CD2 REGULATES PATHOGENESIS OF ASTHMA INDUCED BY HOUSE DUST MITE EXTRACT

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ABSTRACT

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The inflammatory immune response that is characteristic of allergic asthma is triggered by exposure to allergens such as house dust mice extract (HDME) and is driven by CD4+Th2 lymphocytes that secrete large quantities of Th2 cytokines including interleukin (IL)-4, IL-13, and IL-5. Surface expression of CD2 and its ligand CD58, is increased on the monocytes and eosinophils of asthma patients, which correlate with elevated serum IgE levels, suggesting that CD2 may contribute to allergic airway inflammation. Using a murine model of asthma, we observed that HDME-exposed Balb/c mice have increased airway hyper responsiveness (AHR), cellular infiltration, goblet cell hyperplasia, and elevated levels of Th2 cytokines in the lungs, as well as increased serum IgE levels as compared to the control mice. In contrast, AHR, and cellular infiltration were significantly reduced in HDME-exposed *Cd2*$^{-/-}$ mice. Interestingly, *Il13* but not *Il4* or *Il5* gene expression in the lungs was significantly reduced in HDME-exposed *Cd2*$^{-/-}$ mice. Of note, the gene expression of polymeric mucins, *Muc5b* and *Muc5ac* were significantly reduced in the lungs of HDME-exposed *Cd2*$^{-/-}$ mice. Furthermore, gene expression of various protective micro RNAs were increased in the lungs of HDME-exposed *Cd2*$^{-/-}$ mice. Lastly, in a mouse model of neutrophilic asthma, *Cd2*$^{-/-}$ mice exhibit significantly reduced AHR and *Il13* and *Il17a* gene expression. Given the established role of IL-13 in promoting goblet cell hyperplasia and mucus production during allergic asthma, and various micro RNAs regulating different aspects of asthma symptoms, our studies reveal a unique role for CD2 in both Th2-high and Th2-low asthma.

I dedicate this thesis to my Mother, Father, Sister, Brother & Niece.

ACKNOWLEDGMENTS

The word research is derived from the term “recherche” in Middle French which means “to go about seeking”. My intention when I started this program was to find a guide and mentor who could help me pick what I wanted to go about and seek. I have been extremely lucky to have found an amazing scientist and mentor. First and foremost, a thank you to my mentor and advisor Dr. Rupali Das. Thank you for the endless support, encouragement and stimulating hour-long scientific discussions. Thank you for holding my hand, every time I needed help with the smallest things, and thank you for answering the millions of questions I would ask even though it was borderline annoying. You have molded me into thinking like a scientist, and your passion for science has transferred into me to the point of making the decision to continue pursuing research in the future. Thank you for always being my number one supporter and lifelong cheerleader. You have given me the gift of being confident in my abilities and potential, which I will carry along with me in this crazy journey called life. I am proud to say I have made a lifelong mentor and friend from this experience. I am indebted to you for giving me something to go about seeking. It was an honor and privilege with a scientist of your caliber.

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nature of the lab, which was very beneficial to my progress. Lastly, I must express my gratitude to all the members of Dr. Subramanian's lab for their technical assistance where appropriate.

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PREFACE

The primary motivator, and basis of my research stems from clinical stories and experiences I have has as a child with allergic asthma and brought me to the ongoing search for a novel therapy for asthma. Dr. Rupali Das has enlightened me on the emerging potential of immunotherapy, and its successes and deficits. I performed these studies with hopes of one day contributing to advancement and discovery of more powerful and precision-based medicine.

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KEY TO ABBREVIATIONS

3'UTR	3'-untranslated region
AHR	Airway hyperresponsiveness
ANOVA	Analysis of variance
APC	Antigen presenting cell
ASMC	Airway smooth muscle cell
Asp	Aspergillus fumigatus
BALF	Broncho alveolar lavage fluid
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
c-di-GMP	Cyclic diguanylate
CRTH2	Chemoattractant-receptor homologous molecule expressed on Th2 cells
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
EGFR	Epidermal growth factor receptor
Ers	Elastance (airway)
FcεRI	Fc epsilon receptor I
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HDME	House dust mite extract
H&E	Hematoxylin & eosin
ICOS	Inducible T-cell costimulator

IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
IL-13Rα1	Interleukin 13 receptor alpha 1
IL-13Rα2	Interleukin 13 receptor alpha 2
iNKT	Invariant natural killer T cell
M1	Classically activated macrophage
M2	Alternatively activated macrophage
Mch	Methacholine
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
miRNA	Micro RNA
MMP	Matrix metalloprotease
MUC	Mucin
NK	Natural killer cell
OVA	Ovalbumin
PAS	Periodic acid-schiff
PBMC	Peripheral blood mononuclear cells
qPCR	Quantitative real-time polymerase chain reaction
Rrs	Resistance (airway)
Rn	Newtonian resistance (airway)
TCR	T cell receptor

T_{FH}	Follicular helper T cell
TGF	Transforming growth factor
Th	T helper cell
TNFR	Tumor necrosis factor receptor
T_{reg}	Regulatory T cell
TSLP	Thymic stromal lymphopoietin

CHAPTER 1

OVERALL INTRODUCTION

Asthma is a chronic inflammatory disorder that affects more than 300 million people worldwide[1]. Individuals with asthma not only suffer from low quality of life, but also are prone to premature death. The cost of asthma around the world is increasing and contributes to the increased global economic burden. Due to the high costs of asthma treatment and its role in limiting social aspects of life, it can be deemed a major public health issue[2].

