF) was collected on day 19 three days after control IgG- or 4C12 injection followed by ovalbumin aerosolization. (L) The percentage of eosinophils in total cells recovered and (R) the absolute number of eosinophils recovered is shown (* = p<.05, student’s T-test) (n= 3)
2.1.7 TL1A-Ig expands CD4+ Tregs in ovalbumin-sensitized mice

Figure 26: Acute model of allergic lung inflammation with TL1A-Ig. Similar to the acute model of allergic lung inflammation described above, ovalbumin-sensitized mice were injected i.p. on days 11, 12, and 13 with either 100 µg mouse isotype control IgG or 100 µg TL1A-Ig. Mice were subsequently aerosolized with 0.5% w/v ovalbumin in PBS for one-hour on day 16. Mice were sacrificed and analyzed on day 19.

4C12, the hamster anti-mouse antibody that has agonistic properties for TNFRSF25, is able to expand CD4+FoxP3+ Tregs in both naïve and ovalbumin-sensitized mice. To confirm that this result is not an off-target effect of the specific antibody clone but an effect of in vivo TNFRSF25 signaling, a TL1A fusion protein was developed in the lab (Khan SQ, Podack ER, unpublished). The fusion protein consists of the extracellular signaling domain of mouse TL1A, the natural ligand for TNFRSF25, connected to the Fc portion of mouse IgG1 (including the hinge-region). TL1A-Ig, due to its shorter biological half-life (Khan SQ, Podack ER, unpublished), requires 100 µg injected i.p. on three consecutive days to get an equivalent expansion of Tregs as a single i.p. injection of 20 µg 4C12. A slightly modified therapeutic protocol was developed based on this finding (Figure 26). TL1A-Ig or mouse isotype control IgG was administered to ovalbumin-sensitized mice on days
11, 12, and 13, with subsequent aerosolization on day 16, and sacrifice and analysis on day 19. TL1A-Ig-treated mice showed an expansion of CD4+FoxP3+ Tregs in the peripheral blood to levels similarly observed when 4C12 was given on day 12 (Figure 27). Analysis of the lungs and spleens showed CD4+FoxP3+ Tregs are also significantly expanded in the tissues by TL1A-Ig, compared to control IgG (Figure 27).

2.1.8 TL1A-Ig expanded Tregs is associated with suppressed airway eosinophilia and goblet cell hyperplasia after aeroallergen exposure

Analysis of bronchoalveolar lavage fluid (BALF) recovered cells from TL1A-Ig-treated mice sacrificed on day 19 showed results similar to that observed with treatment with anti-TNFRSF25 agonistic antibody, 4C12. The percentage and number of eosinophils recovered in total cells from the BALF was significantly lower in TL1A-Ig-treated mice, compared to control IgG-treated mice (Figure 28).

Figure 27: (Left) Analysis of the peripheral blood mononuclear cells of control IgG- and TL1A-Ig-treated mice to determine the percentage of FoxP3(+) cells within the CD4(+) gate. Blood was drawn on day 11 (prior to i.p. injection), 12, 13, 14, and 15. (Black arrows indicates when control IgG or TL1A-Ig was injected). (* = p<.05, student’s T-test) (n= 3 – 6). (Right) Total frequencies of FoxP3(+) cells within the CD4(+) gate for the lungs and spleens of mice analyzed on day 19. (** = p<.05, 1-way ANOVA, Tukey post-test, with respect to group indicated) (n= 6 – 8)
Figure 28: Bronchoalveolar lavage fluid (BALF) was collected on day 19, three days after aerosolization. (Left) The percentage of eosinophils in total cells recovered and (Right) the absolute number of eosinophils recovered is shown (* = p<.05, 1-way ANOVA, Tukey post-test, with respect to group indicated) (n= 3 – 8)

To evaluate if histopathological parameters were also affected by TL1A-Ig-mediated expansion of Tregs prior to allergen aerosol, formalin-fixed lung samples from mice analyzed on day 19 were embedded in paraffin and sectioned for H&E and PAS staining. H&E stained slides show significant

Figure 29: Images from formalin-fixed, paraffin-embedded lung samples from ovalbumin-sensitized/aerosolized mice treated with mouse isotype control IgG or TL1A-Ig analyzed on day 19. Representative images of (Top Row) Hematoxylin and Eosin stained or (Bottom Row) Periodic Acid-Schiff stained slides are shown (200x)
perivascular and peribronchial cellular accumulation in control IgG-treated mice, with a slight reduction in TL1A-Ig-treated mice (Figure 29). PAS-staining, a marker for mucus production in goblet cells, showed a large number of PAS positive cells within the bronchiolar epithelium in control IgG-treated mice. Similar to 4C12-treated mice, TL1A-Ig-treated mice show significant reductions in the number of PAS positive cells within the bronchiolar epithelium compared to control IgG-treated mice (Figure 29 and Figure 30). CD4+FoxP3- T-cells population was not significantly increased in TL1A-Ig-treated mice when compared to mouse isotype control IgG-treated mice, similar to what was observed in 4C12-treated mice compared to hamster isotype control IgG-treated mice (Figure 31), suggesting TL1A-Ig, like 4C12, administered during the non-inflammatory setting specifically expands Tregs without any significant off-target effects.
2.1.9 CD4+CD25+ depletion of Tregs by PC61

To determine the importance of CD4+CD25+ putative Tregs in the suppression of allergic lung inflammation by 4C12-mediated expansion of Tregs, anti-CD25 (IL-2Rα) depleting antibody, PC61, was used to deplete CD25+ cells in vivo. This experimental approach has been used by number of groups to deplete Tregs in mouse models of allergic asthma (100, 241, 242). Briefly, mice were sensitized with ovalbumin adsorbed to ALUM on day 0, followed a by a boost on day 5. On days 8 and 9, PC61 or rat isotype control IgG was administered by i.p. injection. On day 12 mice were given either 4C12 or hamster isotype control IgG, followed by ovalbumin aerosol exposure on day 16, and sacrificed and analyzed on day 19 (Figure 32).
Peripheral blood was monitored throughout to confirm that PC61 administration properly depleted Tregs (Figure 33A).

**Figure 33:** A) Analysis of the peripheral blood mononuclear cells of control IgG- and PC61-treated mice to determine the percentage of CD25(+) FoxP3(+) cells within the CD4(+) gate from blood drawn on day 11. (** = p<.05, student’s T-test) (n= 4 – 6). Time-course following B) total percentage of FoxP3(+) cells in the CD4(+) gate or C) CD25(low/negative)FoxP(+) cells in the CD4(+) gate. Mice shown have been treated with either rat IgG then hamster IgG (IgG/IgG), rat IgG then 4C12 (IgG/4C12), PC61 then hamster IgG (PC61/IgG), or PC61 then 4C12 (PC61/4C12). (* = p<.05, 1-way ANOVA, Tukey post-test, IgG/IgG and IgG/4C12 vs. PC61/IgG and PC61/4C12) (**) = p<.05, 1-way ANOVA, Tukey post-test, IgG/4C12 and PC61/4C12 vs. PC61/IgG and PC61/IgG) (n= 4 – 6)

As expected, PC61 administration depleted CD4+CD25+FoxP3+ Tregs by ~68% in the peripheral blood by day 11, though a percentage of CD4+CD25low/negative FoxP3+ Tregs remained. Three days after 4C12 administration, CD4+FoxP3+ population expanded to 15% of the CD4+ gate in the peripheral blood of PC61 pre-treated mice, while control IgG-treated mice continued to have a low percentage of CD4+FoxP3+ in PC61 pre-treated mice. 4C12 administration in PC61-depleted mice expanded the residual CD4+CD25+FoxP3+ Tregs that survived depletion, as well as CD4+CD25low/negative FoxP3+ Tregs in the peripheral blood (Figure 33B and Figure 33C). Analysis of spleens and lungs of mice on day 19, three days after ovalbumin aerosol, showed that control IgG-treated mice with PC61
pre-treatment (PC61/IgG) showed a reduced percentage of CD4+FoxP3+ Tregs, compared to control IgG-treated non-PC61-depleted mice (IgG/IgG) (Figure 34). Mice treated with 4C12 after PC61-depletion (PC61/4C12) showed significant increases in the percentage of Tregs in both the spleens and lung compared to PC61-depleted control IgG-treated mice (PC61/IgG) (Figure 34). This level of expansion of Tregs in PC61-depleted mice by 4C12 was unexpected, and indicates that an alternative Treg depletion strategy is needed.

Figure 34: (Left) Splenocytes and (Right) lung-derived lymphocytes were analyzed on day 19 for the percentage of CD25(+) FoxP3(+) and CD25(low/negative) FoxP3(+) within the CD4(+) gate. Mice shown have been treated with either rat IgG then hamster IgG (IgG/IgG), rat IgG then 4C12 (IgG/4C12), PC61 then hamster IgG (PC61/IgG), or PC61 then 4C12 (PC61/4C12).

2.2 TNFRSF25 SIGNALING IN A CHRONIC MODEL OF ALLERGIC ASTHMA AND AIRWAY REMODELING

We have shown evidence that in the acute allergic lung inflammation model in vivo pre-expansion of CD4+FoxP3+ regulatory T-cells (Tregs) using TNFRSF25 agonists could prevent subsequent allergic lung inflammation. As discussed previously in the background, the ideal mouse model for evaluating the efficacy of a therapy for allergic asthma is one that involves repeated exposure of sensitized mice to allergen. There exists a number of different
chronic models of allergic asthma that reflect both the Th2-biased allergic lung inflammation as well as airway remodeling observed in asthmatic patients.

Figure 35: Hypothesized model for TNFRSF25-mediated expansion of T-regulatory cells in a chronic model of allergic lung inflammation and airway remodeling

To further assess the possible clinical application of TNFRSF25-mediated expansion of Tregs in allergic asthma (Figure 35), a chronic model of allergic lung inflammation and remodeling was developed in the lab; a model largely based on one developed in the Lab of Dr. Clare Lloyd, which was previously used to evaluate the clinical efficacy of infusing Tregs after established lung inflammation in mice (102, 137).
2.2.1 Anti-TNFRSF25 agonistic antibody mediates expansion of CD4+FoxP3+ T-regulatory cells in a chronic model of allergic lung inflammation

Most importantly, it needed to be determined if anti-TNFRSF25 agonistic antibody, 4C12, could expand Tregs in a chronic model of allergic lung inflammation. To examine this, 4C12 or control IgG was given after established chronic lung inflammation. The protocol for our chronic model starts with sensitization of the mice with ovalbumin adjuvanted with ALUM (on day 0 and 12), followed by 5 daily one-hour aerosolization of ovalbumin in PBS beginning on day 18 and then three times per week for two weeks (Figure 36). After one-week rest, mice are injected i.p. on day 41 with 20 µg of anti-TNFRSF25 agonistic antibody (4C12). Cells from FoxP3-RFP reporter mice (courtesy of Dr. Flavell, (244)) were used to monitor the level of CD4+FoxP3+ Tregs in the peripheral blood, spleen, lung, and lymphnodes.
4C12 injected on day 41 stimulated expansion of Tregs in the peripheral blood, spleen, lung, and bronchial lymph nodes (Figure 37 and Figure 38).

**Figure 37:** Expansion of peripheral blood Tregs in a chronic model of allergic lung inflammation. Analysis of the peripheral blood mononuclear cells of control IgG- and 4C12-treated mice to determine the percentage of FoxP3(+) cells within the CD4(+) gate. Blood was drawn on days 22, 29, 41, 45, 46, 53, and 60 (* = p < 0.05, student's T-test) (n = 5 – 11).

**Figure 38:** Representative flow plots of FoxP3 staining within CD4(+) gate for lungs, spleen, and bronchial lymph nodes from naïve, control IgG- and 4C12-treated mice analyzed on day 49 (early). Numbers within the quadrants indicate the percentage of cells stained positive for FoxP3.
The peak of Tregs in peripheral blood lymphocytes occurred on day 45, four days after 4C12 administration, with significant expansion of Tregs in the lung, spleen and bronchial lymphnode by day 49 (Figure 39A). A similar expansion of CD4+FoxP3+ Tregs was observed using TL1A fusion protein (TL1A-Ig) on day 49 (Figure 40). On day 61, the end-point of the experiment, there was not a significant difference observed in the percentage of Tregs in peripheral blood lymphocytes, lung, or spleen (Figure 39B).
2.2.2 TNFRSF25 signaling results in situ proliferation of Tregs in lungs and bronchial lymph nodes, with a concomitant increase in antigen-experienced and activated Tregs

On day 49, eight days after administration of 4C12 or control IgG and after two aerosol exposures, the lungs and bronchial lymphnodes were analyzed to further characterize the activation and proliferation states of the expanded Tregs. Ki67, an intracellular marker for a proliferating cell, was measured within the CD4+FoxP3+ Treg population in both the lung and bronchial lymphnodes of control IgG-treated and 4C12-treated mice. The percentage of Ki67+ Tregs was significantly increased in the lungs and bronchial lymphnodes of 4C12-treated mice, when compared to control IgG-treated mice (Figure 41). This would indicate that part of the increased percentage and number of Tregs within the lung and bronchial lymphnode could be attributed to in situ proliferation. The presence of a large circulating population of Tregs in the peripheral blood indicates that increased homing of Tregs to sites of inflammation likely also contributes to the significant Treg presence within both the lungs and bronchial lymphnodes.

Additional phenotyping of lung infiltrating Tregs on day 49 showed that 4C12-treated mice had an increased number of lung CD44+ Tregs as well as
lung CD69+ Tregs (Figure 42), markers for antigen experience and recent activation by antigen, respectively. On day 61, no differences were observed in Ki67, CD69, or CD44 staining on Tregs between the treatment groups (data not shown).

2.2.3 Airway eosinophilia associated with chronic allergic lung inflammation is reduced in mice treated with TNFRSF25 agonist

Presence of eosinophils in the alveolar space of the lung is characteristic of allergic lung inflammation, and is easily measured by counting cells recovered from bronchoalveolar lavage. During the one-week rest period, eosinophil levels in BALF collected from mice on day 34 declined and remained low in 4C12-treated mice even when resuming ovalbumin-aerosolization on day 45 (Figure 43A and Figure 43B). In contrast, in control IgG-treated mice, renewed aerosolization mediated increased eosinophils in BALF, which was associated with increased NKT (CD3+ NK1.1+) levels in the spleen. In 4C12-treated mice NKT-cell frequency remains significantly lower, similar to the level in ovalbumin-naïve controls (Figure 43C).
2.2.4 Therapeutic expansion of T-regulatory cells results in suppressed Th2 cytokine responses

Th2 cytokine levels in lungs were determined by real-time PCR on mRNA extracted from flash-frozen lungs. At day 49, eight days after injection of either control IgG or 4C12 and after two aerosol exposures, control IgG-treated mice had increased levels of mRNA transcripts for IL-5, IL-13, and CCL-11 (eotaxin-1), compared to non-aerosolized controls (Figure 44A). At the same time point, 4C12-treated mice showed significantly lower levels in IL-5, IL-13, and CCL11 with a significant increase in FoxP3 transcripts, corresponding to the increase of FoxP3+ cells observed by flow cytometry of lung parenchymal lymphocytes compared to IgG-treated mice (Figure 44A). On day 61, twenty days after treatment, IL-4, IL-5, IL-13, IL-25 and CCL11 (eotaxin-1) transcript levels were still increased in control IgG-treated mice compared to non-aerosolized control. In contrast, 4C12-treated mice demonstrated significant reductions in the transcript levels for all of the Th2-
associated molecules (Figure 44B). No differences were noted in levels of IL-33, TSLP, IL-10, Ebi-3 (a component of IL-35), and TGF-β (data not shown). At this late time point, FoxP3 expression levels were not significantly different between the treatment groups consistent with the flow cytometric data. Taken together, the cytokine data support the hypothesis that the chronic model of allergic lung inflammation is associated with a Th2-dominated inflammatory response. 4C12-mediated in vivo expansion of Tregs significantly reduced allergic inflammation even after chronic inflammation had been established.

Figure 44: Total RNA was isolated from the lungs and cells recovered in the bronchoalveolar lavage fluid (BALF) and used in real-time RT-PCR. Expression of key inflammatory and regulatory molecules in the 4C12- or control IgG-treated mice analyzed at A) day 49 and B) and C) day 61, is shown relative to non-aerosolized naïve mice. (* = p<.05, student t-test) (n= 3 – 7)
Messenger RNA extracted from BALF recovered cells were also evaluated for quantitative expression of Th2 and regulatory-associated cytokines. On day 61, IL-5 and IL-13 transcripts were up-regulated in BALF recovered cells in control IgG-treated mice with chronic allergen challenge, while both cytokines were down-regulated in 4C12-treated mice. BALF recovered cells are enriched for lymphocytes compared to whole lung mRNA, which includes large numbers of endothelial and epithelial elements. Differences between BALF mRNA transcripts varying at low levels in the lymphocyte compartment may be easier to quantify using BALF cells due to the improved signal-to-noise ratio. Specifically, this was observed with IL-10, TGF-β, and Ebi-3, three molecules known to have roles in the suppression of inflammatory responses by Tregs and other cell types (112, 115, 235, 247). All three regulatory cytokines were significantly increased the BALF collected on day 61 in mice treated with 4C12, compared to BALF collected on the same day in control IgG-treated mice (Figure 44C).