The pathogenesis of asthma involves both the cells of the innate and the adaptive arms of the immune system. The immune cells involved in asthma pathogenesis result in hallmark characteristics of asthma such as coughing, wheezing, shortness of breath due to airway hyperresponsiveness (AHR), bronchoconstriction, goblet cell hyperplasia and airway remodeling[1]. Asthma has been traditionally defined as a T helper 2 (Th2) cell mediated disease resulting in airway eosinophilia[3]. However, further research in human asthma patients found that there exists another endotype of asthma that demonstrates airway neutrophilia that is induced by T helper 17 (Th17) cells, and also in some cases, a mixed granulocytic asthma phenotype with the presence of both eosinophils and neutrophils in the airways[4, 5, 6]. These discoveries led to a consensus in the field that indeed asthma is a complex heterogeneous disease that can be induced by multiple mechanistic pathways, and the various endotypes and phenotypes of asthma need to be studied more extensively in order to make treatment more precise and effective[7, 8].

Traditionally, asthma was categorized into two different forms: non-allergic and allergic phenotypes. Non-allergic asthma can be classified into exercise-induced asthma, irritant-induced asthma or obesity-related asthma[9]. Allergic asthma is the most common form of asthma and is caused by sensitization to allergens. Examples of common allergens that can trigger asthma

include pollen, mold, cockroach, pet dander, and dust mites[10]. Current literature has shed more light into the immunopathogenesis of asthma and has better classified asthma into two different endotypes[3, 4] according to the most common mechanistic pathways: Th2-high and Th2-low asthma shown in Figure 1.

Th2-high endotype

The Th2-high endotype of asthma involves CD4+ Th2 cells as key players and results in airway eosinophilia. This endotype can be a result of both allergic and non-allergic eosinophilic asthma[9]. In allergic Th2-high asthma, an allergen is first taken up by an antigen presenting cell such as a dendritic cell. The dendritic cell digests the allergen and presents the most immunogenic portion to a naïve CD4+ T helper cell (Th0) by the major histocompatibility complex (MHC) II protein. When the T-cell receptor (TCR) of the Th0 cell recognizes the allergen presented by the MHC class II molecule on the dendritic cell, it differentiates in to a Th2 which is now capable of producing cytokines such as interleukin (IL)-4, IL-5, and IL-13[3,11]. These Th2 cytokines then have their own functions in the causation of the hallmark asthma symptoms. In non-allergic Th2 high asthma, it is not an allergen that initiates an inflammatory response, but an irritant that can disrupt the epithelial barrier lining the airways. Irritants that can disrupt the barrier include diesel fumes, ozone, and smoke and cause the release of different cytokines such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP)[12, 13]. These cytokines, known as alarmins, can induce the production of Th2 cytokines from a type of innate cell known as innate lymphoid cells type 2 (ILC2)[13]. ILC2s secrete Th2 cytokines such as IL-4 and IL-13 which in turn induces Th0 cells to become Th2 cells[3,11]. The Th2 cells make IL-5 which will recruit eosinophils into the airways[14]. Exercise induced asthma is another type of non-allergic asthma that results in a Th2-high endotype.

This form of asthma occurs when excessive exercise causes an increase in evaporative force on epithelial cells, leading to water loss. The water loss causes proinflammatory mediators to be released and results in the activation and degranulation of mast cells. Signaling molecules and cytokines released following mast cell degranulation induces AHR and airway remodeling[15]. This type of asthma also is caused by genetic predisposition of loss of function of aquaporin channels, resulting in the dehydration of the cells[15].

Th2-low endotype

The Th2-low endotype of asthma involves Th17 and Th1 cells commonly resulting in airway neutrophilia[4, 5, 6]. It develops more later in life, most commonly in the elderly and is less likely to be of an allergic form[13]. Although non-allergic Th2-low endotypes are most common, the allergic forms of Th2-low endotype has been seen in a subset of patients and most commonly arises from a Th2-high allergic asthma phenotype. This can occur due to the suppression of the Th2 pathway through the use of drugs, that causes a switch in the asthma endotype[16]. Non-allergic forms of Th2-low asthma include irritant induced asthma and obesity-related asthma[17]. Irritants such as smoke, ozone, or diesel gas fumes induce asthma and are typically mediated by a combination of Th1/Th17 cytokines[17, 18, 19, 20, 21]. Non-allergic Th2-low asthma can also be caused by microbes or viruses which result in airway neutrophilia and again involve Th1 and Th17 cells.

Role of immune cells in asthma

Given the high expression of different immune cells of the innate and the adaptive arm of the immune system in the lungs of asthmatic patients, the specific roles of each type of immune cells have been extensively studied as well to understand the immunopathogenesis of asthma better.

Immune cells playing a major role in asthma are shown in Figure 2 and Figure 3 according to the asthma endotype.

Dendritic cells (DCs) are known as the professional antigen presenting cells in our immune system. In allergic asthma, several groups have reported that the sensitization phase of the allergen starts with naïve T cells being primed with the help of dendritic cells in mouse models[22, 23, 24, 25]. Dendritic cells express the protein major histocompatibility complex (MHC) II, which presents the digested allergen to a naïve T cell[26]. There are 3 types of DCs classified according to different origins and they include conventional DCs (cDCs), plasmacytoid DCs (pDCs), and monocyte-derived DCs (moDCs)[27, 28]. Out of the 3 types of DCs, the cDCs are associated with naïve CD4 T cell differentiation into Th2 allergen specific cells[29]. CD4 T cells are the initiators of the allergic Th2 high endotype of asthma.