2.2.5 Reduction of airway mucus and reversal of airway remodeling

Histopathological changes were evaluated in lungs from mice treated with control IgG or 4C12 on day 41 and day 61 of the protocol. Hematoxylin and eosin stained lung sections obtained on day 61 show general perivascular and peribronchial cellular infiltration of the lung parenchyma in control IgG-treated mice (Figure 45). Additionally, periodic acid-Schiff (PAS) staining of lung sections, a marker for mucus hypersecretion/goblet cell metaplasia, was increased on day 61 in control IgG-treated mice, consistent with chronic lung inflammation (Figure 45).
Qualitative observations and objective quantification (using ImageJ) of histological slides from 4C12-treated mice reflected a reduction of perivascular and peribronchial cellular infiltration as well as the amount of PAS staining in the bronchial epithelium as compared to IgG-treated mice, while no difference was apparent between naïve and 4C12-treated mice (Figure 45 and Figure 46).

One characteristic of chronic allergic asthma with many pathophysiological sequelae is airway remodeling (2), observed as increased subepithelial deposition of collagen. To measure the amount of collagen in the subepithelial space, we stained with Sirius Red-Picric acid (248) and quantified the percentage area of Sirius Red staining using ImageJ software (derived from (249)).
The qualitative and quantitative results show that chronically challenged mice had significant increases in collagen deposition by day 41, compared to naïve controls (Figure 46 and Figure 47). Treatment with control IgG had no effect on already established collagen deposition, but 4C12-treated mice showed a
significant reduction of collagen on day 61 compared to day 41 and to control IgG-treated mice on day 61 (Figure 46 and Figure 47). Thus, in vivo Treg expansion by anti-TNFRSF25 agonistic antibody treatment during chronic allergic lung inflammation is associated with a significant reversal of airway remodeling.

2.2.6 Suppression of eosinophilia is not dependent on IL-10 signaling

The qPCR data of mRNA from bronchoalveolar lavage fluid cells as well as previous studies regarding the role of Tregs in airway inflammation and allergy (103, 112, 120, 247) suggests that IL-10 plays an important role in the suppression of allergic lung inflammation. To test this hypothesis in our treatment model, IL-10 receptor blocking antibody (1B1.3A, BioXcell), was administered at a similar dose and frequency as used in another model of Treg-mediated suppression of allergic asthma (103).

First, the bioactivity of IL-10 receptor blocking antibody, clone 1B1.3A, was evaluated. RAW264.7 cells, a macrophage-like cell line, up-regulates surface expression of CD40 upon stimulation with LPS, a process blocked by IL-10 signaling (250). To test the biological activity of 1B1.3A to block IL-10

Figure 48: RAW264.7 cells were analyzed for CD40 surface expression after being cultured in media only (unstimulated), LPS+rat IgG, LPS+IL-10+rat IgG, and LPS+IL-10+1B1.3A for 12 hours. (* = p<.05, 1-way ANOVA, Tukey post-test, with respect to groups indicated)
signaling in cells, in vitro cultures of LPS-stimulated RAW264.7 cells were incubated for 12 hours with either IL-10 and rat isotype control IgG, IL-10 with 1B1.3A, or rat isotype control IgG only, in culture media. Surface expression of CD40 was analyzed by flow cytometry, in triplicate, for each of the treatment groups and for un-stimulated RAW264.7 cells. As expected, LPS-stimulation greatly increased the amount of surface CD40 expression on RAW264.7 cells, compared to un-stimulated cells, while the presence of IL-10 in the culture was able to suppress the surface up-regulation of CD40. When IL-10 receptor blocking antibody, 1B1.3A, was present, IL-10 activity was blocked and RAW264.7 cells had increased CD40 surface expression in response to LPS stimulation (Figure 48).

![Diagram](image)

**Figure 49:** The protocol for IL-10 receptor blocking is similar to the therapeutic protocol for chronic allergic lung inflammation and airway remodeling as in Figure 1, but IL-10 receptor blocking antibody, 1B1.3A, or rat isotype control IgG was administered i.p. 30 minutes prior to each aerosolization, starting on day 45 and continued till day 58.
In a modified version of our therapeutic model chronic allergic lung inflammation, IL-10 receptor-blocking antibody (1B1.3A, BioXcell), or rat IgG isotype control antibody (BioXcell), was injected prior to each aerosolization in the last two weeks (Figure 49).

Figure 50: Analysis of the peripheral blood mononuclear cells mice treated with hamster isotype control IgG followed by mouse isotype control IgG (IgG+IgG), 4C12 followed by mouse isotype control IgG (4C12+IgG), and 4C12 followed by 1B1.3A, was done to determine the percentage of FoxP3(+) cells within the CD4(+) gate. Blood was drawn on day 50. (* = p<.05, student’s T-test) (n= 2 – 3)

Peripheral blood monitoring of Tregs as well as analysis of lungs and spleens showed no differences in Treg expansion with administration of IL-10 receptor blocking antibody in 4C12-treated mice (Figure 50). Analysis of the percent and number of eosinophils recovered in the BALF on day 61 revealed that eosinophilia did not differ between mice treated with a combination of 4C12 and 1B1.3A versus a combination of 4C12 and control rat IgG (Figure 51). Both of these groups had significantly reduced eosinophilia compared to mice treated with the dual control antibody combination (hamster IgG and rat IgG) (Figure 51). These data indicate that antibody mediated blockade of IL-10 receptor does not affect the observed inhibition of airway eosinophilia by 4C12-expanded Tregs, suggesting other suppressive mechanisms.
2.2.7 Manipulation of TNFRSF25 signaling does not confer enhanced suppression compared to TNFRSF25-mediated Treg expansion therapy alone

Our previous hypothesis for TNFRSF25 signaling in allergic asthma focused on its role in the co-stimulation of NKT-cells and CD4+CD44+FoxP3- Teffector cells (Teff) (149). These previous studies in the lab used an anti-TL1A blocking antibody (hamster anti-mouse IgG, clone L4G6) as a therapy to suppress allergic lung inflammation, primarily blocking TNFRSF25 signaling on NKT-cells, while likely playing a role in blocking co-stimulation of CD4+ Teff and relieving the suppression on de novo Treg differentiation and activity (149, 192, 196, 246). By combining the therapeutic expansion of Tregs by 4C12 during the non-aerosol period with L4G6-mediated blocking of co-stimulation during the aerosol period, we evaluated if manipulating TNFRSF25 signaling could capitalize on the multiple roles TNFRSF25/TL1A has in allergic asthma.

We theorized that this therapeutic approach would enhance the suppression by 4C12-mediated transient expansion of Tregs by blocking co-stimulation of NKT-cells and CD4+ Teff during aerosolization. A modified
version of the therapeutic protocol for a chronic model of allergic lung inflammation was utilized to evaluate this strategy (Figure 52).

Figure 52: Modulation of TNFRSF25 signaling in a chronic model of allergic lung inflammation. Similar to the therapeutic protocol in a chronic model of allergic lung inflammation, with the added intervention of i.p. injecting either 50 µg control IgG or 50 µg anti-TL1A blocking antibody (L4G6) on days, 48, 53, and 58, 10 minutes after the end of aerosolization on each day.

Specifically, in addition to 4C12 administration on day 41, L4G6 was administered three times over the last two weeks of aerosolization, on days 48, 53, and 58, approximately 10 minutes after the end aerosolization. BALF eosinophilia was evaluated on day 61 as a measure of the severity of allergic lung inflammation. In 4C12/IgG- and 4C12/L4G6- treated mice, there was not a significant difference in the percentage or number of eosinophils in the total cells recovered in the BALF (Figure 53). Comparison of the BALF levels of eosinophils in both of these groups to control IgG-treated mice showed that
both 4C12/IgG and 4C12/L4G6-treated mice significantly reduced BALF eosinophilia (Figure 53).

Figure 53: Bronchoalveolar lavage fluid (BALF) was collected on day 61. The percentage of eosinophils in total cells recovered and the absolute number of eosinophils recovered is shown for mice treated with control IgG, combination of 4C12 and control IgG, or combination 4C12 and L4G6. (* = p<.05, 1-way ANOVA, Tukey post-test, with respect to group indicated) (NS = p<.05 Tukey post-test, with respect to group indicated) (n= 6 – 7)

2.3 **ANTIGEN-SPECIFIC T-CELL RESPONSES IN ALLERGIC ASTHMA**

Antigen-specific T-cell responses, both CD4+ effector and regulatory cells, are quite important to determining if immune responses to antigens will result in immunopathology or if the immune system will be properly controlled by immunoregulatory mechanisms (113, 117, 251, 252). Antigen-specific responses are often difficult to monitor and evaluate in CD4+ T-helper subsets in humans due to the complications associated with the development of MHC Class-II restricted antigen specific-tetramers. In mice, a number of tools have been developed and characterized that allow for the proper identification of MHC Class II-restricted antigen-specific T-cell receptors on CD4+ T-cells. We have used a variety of approaches to understand the changes in the ovalbumin-specific T-cell responses with TNFRSF25-mediated expansion of regulatory T-cell (Tregs) in both the acute and chronic allergic lung inflammation setting.
2.3.1 Ovalbumin/ALUM sensitization and ovalbumin-aerosolization induces both antigen-specific effector and regulatory cell-subsets

Disease models relating to atopy or autoimmunity indicate that sensitization to the antigen results in the induction of both antigen-specific effectors as well as antigen-specific regulatory cells (253).