High amounts of **CD4 T cells** have been found in the lungs of asthma patients compared to normal patients[30]. They are activated and differentiate into Th2 cells when presented with an antigen/allergen. This event occurs during the first exposure to allergen or the sensitization phase, however, there is no immune response against the foreign body[31]. During second exposure to allergen or the challenge phase, allergen specific Th2 cells in the lungs are activated and produce copious amounts of Th2 cytokines such as IL-4, IL-5 and IL-13, which causes hallmark asthma symptoms[32].

IL-4 is a known activator of **B cells** resulting in the production of immunoglobulins, in this case IgE. Once B cells are activated by IL-4 produced by Th2 cells, they differentiate into plasma cells and induce class switch of immunoglobulins to allergen specific IgE. They release the IgE into the

blood stream and once the IgE comes across a mast cell, they bind with high affinity to the receptor FcεRI[33]. B cells have also been shown to have a role in the priming of T follicular helper (T_{FH}) cells into effector Th2 cells in mouse models of allergic asthma[34].

Along with high numbers of CD4 T cells, **CD8 T cells** have also been reported to be found in high numbers in the bronchoalveolar lavage fluid (BALF) and sputum of asthma patients[35]. The role of CD8 T cells are overlooked in an allergic asthma setting due to its main function which is cytotoxicity. However, unlike their conventional function in cytotoxicity, CD8 T cells have been reported to produce Th2 cytokines IL-4, IL-5 and IL-13 in asthma patients[36]. They have also been reported to be in higher numbers in steroid resistant asthma, which is normally associated with airway neutrophilia[37]. It is also possible that in severe eosinophilic asthma, CD8 T cells are the cause of steroid resistance. Due to their role in Th2 cytokine secretion, they could be playing a role in immune cell recruitment and airway remodeling and their steroid resistance could be providing them with a more prominent role in asthma than expected. Given that CD4 T cells are more sensitive to steroid treatment, the steroid resistance of CD8 T cells could be replacing the role of CD4 T cells, in terms of Th2 cytokine production.

Macrophages, traditionally known as phagocytes, play a major role in tissue homeostasis and guard the mucosa to maintain the homeostasis. The maintenance is carried out by specific functions the macrophages have, one of them being that they are tissue resident. Macrophages are also highly plastic and can communicate with other immune cells when activated by secreting cytokines and chemokines[38]. Macrophages have two general phenotypes known as M1 or M2. M1 macrophages are traditionally named the classically activated macrophages driven by Th1 cytokines such as

IFNγ and TNF-α. M2 macrophages on the other hand are called the alternatively activated macrophages driven by Th2 cytokines such as IL-4 and IL-13[39]. Given their plasticity and other functions in contacting other cells of the immune system, macrophages can either be proinflammatory or anti-inflammatory. Many reports have shown macrophages ameliorate asthma symptoms[40] while others have shown that macrophages exacerbate asthma symptoms by releasing cytokines to recruit and activated other immune cells[41]. The function of macrophages in allergic asthma is controversial due to different groups reporting different functions observed, but it is agreed in the field that M2 macrophages are associated with allergic asthma patients in comparison to normal patients[42].

Mast cells are part of the innate immune system and multiple reports have shown that they are found in the airway smooth muscle of asthma patients in comparison to normal patients[43]. Higher number of mast cells have been found in the lungs of allergic asthma patients compared to patients with non-allergic asthma[44]. Higher number of mast cells in the lungs has also been associated with the Th2 high endotype of asthma[45]. Mast cells are activated and degranulate when allergen specific IgE antibodies crosslinked with the high affinity receptor FcεRI bind with the allergen[33]. Mast cell degranulation releases mediators that include histamine, enzymes, leukotrienes, and cytokines amongst multiple other mediators that causes bronchoconstriction, airway hyperresponsiveness, and goblet cell hyperplasia[33]. Mast cells also release chemokines that can recruit other immune cells, most importantly eosinophil chemotactic factor (ECF) which recruits eosinophils into the airway microenvironment[33].

Eosinophils are the hallmark immune cells associated with allergic Th2 high endotype of asthma[46]. In a Th2 high endotype of allergic asthma, eosinophils are the result of IgE mediated activation and degranulation of mast cells which in turn release chemokines to recruit them[33]. In a non-allergic Th2 high endotype of asthma, irritants will disrupt the epithelial layer of the airway mucosa causing the epithelial cells to release alarmins such as IL-25, IL-33 and TSLP. These alarmins work on innate lymphoid cells (ILCs), which produce Th2 cytokines such as IL-4, IL-5, and IL-13[12, 13]. IL-5 is a known recruiter of eosinophils and is the reason for eosinophilia in the non-allergic Th2 high endotype of asthma[14]. Eosinophils also degranulate after activation and release mediators such as leukotrienes, enzymes, growth factors and cytokines[33]. They are associated with disease severity and due to their release of enzyme elastase which breaks down elastin in the airway and lungs, can cause stiffness in the airways[46].

The most important innate immune cell that plays a major role in Th2-low non-allergic asthma is known as a **neutrophil**[4, 5, 6]. Neutrophils play a major role in microbial or irritant induced asthma[17]. They are recruited by the cytokine IL-17A, which is produced by Th17 or ILC3s at the site of irritant or microbe exposure[47, 48]. Similar to mast cells and eosinophils, neutrophils also degranulate and release multiple mediators. The mediators include enzymes, cytokines, leukotrienes and histamine. Like eosinophils, neutrophils also release elastase as well as collagenase and gelatinase such as matrix metalloprotreinase-9 (MMP-9)[49]. These enzymes break down collagen and extracellular matrix resulting in tissue remodeling.

Fairly new as an established player in asthma are another type of innate immune cell known as **innate lymphoid cells**. There are three subtypes of ILCs known as ILC1, ILC2 and ILC3. Each of

these subtypes are similar in function to Th1, Th2, and Th17 cells in terms of their cytokine production profiles. ILC1s can be subdivided into **natural killer cells** and ILC1s. Natural killer cells are the subtype of ILC1s that were discovered first followed by the discovery of ILC1s. Natural killer cells and ILC1s both produce Th1 cytokines such as IFNy and TNFα. ILC2s produce Th2 cytokines IL-4, IL-5 and IL-13 whereas ILC3s produce Th17 cytokines such as IL-17 and IL-22[47]. The role of ILC2s specifically has been established in alarmin induced non-allergic Th2 high endotype of asthma. Epithelial cells release alarmins after undergoing damage and stress, which in turn activates the ILC2s to produce high amounts of Th2 cytokines. ILC1s and ILC3s have been implicated to play a role in neutrophilic asthma due to their function in regulating Th1 and Th17 mediated inflammation[47].

Innate and adaptive immune cells both play major roles in the pathogenesis of asthma, however, there are cells in the immune system that play both innate and adaptive immune roles. One such cell known as **invariant natural killer T (iNKT)** cell, has been implicated to play a role in asthma pathogenesis. Invariant natural killer T cells are innate T cells that express an invariant TCR that recognizes only glycolipid antigens, and upon activation can release high amounts of Th1 and Th2 cytokines due to intracellular storage of pre-made mRNA[50]. These cells have been reported by multiple groups to be expressed highly in sputum and BALF of asthma patients[51, 52, 53], and other groups have observed no significant increase in iNKT cells in asthma patients[54, 55]. Due to the contradictory reports, there is controversy surrounding its role in asthma pathogenesis. There is, however, a consensus in the field that perhaps iNKTs play a role early in the disease progression and exacerbation[56]. If that is true or not, is yet to be determined in animal models of asthma and human asthma.

Current therapies in asthma

Traditional therapy for asthma includes inhaled or systemic corticosteroids, bronchodilators, and anti-leukotrienes[22]. However, given the heterogeneity of asthma and its various endotypes, traditional therapy is not always effective for all patients. About 5-10% patients don't respond to all kinds of traditional asthma therapy and have been deemed "severe asthmatics" [4]. In the case of these patients, the use of "precision medicine" is a better route to follow in terms of the asthma phenotype and endotype they present with[57]. Specific immunotherapy such as monoclonal antibodies targeting key players in asthma such as cytokines, IgE, or cell surface receptors on immune cells have been developed. The first one to be developed was an anti-IgE monoclonal antibody for patients with severe allergic asthma[58]. It has been extremely effective in reducing hallmark asthma symptoms such as airway inflammation, eosinophil numbers, airway remodeling and IgE levels in these patients[59, 60]. However, anti-IgE has only been effective in severe asthma patients with proven sensitization to perennial allergen and high serum IgE levels[58]. Monoclonal antibodies against Th2 cytokines such as IL-4, IL-5 and IL-13 or their receptors have also been developed to tackle asthma patients with a Th2-high endotype. Monoclonal antibodies targeting IL-5 or its receptor have shown reduction in asthma exacerbations and oral corticosteroid use with improved quality of life for patients with severe eosinophilic asthma[61, 62, 63, 64, 65, 66, 67]. A monoclonal antibody targeting IL-4R alpha chain that is a shared in a common receptor for cytokines IL-4 and IL-13 have been also successful in reducing exacerbations, improved lung functions and tolerance to corticosteroid withdrawal[68, 69]. Monoclonal antibodies targeting IL-13 receptors, however, have not been as successful as IL-4 or IL-5[70]. Two recent anti-IL-13 clinical trials were unsuccessful in controlling severe asthma and were discontinued and a current one is in phase I of clinical trial[71, 72, 73]. Given that IL-13 is a major effector cytokine in the Th2-high

endotype of asthma, it is critical to develop a target for IL-13 in order to reduce mucus production, airway hyperresponsiveness, and irreversible airway fibrosis and remodeling in patients. Other monoclonal antibodies under clinical trials are targeting alarmins such as IL-33 and TSLP with anti-TSLP showing improved lung function and reduced exacerbations[74]. A monoclonal antibody against the chemoattractant-receptor homologous molecule expressed on Th2 cells (CRTH2) has been in the process of being tested in two clinical trials. It is known to recruit eosinophils and basophils, as well as initiate the release of Th2 cytokines[75]. It is a promising candidate for Th2-high endotypes of asthma, but its effectiveness is yet to be determined. Collectively, these precision medicine-based therapies have shown great potential in reducing asthma symptoms but have all been targeting single molecules that play a role in asthma. Novel precision medicine-based therapies that can target a molecule upstream of Th2 cytokine production are yet to be tested in the context of asthma.