![Diagram of experimental setup](image)

Figure 54: Ovalbumin-specific Teff and Tregs are induced in an acute model of allergic lung inflammation. CD4(+), CD25(-), GITR(low) naïve OT-II splenocytes were i.v. transferred into CD45.1 congenic mice on day -2. Mice were then i.p. immunized on day 0 and 5 with ovalbumin/ALUM, and aerosolized with 0.5% w/v ovalbumin in PBS on day 16 for one-hour. Mice were sacrificed and analyzed on day 19.

To determine if ovalbumin-specific T-cells are similarly induced in our acute model of allergic lung inflammation, we used splenocytes from transgenic mice with MHC Class-II restricted T-cell receptors specific for ovalbumin (OT-II, Jackson Labs) as a source for CD3+ Vβ5.1/5.2+ CD4+ CD25- GITRlow naïve T-cells. Cells were adoptively transferred by tail vein injection into congenically marked CD45.1 mice (OT-II mice are CD45.2+) on day -2. On day 0 and 5, mice were immunized with ovalbumin adjuvanted with ALUM. Mice were then aerosolized on day 16 with ovalbumin in PBS, and sacrificed on day 19 for analysis of tissues and bronchoalveolar lavage fluid (BALF) (Figure 54). Analysis of BALF eosinophils showed no significant difference in percentage or number compared to historical controls, which were
significantly increased when compared to non-aerosolized mice (data not shown).

CD45.2+ OT-II T-cells were detected in both spleens and lung, at about 1% and 2% in total CD4+ cells, respectively (Figure 55A). In addition, CD4+FoxP3+ OT-II induced-Tregs (iTregs) were detected in both the spleen and lungs, with an increased frequency of FoxP3+ OT-II iTregs in the lungs compared to non-OT-II Tregs (CD4+CD45.1+FoxP3+) (Figure 55B). The non-OT-II Treg population also includes a percentage of ovalbumin-specific iTregs, but is not easily identified from nTregs or non-ovalbumin-specific iTregs in this system. Proliferation in CD4+FoxP3- OT-II T-cells and OT-II
iTregs was determined using Ki67 as a marker. Analysis of the lungs and spleens on day 19 showed that both CD4+FoxP3- OT-II T-cells and OT-II iTregs populations had a higher percentage of cells undergoing proliferation compared to CD4+CD45.1+ non-OT-II T-cells (Figure 56) as well as lungs and spleens from naïve mice (data not shown).

Figure 56: Total frequency of Ki67(+) cells within the CD4(+) FoxP3(+) and CD4(+) FoxP3(-) T-cell population from the spleens and lungs of mice analyzed on day 19.

This data suggests that during the sensitization and aerosolization of ovalbumin in an acute model of allergic inflammation, both ovalbumin-specific CD4+ Teff and Tregs are induced and subsequently proliferate in both the lung and spleen.

2.3.2 TNFRSF25-mediated expansion of Tregs results in an antigen-specific responses toward a regulatory phenotype in acute model of allergic lung inflammation

Research tools to identify T-cell receptors that recognize specific antigens have been developed by a number of laboratories and companies, including the laboratory of Dr. Marc Jenkins at the University of Minnesota School of
Medicine, who has graciously donated I-A\textsuperscript{b} tetramers carrying OVA-peptide (254). The OVA:I-A\textsuperscript{b} tetramers are a combination of two different tetramers that recognize overlapping peptides that can detect a majority of the ovalbumin-specific T-cell receptors. Using these tetramers to identify ovalbumin-specific populations within the CD4+ T-cell compartment, we can monitor the changes in the antigen-specific T-cell effectors (FoxP3-) and regulators (FoxP3+) that occurs in an acute model of allergic lung inflammation, and observe how these populations are affected by in vivo TNFRSF25 stimulation.

Figure 57: OVA-specific CD4(+) T-cells were identified in the spleen using peptide:MHC Class-II tetramers specific for T-cell receptors that recognize ovalbumin presented via MHC Class-II, OVA:I-A\textsuperscript{b}. On day 16, prior to aerosolization, splenocytes from control IgG- and 4C12-treated mice were analyzed for the percentage of OVA:I-A\textsuperscript{b} -specific CD4(+) FoxP3(-) Teff and CD4(+)FoxP3(+) Tregs within the total CD4(+) gate. (* = p<.05, student’s T-test) (n= 6)

TNFRSF25 stimulation in mice with 4C12, after ovalbumin/ALUM prime and boost, was able to significantly increase the percentage of ovalbumin-specific Tregs (CD4+FoxP3+) in the spleen compared to mice treated with control IgG (Figure 57), but no differences were noted in the lung at the time (data not shown). On day 19, three days after aerosol challenge with ovalbumin in PBS, a significant increase in the percentage of OVA:I-A\textsuperscript{b} -specific Tregs was observed in the spleens and lungs of 4C12-treated mice, compared to control IgG-treated mice. Additionally, OVA:I-A\textsuperscript{b} -specific Teff (CD4+FoxP3-) were
significantly reduced in 4C12-treated mice, compared to control IgG-treated mice (Figure 58A and Figure 58B). Another way to analyze antigen-specific T-cell responses is to determine the antigen-specific Teff to Treg ratio, a strong measure for following clinical responses to treatment in allergy (109). Analysis of control IgG-treated mice at day 19 shows that the OVA:I-A\textsuperscript{b} -specific Teff to Treg ratio is greatly weighted toward the OVA:I-A\textsuperscript{b} -specific Teff population in mice with significant allergic lung inflammation. In mice treated with 4C12, the lung-derived lymphocytes show a skewing of the OVA:I-A\textsuperscript{b} -specific Teff to Treg ratio towards the ovalbumin-specific Treg population (Figure 58C).

![Figure 58:](image)

2.3.3 TNFRSF25 signaling expands non ovalbumin-specific Tregs more than ovalbumin-specific Tregs

The previous work done by Dr. Schreiber and Dr. Wolf showed that TNFRSF25-mediated expansion of Tregs required T-cell receptor engagement (240). Applying that knowledge to our acute model of allergic
lung inflammation would indicate that ovalbumin-specific and self-specific Tregs would be expanded with TNFRSF25 signaling in ovalbumin-sensitized mice, if residual ovalbumin was presented at sufficient levels at the time of signaling. A question that stems from this conclusion is if TNFRSF25-mediated expansion of Tregs in ovalbumin-sensitized mice, preferentially expands either ovalbumin-specific Tregs or non-ovalbumin specific Tregs. To answer this question, we used the tetramers we have available to compare the expansion of ovalbumin-specific Tregs with respect to the total Treg compartment.

<table>
<thead>
<tr>
<th>Expansion of ovalbumin-specific Tregs</th>
<th>Expansion of all Tregs</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \frac{\text{ovalbumin-specific Tregs % in 4C12 mice}}{\text{ovalbumin-specific Tregs % in IgG mice}} )</td>
<td>( \frac{\text{Total Tregs % in 4C12 mice}}{\text{Total Tregs % in IgG mice}} )</td>
</tr>
</tbody>
</table>

**Table 2: Calculating ovalbumin-specific vs. non-specific Treg expansion**

Using the formula described in Table 2, the relative expansion of ovalbumin-specific Tregs compared to total Treg expansion by TNFRSF25 signaling was determined in the acute model of allergic lung inflammation. In the spleens of mice analyzed on day 19, 4C12-treated mice showed a 1.75 fold increase in ovalbumin-specific Tregs compared to control IgG-treated mice. The fold increase of total Tregs in 4C12-treated mice over control IgG-treated mice was 2.5, a significant increase compared to the expansion of ovalbumin-specific Tregs by 4C12 (Figure 59). This finding suggests that in the acute allergic lung inflammation setting, there is a slight preference for the
expansion of non-ovalbumin-specific Tregs by 4C12, though not by a large margin.

2.3.4 Poor up-regulation of metabolic machinery in ovalbumin-specific CD4+ Teff after TNFRSF25-mediated Treg expansion

To evaluate what effects TNFRSF25-mediated expansion of Tregs may have on ovalbumin-specific CD4+ Teff in the acute model of allergic lung inflammation, lung-derived lymphocytes from mice sacrificed on day 19 of the protocol were stained for CD71 surface expression, a transferrin receptor that is highly up-regulated after CD4+ Teff cell activation. Failure to up-regulate the receptor to the cell surface has been indicated as a marker for T-cell anergy (255). OVA:I-Ab specific CD4+ Teff cells from allergen-sensitized and challenged mice treated with 4C12 showed significantly less CD71 surface expression when compared to OVA:I-Ab specific CD4+ Teff cells from mice treated with control IgG (Figure 60). Though only one measure of the metabolic machinery necessary for increased biological activity, this finding suggests that anergy within the ovalbumin-specific Teff population is a possible mechanism to the suppression of immunopathology by the TNFRSF25-mediated expansion of Tregs. A similar observation of antigen-
specific Tregs suppressing antigen-specific CD4+ T-memory cells (central and effector) has previously been observed in studies of human peripheral blood mononuclear cells after BCG vaccination (256).