APPENDIX

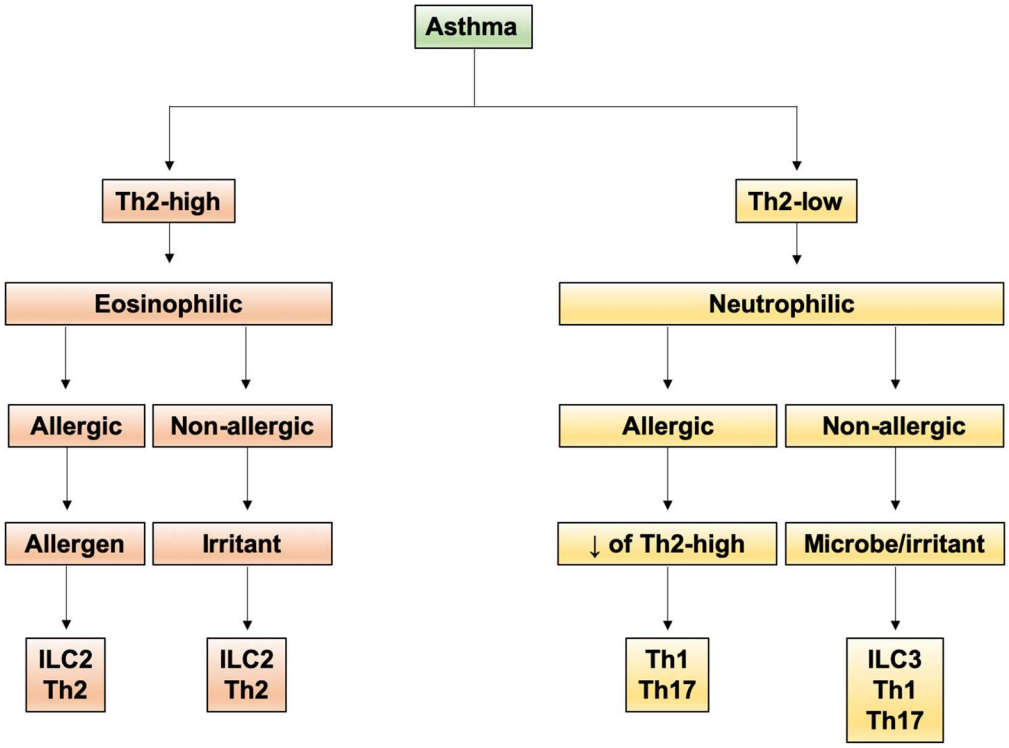


Figure 1. Classification of asthma subsets. Due to the help of proteomic and genomic sequencing of asthma patients, asthma has been divided into two different endotypes; Th2-high and Th2-low. Th2-high asthma is normally characterized by airway eosinophilia whereas Th2-low asthma is characterized by airway neutrophilia. Th2-high eosinophilic asthma can be allergic and non-allergic and is differentiated by the antigen that initiates the asthma pathogenesis. For allergic asthma, the antigens can be allergens such as dust mite, pollen, mold, cockroach and animal dander. In non-allergic asthma, the antigens are irritants such as ozone, diesel fumes and smoke. Th2-low neutrophilic asthma can be a result of allergic or non-allergic asthma. The allergic Th2-low asthma is induced by a suppression of Th2-high eosinophilic asthma by the use of drugs such as corticosteroids. Non-allergic Th2-low asthma can be induced by irritants and microbial infections in the airways. Immune cells that play a role in Th2-high asthma are ILC2s and Th2 cells. Th1 and Th17 cells play a role in allergic Th2-low asthma but in non-allergic Th2-low

Figure 1. (cont'd)

asthma, both Th1 and Th17 along with ILC3s play a role.

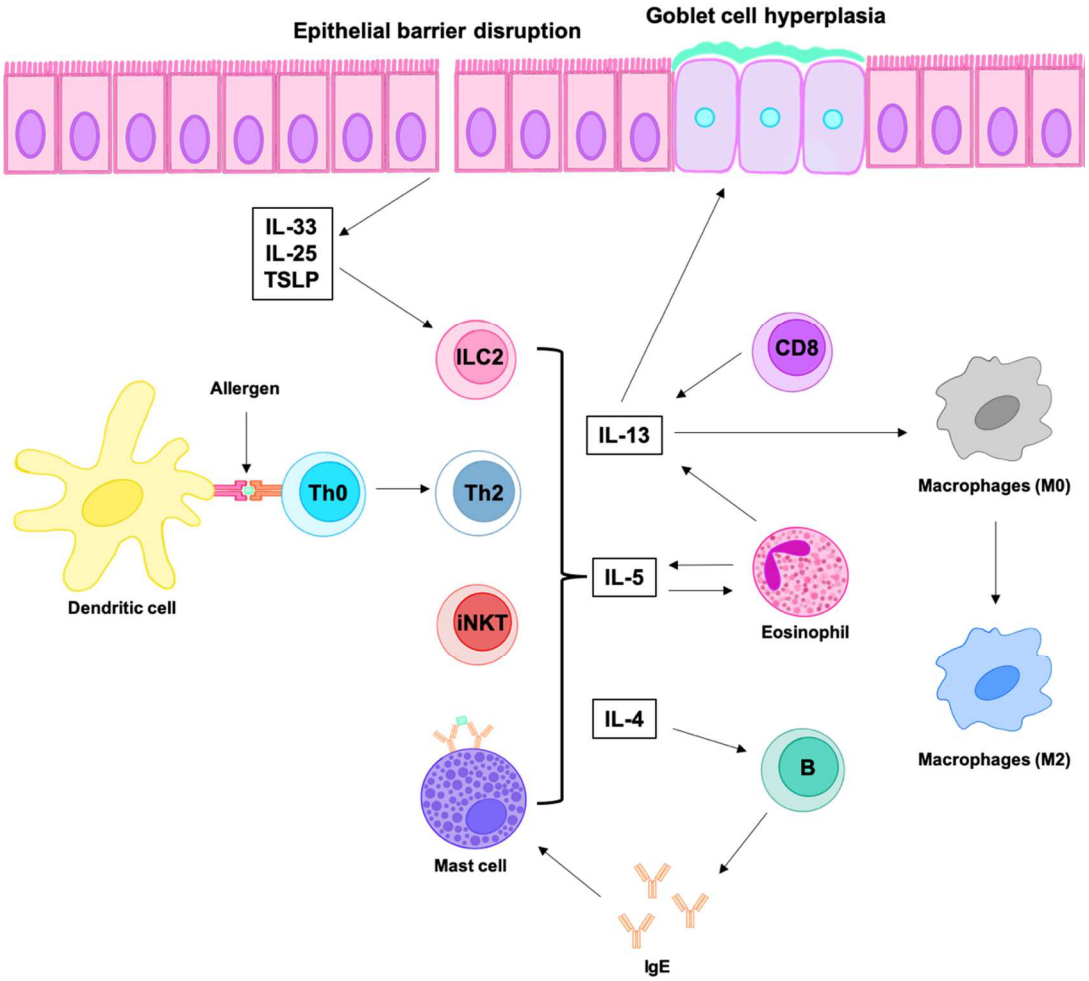


Figure 2. Immune cells in Th2-high asthma. In allergic Th2-high asthma, dendritic cells initiate the sensitization to an allergen by presenting it to naïve T cells (Th0). The naïve T cell differentiates into a Th2 cell. Th2 cells can produce IL-4, IL-5, and IL-13. Along with Th2 cells, CD8 T cells, mast cells and iNKT cells can also produce Th2 cytokines. IL-4 causes B cells to make IgE, which binds to mast cells and causes degranulation. IL-5 recruits eosinophils and IL-13 causes goblet cell hyperplasia. IL-13 can also alternatively activate macrophages to an M2 phenotype. If it is a non-allergic Th2-high asthma, airway epithelia are disrupted by irritants which induces the airway epithelial cells to make alarmins, IL-33, TSLP and IL-25. The alarmins induce ILC2s to make Th2 cytokines and initiate the same pathway as Th2 cells.