2.3.5 Ovalbumin-specific Tregs are transiently and significantly expanded by TNFRSF25 signaling in a model of chronic allergic lung inflammation

To determine if the findings in the acute model would be validated in our chronic model, ovalbumin-specific T-cell populations in different tissue compartments were also analyzed in the chronic model of allergic lung inflammation.

Tetramer analysis of splenocytes procured from mice on day 61 showed an almost 5-fold increase of OVA:I-A\(^b\) -specific iTregs in 4C12 treated over naïve mice and a two-fold increase over control IgG-treated mice (Figure 61A and Figure 61B). OVA:I-A\(^b\) -specific effector T-cells showed a 4-fold elevated frequency in ovalbumin-challenged control IgG-treated mice compared to naïve controls, and in 4C12-treated mice, OVA:I-A\(^b\) -specific effector T-cells expanding only two-fold (Figure 61A and Figure 61C).
Figure 61: A) Representative flow plots for OVA:I-Ab staining (OVA-specific) splenocytes procured from naïve, control IgG- and 4C12-treated mice on day 61 (final). Flow plots were pre-gated on NK1.1(-) CD11c(-) CD11b(-) Gr-1(-) CD8(-) CD19(-) CD3(+) CD4(+) cells. B) Total frequencies of OVA:I-Ab (+) FoxP3(+) cells (OVA-specific Treg) and C) OVA:I-Ab (+) FoxP3(-) cells (OVA-specific Teff) within the CD4(+) gate. D) Ratio of OVA-specific Teff to Treg (* = p<.05, 1-way ANOVA, Tukey post-test, with respect to group indicated) (n= 4 – 6)

TNFRSF25 stimulation by 4C12 on day 41 resulted in a reduction of the ovalbumin-specific Teff to Treg ratio in spleens as observed on day 61 from 14.22 in control IgG-treated mice to 4.99 in 4C12-treated mice (Figure 61D) (Table 3). Analysis of lung-resident T-cells with tetramers resulted in frequencies too low for reliable detection. In the acute model of allergic lung inflammation, spleen and lung resident ovalbumin-specific T-cells showed similar changes with TNFRSF25 agonist treatment, thus a similar trend likely
occurred in the ovalbumin-specific T-cell populations in the spleen and lung of mouse administered TNFRSF25 agonist in a chronic model of allergic lung inflammation.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>OVA:I-A^b Teff to Treg Ratio (mean ± SEM)</th>
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<tbody>
<tr>
<td>Naïve</td>
<td>4.97 ± 0.86</td>
</tr>
<tr>
<td>IgG</td>
<td>14.22 ± 4.21</td>
</tr>
<tr>
<td>4C12</td>
<td>4.99 ± 1.31</td>
</tr>
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Table 3: Ovalbumin-specific Teff to Treg Ratio

2.3.6 Blunting of IL-5 antigen-specific effector response in chronic model of allergen challenge

IL-5 mRNA expression levels in the BALF recovered cells and lungs of 4C12-treated mice were suppressed on day 61. To confirm these results and to further investigate changes in the antigen-specific immune responses in our therapeutic model for chronic allergic lung inflammation, single-cell suspensions from mesenteric lymph nodes of chronically challenged mice were harvested on day 61 and evaluated for intracellular IL-5 production in response to in vitro re-stimulation with OVA_{323-339} peptide. Intracellular staining for IL-5 in CD3+CD4+ T-cells from these re-stimulated cells was significantly increased in control IgG-treated mice compared to re-stimulated mesenteric lymph node-derived cells from naïve mice. In 4C12-treated mice, the intracellular IL-5 staining in CD3+CD4+ T-cells re-stimulated with OVA_{323-}
peptide, was significantly reduced compared to control IgG-treated mice (Figure 62A and Figure 62B).

The results from the re-stimulation experiments indicate that anti-TNFRSF25 agonistic antibody mediated expansion of Tregs is able to suppress Th2 antigen-specific responses, specifically by blunting IL-5 cytokine production in response to MHC Class-II restricted OVA323-339 peptide. Intracellular staining for IL-4 and IL-13 was attempted, but failed to detect a difference between groups (data not shown).
CHAPTER 3: DISCUSSION

Treg immunotherapy in mouse models of allergic asthma have been established by a number of groups and evidence suggests it to be efficacious in preventing immunopathology associated with subsequent allergen challenge (102, 103, 105). TNFRSF25 stimulation is novel because a single-dose of agonist is able to specifically in vivo expand antigen-specific and non-specific Tregs that can home to the site of inflammation (lung), with in situ proliferation occurring within the lung, and suppress subsequent allergic inflammation (240). Current Treg immunotherapies involve adoptive transfer of T-regulatory cells, a process encumbered with technical difficulties and considerable expense. Recently, other groups have developed protocols for in vivo Treg expansion including administration of an IL-2/anti-IL-2 monoclonal antibody complex (JES6-1), components of Streptococcus pneumoniae (T3P+Ply), and a TLR7 agonist (R848) (118, 120, 257). All three approaches show promise, but further characterization of the durability of suppression and changes in antigen-specific responses would be needed to compare against standard asthma immunotherapy: specific-antigen immunotherapy (SIT).

A concern that arises when using monoclonal agonistic antibodies is if the observed effects are a result of signaling of the specific receptor, or an off-target effect of the monoclonal antibody used. Subsequent to the development of 4C12, a homohexameric fusion protein of TL1A fused to the Fc portion of mouse IgG1 was designed, produced, and characterized in the lab (Khan SQ, Podack ER, unpublished). Using this molecule in both the acute and chronic models of allergic lung inflammation, similar results to anti-
TNFRSF25 agonistic antibody (4C12) administration was observed. These results support the hypothesis that specific TNFRSF25 signaling is responsible for the expansion of Tregs to therapeutic levels, and not a consequence of unexpected off-target effects of clone 4C12.

To evaluate if the suppression of allergic lung inflammation was dependent on Treg suppression, PC61-mediated depletion of CD4+CD25+ Tregs prior to TNFRSF25 signaling was attempted in the acute model of allergic lung inflammation. Interestingly, TNFRSF25 signaling was able to expand CD4+CD25low/negative FoxP3+ Tregs to a level in the peripheral blood comparable to non-PC61-depleted, 4C12-treated mice. This result made interpreting BALF eosinophil data difficult, because the total Treg population was not depleted by PC61. The expansion of CD4+CD25low/negative FoxP3+ Tregs was not entirely unexpected based on the results from Schreiber et al. which showed TNFRSF25 agonist expansion of Tregs expanded the CD25low/negative population more robustly than the CD25hi population (240). An alternative approach to determine if Treg expansion is essential for the suppression of allergic lung inflammation by 4C12 is needed and likely requires specific and efficient depletion of CD4+FoxP3+ cells, rather than just CD4+CD25+ cells. One alternative would be a genetically modified mouse with the human diphtheria toxin receptor co-expressed on cells with high levels of FoxP3 transcript (DEREG mouse) (258, 259). Both animals have been shown to efficiently and selectively deplete FoxP3+ cells in vivo, and have already been used in various tolerance and disease models (260-262).
In the chronic model of allergic lung inflammation, we sought to address the question of whether in vivo expansion of Treg cells by administration of a TNFRSF25 agonistic antibody (4C12) could reduce chronic lung inflammation, based on the known efficacy of this agent in preventing acute lung inflammation. In particular, we wanted to investigate the durability of transiently expanded Tregs in reducing inflammation after subsequent antigen-challenge over a prolonged period and at a time when airway immunopathology included indication of airway tissue remodeling. Current therapies, such as β-agonists and corticosteroids effectively reduce asthmatic symptoms (111) but do not reverse airway remodeling in patients (263, 264). In this study, TNFRSF25-mediated expansion of Tregs was associated with a reversal of airway remodeling as measured by collagen deposition in the subepithelial space in the central bronchiole. The specific mechanism for the reversal of airway remodeling is unknown, but it is likely that there is not a single specific molecular or cellular mechanism. A combination of the suppression of Th2 cytokines, specifically the fibrogenic factor IL-13, by Tregs expanded by TNFRSF25, followed by the induction of the normal mechanisms of lung repair, possibly through FGF10 from type II pneumocytes, a previously described mechanism in the reversal of airway fibrosis in models of pulmonary fibrosis (265, 266). Another parallel pathway for the reversal of lung fibrosis may involve blocking neovascularization in the lungs by Tregs via DLL4-Notch signaling (104). An interesting extension of investigating mechanisms for the reversal of airway remodeling is to determine if Tregs and the epithelium at mucosal surfaces directly interact.
There are a number of mechanisms implicated in immune suppression by Tregs. IL-10 signaling is a possibly mechanism and is important in Treg-mediated tolerance induction as well as therapeutic suppression of allergic asthma by Tregs. Our studies using IL-10 receptor blocking antibody indicate that blocking IL-10 signaling does not significantly change the durable suppression of BALF eosinophilia by transiently expanded Tregs. Other potential mechanisms include direct suppression of mast cell and NKT-cells, induction of indoleamine 2,3 dioxygenase, CTLA-4, or production of TGF-β in the local immune environment (155, 225, 238, 267). Using mRNA extracted from whole lung, real-time RT-PCR was used to examine if there was differential expression of indoleamine 2,3 dioxygenase in 4C12-treated mice versus control IgG-treated mice. Unfortunately no differences were noted in either the acute or chronic models (data not shown), though it is likely that the differential expression of this molecule occurs on cell populations that are at lower frequencies in lungs. An alternative approach to real-time RT-PCR for analyzing changes in molecule expression likely requires analysis of these suppressive molecules on a single-cell basis from both the lungs and draining lymphnodes. Further studies to understand the mechanism of suppression include phenotyping of lung-residing and bronchial lymph node-derived dendritic cells for co-stimulatory and regulatory markers, such as CD80/86 and indoleamine 2,3 dioxygenase.