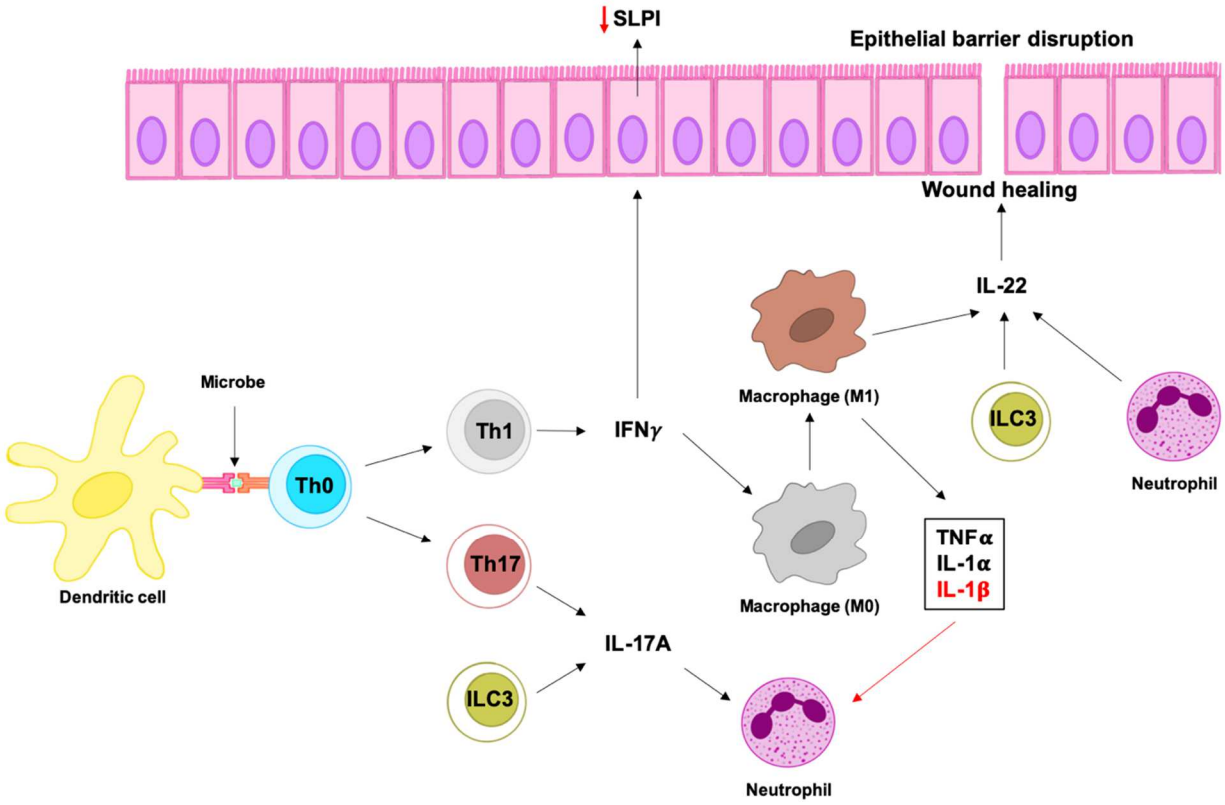


Figure 3. Immune cells in Th2-low asthma. Dendritic cells initiate the sensitization to an allergen by presenting it to naïve T cells (Th0). The naïve T cell differentiates into Th1 and Th17 cells depending on the antigen presented, in this case microbial antigen. Th1 cells can produce IFNγ, which works on epithelial cells and reduces the production of secretory leukocyte protease inhibitor (SLPI). IFNγ can also classically activate macrophages to an M1 phenotype. The M1s can make large amounts of proinflammatory cytokines such as TNFα, IL-1α and IL-1β. IL-1β can also recruit neutrophils along with IL-17A that is produced by both Th17 cells and ILC3s. The M1s, ILC3 and neutrophils can also make IL-22, which can initiate wound healing in the disrupted epithelia. Non-allergic Th2-lo asthma is normally associated with microbes, irritants and obesity. However, allergic Th2-low asthma results from suppression of Th2-high asthma most commonly by medication and in turn switching the phenotype to a Th2-low endotype.

Table 1. Cytokines involved in Th2-high vs Th2-low endotype of asthma.

Phenotype/Endotype	Cytokine	Secreted by
Eosinophilic/Th2-high	IL-4	Th2,ILC2,iNKT,MC
	IL-5	Th2,ILC2,Eosinophils,MC
	IL-13	Th2,ILC2,iNKT,MC
Neutrophilic/Th2-low	IFN-γ	Th1,NK,ILC1,iNKT
	TNF-α	Th1,Macrophages,MC
	IL-1α	Macrophages, Neutrophils
	IL-1β	Macrophages, MC
	IL-17A	Th17,ILC3,Neutrophils
	IL-17F	Th17,ILC3

Th1, T helper 1 cell; Th2, T helper 2 cell; Th17, T helper 17 cell; NK, Natural killer cell; iNKT, invariant Natural killer T cell; ILC1, Innate lymphoid cell 1; ILC2, Innate lymphoid cell 2; ILC3, Innate lymphoid cell 3; MC, Mast cell.

CHAPTER 2

CD2 EXPRESSION IS UPREGULATED IN THE LUNGS OF ASTHMATIC MICE AND HUMAN PATIENTS

Introduction

The most common form of asthma is allergic asthma, which is caused by sensitization to environmental antigens, or allergens[10]. Most people living in North and South America, Australia and Europe have been reported to be sensitized to house dust mite as these countries have the environment for dust mites to thrive[76]. In experimental mouse models of asthma, people have extensively used ovalbumin (OVA), which is derived from chicken egg, as an allergen[77]. However, asthma caused by dust mites have been studied in recent years due to its clinical relevance and the mechanistic endotype has been categorized to a Th2-high endotype with high airway eosinophilia[78].

The Th2-high endotype of asthma consists of multiple immune cells that play a role in disease progression, the most important being the CD4+ T helper 2 (Th2) cells[3]. Upon antigen presentation by dendritic cells, naïve T (Th0) cells differentiate into Th2 cells, which in turn make copious amounts of cytokines such as IL-4, IL-5 and IL-13[3, 11]. Each of these cytokines play critical roles in disease progression. The production of these Th2 cytokines, however, is preceded by T cell activation. T cell activation has been extensively studied and two important steps have been defined. The first signal of T cell activation is the engagement of the T cell receptor (TCR) to the major histocompatibility complex (MHC)[79]. Antigen presentation by the MHC molecule to the TCR is followed by the second signal, known as co-stimulation[79]. There are several co-stimulatory molecules expressed on T cells, of which CD28 is the most well studied. CD28 binds to its ligand CD80 or CD86 on antigen presenting cells (APC), providing further adhesion and signaling for T cell activation. Other co-stimulatory molecules and their ligands such as cytotoxic T-lymphocyte-

associated protein (CTLA-4)/CD28 and inducible T-cell costimulator (ICOS)/ICOS-L, which are part of the B7 family of receptors, as well as OX40/OX40L, CD40/CD40L and 4-1BB/4-1BB-L, which are part of the tumor necrosis factor receptor (TNFR) family have been studied in the context of T cell activation. CD28-CD80/86 and ICOS-ICOS-L ligation plays a role in T cell activation whereas, CD28/CTLA-4 interaction negatively regulates T cell activation. These costimulatory molecules have also been studied in the context of asthma[80] and are shown in Figure 4.