Recently, a new family of cell types has been implicated in the development and progression of allergic asthma: innate-like cells (ILCs). The ILC family represents a variety of different cell types, but nuocytes and lung
natural helper cells, in particular, have been found to have a significant influence on lung immunity (78, 268, 269). ILCs were not analyzed in our therapeutic models, but changes in expression of IL-25 and other Th2 cytokines after TNFRSF25-mediated expansion of Tregs suggests that these populations could have been affected, since ILCs have been found to be significant sources of these cytokines (270). Systematic phenotyping of lung-resident cells would be informative in determining if ILCs are affected by expanded Tregs in the allergic asthma model. Further studies with ILCs would include surface staining for TNFRSF25 and TL1A, since previous work identifying cell-types that expressed TNFRSF25 or TL1A did not specifically analyze ILCs. If any of these cell-types do express either of these molecules, new cellular mediators of TNFRSF25 signaling could be identified.

Using ovalbumin-specific tetramers to monitor antigen-specific populations, we found that in vivo TNFRSF25 stimulation was able to expand both ovalbumin-specific and non-specific Treg populations with a preference for non-specific Tregs. The expansion of Tregs was also associated with a significant reduction in the ovalbumin-specific Teff population, leading to an ovalbumin-specific Teff to Treg ratio favoring a regulatory phenotype and reduction of IL-5 in response to in vitro stimulation with OVA_{323-339}. Taken together, these data indicates that TNFRSF25 expansion of Tregs can effectively suppress antigen-specific effector responses in the setting allergic asthma. A similar skewing of the antigen-specific Teff to Treg ratio away from effector dominance has also been observed with ex vivo allergen challenge of peripheral blood mononuclear cells from allergic patients responsive to
specific-antigen immunotherapy therapy (SIT) and healthy controls (113, 251, 271), suggesting TNFRSF25-mediated expansion may use similar mechanisms for tolerance induction.

Antigen-specific Tregs are recognized as efficient suppressors of antigen-specific responses, though non-specific “bystander suppression” by nTregs has also been observed by others (121, 238). From our results showing the suppression of antigen-specific T-cell responses, a question that arises is if this reduction in antigen-specific effector responses and suppression of allergic lung inflammation, is primarily by antigen-specific iTregs, or the concurrent and significant expansion of antigen-non-specific Tregs. To address this, a new acute model of allergic lung inflammation has been developed to assess the abrogation of allergic lung inflammation by TNFRSF25 expanded antigen-specific Tregs. The model developed involves reconstitution of acute allergic lung inflammation in Cd4-/- mice by the transfer of CD4+CD25- naïve T-cells from wild-type mice (Figure 63). This model system allows us to control what types of CD4+ T-cells are available for suppression. The transferred CD4+CD25- naïve T-cells will be from DEREG mice (258) which allows for the specific depletion of any cell that induces FoxP3 transcript. After sensitization and depletion of iTregs, different antigen-specific or non-specific Tregs can be transferred, expanded, and evaluated.
Figure 63: CD4\(^{+}\) mice received varying amounts of CD4-enriched splenocytes from wild-type mice (2 x 10\(^6\) or 10 x 10\(^6\) cells by i.v. adoptive transfer; or 10 x 10\(^6\) or 12 x 10\(^6\) cells by i.p. injection) on day -7. On day 0 and 5, mice are i.p. immunized with ovalbumin adsorbed to ALUM, and then on day 16, aerosolized with 2.5% w/v ovalbumin in PBS for one-hour. On day 169, mice are sacrificed, lungs lavaged, and tissues analyzed. The total percent of CD4(+) T-cells within the CD3(+) gate in the A) spleen and B) lung. Bronchoalveolar lavage fluid (BALF) was collected on day 19. The C) percentage of eosinophils in total cells recovered and the D) absolute number of eosinophils recovered is shown for mice given different amounts of CD4-enriched splenocytes.

After the discovery that regulatory T-cells were robustly expanded in vivo by a TNFRSF25 agonist, a question that needed to be addressed was if our previously hypothesized role of TNFRSF25 signaling in allergic lung inflammation was still supported in light of this result. Previously, Fang et al. concluded that effective co-stimulation of Th2 immune responses requires TCR engagement and with the recent observation that agonists for OX40, another TNF superfamily member, can enhance or reduce inflammation depending on the inflammatory setting (148), we concluded that specific
expansion of Tregs through TNFRSF25 signaling likely occurs when inflammatory cytokines and co-stimulation were reduced. This hypothesis was supported by the specific expansion of Tregs and suppression of allergic lung inflammation, with minimal expansion of CD4+ effector T-cells observed in the lungs of mice. This lack of effector T-cell co-stimulation did not disprove previous findings regarding TNFRSF25 role in co-stimulation of effector responses, only modified the paradigm that TNFRSF25 signaling co-stimulates inflammatory responses. TNFRSF25 signaling during allergen aerosol enhanced allergic lung inflammation supporting the previous findings by Fang et al. that TNFRSF25 co-stimulation can enhance inflammatory responses. Interestingly, TNFRSF25 signaling during allergen challenge also expanded Tregs in the lungs, though allergic lung inflammation was still enhanced. This suggests that TNFRSF25 co-stimulation of effectors during allergen exposure can overcome any suppression by expanded Tregs. This is likely due to faster proliferation kinetics by T effectors compared to Tregs, similarly observed in a model of skin-specific autoimmunity and resolution (205).

TNFRSF25 agonists in the presence of antigen co-stimulate both CD4+ effector and regulatory T cell expansion (149, 150, 192, 196). Co-stimulation during chronic lung inflammation results in asymmetric expansion in favor of Tregs, resulting in a significant decrease in the ovalbumin-specific Teff to Treg ratio. The preferential Treg expansion by TNFRSF25 agonists may be explained by several hypotheses: 1) Treg co-stimulation by TNFRSF25 may require lower antigen concentration. In our studies, mice were rested for one
week before agonist administration which may have left antigen levels sufficient for Treg but not for Teff co-stimulation. 2) Treg signals from prior antigenic stimulation may be maintained longer. This hypothesis is similar to hypothesis 1 in that less antigen would be required for co-stimulation. 3) TNFRSF25 induced concurrent self-antigen specific nTreg and allergen-specific iTreg expansion may generate a cytokine milieu favoring Treg expansion, but adversarial to Teff expansion. This is supported by reduced surface expression of transferrin receptor in antigen-specific Teff in 4C12-treated mice in the acute model for allergic lung inflammation. 4) It is also possible that TNFRSF25 co-stimulated Tregs interfere with effector cell expansion directly or via dendritic cells. To distinguish between possibilities additional studies will be necessary. It is likely that features of each of these hypotheses contribute to the preferential expansion of Tregs.

To take advantage of both aspects of TNFRSF25 co-stimulation, manipulation of TNFRSF25 signaling was attempted in the chronic model of allergic lung inflammation using 4C12 (TNFRSF25 agonist) to expand Tregs, followed by L4G6 (TL1A blocking antibody) (149) administration during allergen challenge to block co-stimulation of Teff and NKT-cells. The addition of L4G6 did not enhance suppression mediated by 4C12-expanded Tregs. It is possible that blocking TL1A was insufficient due to an anti-hamster IgG response developed by the mouse. L4G6, the anti-TL1A blocking antibody used in the last two weeks of the protocol, like 4C12, is a hamster IgG, which was administered on day 41. It is likely that after 4C12 administration, the mice mounted a primary immune response to hamster IgG, and soon after the
first L4G6 injection, strong mouse anti-hamster immune responses were elicited, effectively reducing the biological activity of the anti-TL1A blocking antibody. An alternative approach is needed for the manipulation of TNFRSF25 signaling to both expand the Treg population and block effector cell co-stimulation. Using a TL1A-Ig fusion protein (mouse TL1A extracellular domain fused to the Fc portion of mouse IgG1) with L4G6 could help delay the anti-hamster response. Alternatively, a DR3 construct (lacking transmembrane and intracellular domains) or DcR3 fusion proteins could be developed as “sinks” for TL1A in vivo. Other therapeutic strategies to combine TNFRSF25-mediated expansion of Tregs with co-stimulation blocking agents could also be used in a chronic model of allergic lung inflammation to enhance the suppression of immunopathology.

Our model of acute allergic lung inflammation was developed using the C57BL/6 strain, which has been found to be a reliable model of allergic lung inflammation, one of the pathological components of allergic asthma, but does not consistently show measureable airway hyperreactivity, a key clinical measure of patients suffering from asthma (136). The reason we have chosen this strain is because of its consistent and measurable inflammatory response since our therapeutic approach expands Tregs to suppress the Th2-type immune responses. But because acute exacerbations in asthmatics are clinically characterized by enhanced bronchoconstriction in response to allergen, it is important to investigate the physiological effects that TNFRSF25-mediated expansion of Tregs has on the allergic asthma model. To address this, a similar therapeutic model of acute allergic asthma must be
developed in BALB/C mice, a strain that is consistent in replicating airway hyperreactivity in response to airway challenge (135, 136). A model that exhibits all three components of allergic asthma in a C57BL/6 strain of mouse: allergic lung inflammation, airway remodeling, and airway hyperreactivity, was developed by Doherty et al. (132), and would be an ideal model to evaluate physiological effects of TNFRSF25-mediated expansion of Tregs.

Evaluation of TNFRSF25-mediated expansion of Tregs in the clinical setting is a future direction, but requires a number of pre-clinical studies to show safety and efficacy. The next steps toward that goal include testing a rhesus macaque TL1A-Ig fusion protein to evaluate if in vivo TNFRSF25 signaling in non-human primates shows similar expansion of Tregs, and then determine the proper dosing for therapeutic use. Furthermore, studies to evaluate safety and adverse effects in non-human primates are essential to an Investigation New Drug (IND) application. Follow-up efficacy studies using a human allergen, such as house dust mite, in an allergic asthma model in rhesus macaques would be strong evidence for use of TNFRSF25 agonists in patients with allergic asthma. Eventual translation of this approach to humans is most likely in a population of patients with established chronic asthma and where the offending antigen is known. The current mouse model provides an approximation of allergic lung inflammation to seasonal allergens, where symptoms relapse and remit in association with environmental antigens. The unique properties of TNFRSF25 agonists to stimulate Treg proliferation in vivo in an antigen-specific fashion may provide a translatable pathway as an alternative to ex vivo based Treg approaches. Of critical importance to this
approach is the generation of antigen-specific Treg cells and localization of these cells to the lungs. These findings indicate that TNFRSF25 agonists can be an important new compound uniquely capable of reversing airway remodeling and providing durable suppression of inflammatory immunopathology, even for those patients with long-standing disease and airway remodeling.
CHAPTER 4: MATERIALS & METHODS

4.1 MICE

Wild-type C57BL/6 and OT-II (B6.Cg-Tg(TcraTcrb)425Cbn/J) mice were purchased from Jackson Laboratories. FoxP3-IRES-RFP mice were provided by R. Flavell (Yale University, (244)). CD45.1 SJL and Cd4⁻ mice were bred in our animal facility. Mice were used at 6-12 weeks of age and were maintained in pathogen-free conditions at the University of Miami animal facilities. The University of Miami Animal Care and Use Committee approved all the animal procedures used in this study.

4.2 ANTIBODIES AND REAGENTS

All the commercial antibodies that were used for flow cytometry staining were purchased from Biolegend, eBioscience, or BD Biosciences-Pharmingen. The Armenian hamster IgG1 isotype control was purchased from Jackson ImmunoResearch. Armenian hamster hybridoma producing antibody to mouse TNFRSF25 (agonist 4C12) and mouse TL1A (antagonist L4G6) was maintained as described previously (149). 4C12 and L4G6 were produced in hollow fiber bioreactors (Fibercell Systems) and purified from serum-free supernatants on a protein G column (GE Healthcare). TL1A-Ig fusion protein, with the extracellular domain of mouse TL1A fused to the Fc portion of mouse IgG1 (with hinge), was produced in Chinese hamster ovarian cells, and purified for in vivo use (Khan SQ, Podack ER, unpublished). Anti-CD25 (PC61) was purchased from Biolegend. IL-10 receptor-blocking antibody
(1B1.3A) and rat isotype control IgG were purchased from BioXcell. Recombinant mouse IL-10 was purchased from Peprotech. LPS was purchased from Difco. OVA323-339 peptide was purchased at Anaspec.

4.3 ACUTE ALLERGIC LUNG INFLAMMATION MODEL

Mice were sensitized by i.p. injection of 66 µg of ovalbumin (crystallized chicken egg ovalbumin, grade V; Sigma-Aldrich) adsorbed to 6.6 mg of ALUM (aluminum potassium sulfate dodecahydrate; Sigma-Aldrich) in 200 µL of phosphate-buffered saline (PBS) on day 0, with a boost on day 5. On day 12, mice were injected i.p. with either 20 µg Armenian hamster isotype control IgG1 or 20 µg anti-TNFRSF25 agonistic antibody (clone 4C12) in 200 µL PBS. In experiments some experiments, mice were injected i.p. on days 11, 12, and 13 with either 100 µg mouse isotype control IgG1 or 100 µg TL1A-Ig in 200 µL PBS. On day 16, mice were aerosolized for one-hour with 0.5% w/v ovalbumin in PBS using a BANG nebulizer (CH Technologies) into a Jaeger-NYU Nose-Only Directed-Flow Inhalation Exposure System (CH Technologies). On day 19, mice were sacrificed, lungs were perfused with PBS, and bronchoalveolar lavages were obtained. Lung lobes were processed for RNA, for flow cytometry analysis, or for lung histology. Spleens and mesenteric lymphnodes were also procure for subsequent flow cytometry analysis. Mice were bled periodically to monitor peripheral blood mononuclear cells. In some experiments, mice were injected i.p. on day 16 with either 20 µg Armenian hamster isotype control IgG1 or 20 µg anti-TNFRSF25 agonistic antibody (clone 4C12) in 200 µL PBS, 5 minutes prior to
one-hour of aerosolization with 0.25% w/v ovalbumin in PBS. Mice were subsequently sacrificed and analyzed on day 19.

4.4 PC61-DEPLETION IN ALLERGIC LUNG INFLAMMATION

Mice were sensitized to ovalbumin on days 0 and 5, as described above in the acute model of allergic lung inflammation. On days 8 and 9, mice were either injected i.p. with 500 µg rat isotype control IgG1 or 500 µg anti-CD25 depletion antibody (PC61). On day 12, mice were injected i.p. with either 20 µg Armenian hamster isotype control IgG1 or 20 µg anti-TNFRSF25 agonistic antibody (clone 4C12) in 200 µL PBS. On day 16, mice were aerosolized for one-hour with 0.5% w/v ovalbumin in PBS. On day 19, mice were sacrificed, lungs were perfused with PBS, and were processed with spleens for flow cytometry analysis. Mice were bled periodically to monitor peripheral blood mononuclear cells.

4.5 CHRONIC ALLERGIC LUNG INFLAMMATION MODEL

Mice were sensitized by i.p. injection of 66 µg of ovalbumin (crystallized chicken egg ovalbumin, grade V; Sigma-Aldrich) adsorbed to 6.6 mg of ALUM (aluminum potassium sulfate dodecahydrate; Sigma-Aldrich) in 200 µL of phosphate-buffered saline (PBS) on day 0, with a boost on day 12. On days 18 to 22, mice were aerosolized for one-hour each day, with 2.5% w/v ovalbumin in PBS using a BANG nebulizer (CH Technologies) into a Jaeger-NYU Nose-Only Directed-Flow Inhalation Exposure System (CH Technologies). Starting on day 25, mice were aerosolized for two weeks, three times a week, for one hour with 2.5% w/v ovalbumin in PBS. Mice were
kept allergen free from days 34 to 45. On day 41, mice were injected i.p. with
either 20 µg Armenian hamster isotype control IgG1 or 20 µg anti-TNFRSF25
agonistic antibody (clone 4C12) in 200 µL PBS. In experiments some
experiments, mice were injected i.p. on days 40, 41, and 42 with either 100 µg
mouse isotype control IgG1 or 100 µg TL1A-Ig in 200 µL PBS. On day 45,
aerosolization of mice with 2.5% w/v ovalbumin in PBS was restarted at a
frequency of three times a week, for one-hour each time, over a two-week
period. On day 49 and 61, mice were sacrificed, lungs were perfused with
PBS, and bronchoalveolar lavages were obtained. Lung lobes were
processed for RNA, for flow cytometry analysis, or for lung histology. Spleens
and draining bronchial lymph nodes were also procured for subsequent flow
cytometry analysis, with cells derived from mesenteric lymph nodes also used
for ex vivo restimulation. Mice were bled periodically to monitor peripheral
blood mononuclear cells as well as sacrificed at certain points in the protocol
to obtain bronchoalveolar lavage fluid. In some experiments, IL-10 receptor
blocking was accomplished using 1B1.3A (BioXcell). 1B1.3A was dosed at
250 µg injected i.p. in 200 µL PBS, 30 minutes prior to each aerosolization,
starting on day 45, and continued on each aerosolization day till day 58. Mice
were sacrificed on day 61 and lungs lavaged as described above.

4.6 TNFRSF25 SIGNAL MANIPULATION IN CHRONIC MODEL OF
ALLERGIC LUNG INFLAMMATION

Similar to the chronic model of allergic lung inflammation (described above),
mice were sensitized to ovalbumin and aerosolized for three weeks. On day
41, mice were injected i.p. with either 20 µg Armenian hamster isotype control
IgG1 or 20 µg anti-TNFRSF25 agonistic antibody (clone 4C12) in 200 µL PBS. On day 45, aerosolization of mice with 2.5% w/v ovalbumin in PBS was restarted at a frequency of three times a week, for one-hour each time, over a two-week period. On days 48, 53, and 58, mice were injected i.p. with either 50 µg Armenian hamster isotype control IgG1 or 50 µg anti-TL1A antagonistic antibody (clone L4G6) in 200 µL PBS, 10 minutes after the end of aerosolization. Mice were sacrificed on day 61, with bronchoalveolar lavage fluid obtained for cellular analysis.

4.7 BRONCHOALVEOLAR LAVAGE ANALYSIS

Cells recovered from the bronchoalveolar fluid were counted and used for cytospin preparations (≤100,000 cells/slide). At least 400 cells were counted for each cytospin slide stained with Wright-Giemsa stain (Sigma-Aldrich) to determine differential cell counts.

4.8 HISTOPATHOLOGY

Lungs were removed from mice after the bronchial lavage procedure and saline perfusion, and then fixed in 10% neutral buffered formalin. Samples were submitted to the Comparative Pathology Core at the University Of Miami Miller School Of Medicine, where specimens were embedded, sectioned at 5 µm each, and stained with Hematoxylin and Eosin, and Periodic acid-Schiff. Additional sections were cut for staining with Sirius Red-Picric Acid, 0.1% Sirius Red F3B in 1.3% saturated aqueous picric acid, pH 2.0 (Sigma-Aldrich).
4.9 IMAGEJ ANALYSIS OF HISTOLOGY SLIDES
Quantification of Periodic acid-Schiff stained (PAS) lung sections was performed as described previously (240). Quantification of Sirius Red-Picric Acid was performed using MacBiophotonics ImageJ software by conversion to grey scale using the RGB Stack, thresholding the green channel to 55, and measuring the area stained red with Set Measurements: Area and Limit to Threshold (Sirius Red+ Area). To measure the total area (to determine the Total Area subepithelial space), reset threshold to 125 to include entire area, and again measure with Set Measurements: Area and Limit to Threshold. Percent Sirius Red positive is the fraction of the two measurements (Sirius Red+ Area/Total Area in subepithelial space).

4.10 RNA EXTRACTION AND QUANTIFICATION
Total RNA was extracted from flash-frozen lungs and BALF recovered cells, and then reverse transcribed, using the RNeasy Mini Kit and the QuantiTect Reverse Transcription Kit from QIAGEN, respectively. Real-time PCR was performed in triplicate on an ABI 7300 Light Cycler using TaqMan probes from Applied Biosystems. Samples were done in triplicate and normalized to GAPDH in each sample, and relative to naïve controls using the $\Delta\Delta$Ct calculation.

4.11 FLOW CYTOMETRY ANALYSIS
Single-cell suspensions were prepared from peripheral blood, spleen, lymph nodes, and lungs. $10^6$ cells were pre-blocked with anti-mouse CD16/CD32 (Biolegend) and stained with different antibody combinations. Intracellular
staining was performed according to standard procedures. Flow cytometry was performed on a BD FACS Fortessa instrument with subsequent analysis with BD FACSDiva or FlowJo software.

4.12 IL-10R BLOCKING IN RAW264.7 CELLS

RAW264.7 cells were seeded at 3 x 10^5 in 6-well plates and cultured overnight with 10% v/v heat-inactivated fetal bovine serum in Iscove’s Modified Dulbecco’s Media supplemented with 50 µg/mL gentamicin (culture media). The next day, cells were then cultured for 12-hours at 37 degrees Celsius with 5% CO₂, in culture media only, 10 ng/mL LPS, LPS+ 10ng/mL IL-10, or LPS+IL-10+ 10µg/mL IL-10R blocking antibody (1B1.3A) (each in triplicate). After the 12-hour incubation, cells were scraped, washed, viable cells counted by trypan blue exclusion, and then surface stained for CD40 after Fc blocking. Cells were then analyzed by flow cytometry.

4.13 FACS SORTING

Cell sorting was done using a FACSFlowia cell sorter (BD) after enrichment of splenocytes for CD4+ T cells using the EasySep Mouse CD4+ T cell Pre-Enrichment Kit from Stem Cell Technologies.

4.14 OT-II ADOPTIVE TRANSFER

CD4+CD25-GITRlow cells were FACS sorted from OT-II mice. 10^6 of these cells were adoptively transferred i.v. into CD45.1 SJL mice on day –2. On days 0 and 5, mice were sensitized to ovalbumin similarly described in the acute model of allergic lung inflammation. On day 16 mice were aerosolized with 0.5% w/v ovalbumin in PBS for one-hour, and then sacrificed and
analyzed on day 19. Mice were bled periodically to monitor peripheral blood mononuclear cells.

4.15 **IN VITRO STIMULATION WITH OVA\textsubscript{323-339} PEPTIDE**

On day 61 of the chronic model of allergic lung inflammation, mesenteric lymph nodes were harvested, and single-cell suspensions were prepared from challenged mice or ovalbumin-naïve controls. Cells were seeded in FACS tubes at 2 x 10\textsuperscript{6} cells/tube and cultured with 2 µM OVA\textsubscript{323-339} peptide in media for 12 hours, with Golgi-Stop and Golgi-Plug (BD Biosciences). Cells were washed and then surfaced stained with flow antibodies. Cells were then fixed and permeabilized (Intracellular Staining Kit; eBioscience) and stained for intracellular IL-5 with fluorochrome-conjugated anti-IL-5 antibody (eBioscience).

4.16 **PEPTIDE:MHC CLASS II STAINING AND ENRICHMENT**

The peptide:MHC Class II tetramer staining and enrichment was followed as described previously (254). Briefly, single-cell suspensions of splenocytes were stained with OVA:I-\textsuperscript{A\textsubscript{b}} pMHC Class II tetramers conjugated to PE, and enriched using anti-PE antibody conjugated to magnetic microbeads (Miltenyi Biotec) using an autoMACS separator to enrich (Miltenyi Biotec). The enriched fraction was then stained with a cocktail of fluorochrome-labeled antibodies specific for CD3, CD4, CD8, CD19, Gr-1, NK1.1, CD11c, and CD11b, and then gated on CD8- CD19- Gr-1- NK1.1- CD11b- CD11c- CD3+ CD4+ cells.
4.17 CD4⁺⁻ ALLERGIC LUNG INFLAMMATION MODEL
Cd4⁻⁻ mice were either i.v. injected on day -7 with 2 x 10^6 or 10 x 10^6 CD4⁺ enriched splenocytes from wild-type mice, or i.p. injected on day -7 with 10 x 10^6 or 12 x 10^6 CD4⁺ enriched splenocytes from wild-type mice. Mice were then injected on day 0 and 5 with ovalbumin adsorbed to ALUM similarly described above in the acute model of allergic lung inflammation. Mice were then aerosolized with 2.5% w/v ovalbumin in PBS for one-hour on day 16, with sacrifice and analysis of mice on day 19. Mice were bled periodically to monitor peripheral blood mononuclear cells.

4.18 STATISTICS
All graphing and statistical analysis was performed using Graphpad Prism. Unpaired analysis was performed using student's T-test. Multiple variable-analysis was performed using 1-way ANOVA and Tukey post-test. A P-value less than 0.05 was considered significant.
REFERENCES