CD28 deficient mice induced with allergen did not develop asthma, suggesting the importance of its role in the pathogenesis of asthma. ICOS has been shown to intensify the function of CD28 in T cells[81]. Multiple studies in mouse models have shown that ICOS plays a role in both enhancing or inhibiting Th2 responses[82, 83]. Adoptive transfer of ICOS+ T cells after allergen challenge into WT mice resulted in infiltration of T and B cells and local production of IgE[82] whereas, ICOS-deficient mice were not able to produce high levels of IgE in response to allergen[84]. These studies suggested that ICOS plays an important role in asthma pathogenesis. Yet, adoptive transfer of ICOS+ regulatory T cells (T_{reg}) was shown to prevent AHR in allergen-induced mice[83], suggesting the role of ICOS can vary depending on the type of cell it is expressed on. Polymorphism in CTLA-4 in humans has been related to allergy[85]. Similarly, in a mouse model of asthma, administration of a CTLA-4 blocking antibody during the sensitization phase resulted in reduced production of allergen-specific IgE[86]. OX40 and OX40L-deficient mice were shown to have reduced Th2 responses as well as reduced lung inflammation[87, 88]. Relatedly, OX40 was shown to inhibit development of CD4+ FoxP3+ T_{regs}[89, 90]. Lastly, in a mouse model of asthma, 4-1BB blocking antibody reduced AHR and allergen-specific IgE production[91] while in another study, prophylactic administration of 4-1BB blocking antibody prevented the development of AHR, production of

allergen-specific IgE production, and infiltration of eosinophils[92]. Taken together, these studies demonstrate that costimulatory molecules are an attractive target for asthma therapy.

Another important costimulatory molecule that plays a big role in T cell activation is CD2. CD2 is a part of the immunoglobulin superfamily of receptors and is a transmembrane glycoprotein. It has two extracellular Ig like domains and a long proline rich cytoplasmic tail[79]. It binds with high affinity to its ligand CD58 in humans, and CD48 in mice[93, 94]. Previously, CD2 was thought to function solely as an adhesion molecule and provided little to no co-stimulation for T cell activation[79]. However, recent studies have shown that CD2, similar to CD28, can also bind to src kinases such as lyk and fyn, indicating they play a role in T cell signaling[95, 96, 97, 98, 99]. Knockout studies suggested that CD2 and CD28 present redundant roles in T cell function[100]. Yet, recent studies have shown that CD28 or CD2 induced co-stimulation results in distinct signaling responses in T cells[101]. CD28 co-stimulation was shown to produce Th1 cytokines such as IFNγ and IL-2. In contrast, CD2 co-stimulation has been shown to induce a more Th2 response, by producing more IL-4 and has also been shown to induce IgE class switch in a CD40L independent manner[101, 102]. In the case of humans, the ligand for CD2, CD58, has been shown to be highly expressed in the eosinophils of asthmatic patients[102]. Furthermore, increased expression of FcεRI on $CD2^{high}$ monocytes from asthma patients were positively correlated with higher serum IgE levels[103]. Although these studies provide an association between CD2 and asthma, the specific role of CD2 in the context of allergic asthma remains unclear.

Here, we show that asthma patients express higher levels of CD2 and Th2 cytokines, IL-4 and IL-13. Hence, we generated *Cd2*$^{-/-}$ mice on a Balb/c (WT) background in order to study the role of

CD2 in house dust mite extract (HDME) induced asthma. Additionally, we show that CD2, not CD48, is upregulated in WT mice challenged with HDME-induced asthma. Collectively, these observations indicate that CD2 indeed plays a role in disease pathogenesis of a Th2-high endotype of allergic asthma with the use of a clinically relevant allergen. Thus, CD2 may be an important costimulatory molecule that has been previously overlooked, due to the lack of studies on its co-stimulatory functions in different immune cells and presents itself to be a potential target for treatment of Th2-high allergic asthma.

Materials and Methods

Mice

Balb/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). *Cd2*$^{-/-}$ mice on a C57BL/6 (B6) background was a kind gift from Dr. Dorothy Yuan (University of Texas Southwestern Medical Center, Texas). *Cd2*$^{-/-}$ mice on a Balb/c background were obtained by backcrossing B6.*Cd2*$^{-/-}$ mice with Balb/c mice for 9 generations. All animal studies were approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (IACUC). Mice used for experiments were 10-11 weeks old.

Mouse model of mild asthma

Balb/c (wild-type) and *Cd2*$^{-/-}$ age-matched female mice were injected intranasally (i.n) with 25µl of 2µg/µl house dust mite extract (HDME) (28750 EU/vial; Stallergenes Greer, UK) or 25µl of PBS on alternate days for a total of 7 injections. Twenty-four hours after the final HDME challenge, mice were anesthetized for measurement of airway hyperresponsiveness (AHR) and then sacrificed for collection of blood, bronchoalveolar lavage fluid (BALF) and lung tissue for various endpoint analysis.

Human lung samples

Lung samples from control subjects who died from other causes and individuals who died from complications of asthma were obtained from either the International Institute for the Advancement of Medicine (IIAM) or National Disease Resource Interchange (NDRI) and its use was approved by Institutional Review Board at Rutgers University. All donor tissue samples were harvested

anonymously and de-identified. Thus, the use of these samples does not constitute human subjects research.

Quantitative real-time PCR

Lungs were dissociated in TRIzol solution (Invitrogen) using a high-speed homogenizer (Fisher Scientific, Hampton, NH) and total RNA was extracted as per manufacturer's protocol. RNA (2 μg) was reverse transcribed into cDNA using SuperScript III in a 20 μl reaction volume according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed using Quant Studio™ 3 system (Applied Biosystems) with validated Taqman primers and Fast Advanced Master Mix according to manufacturer's instructions. Relative gene expression data (fold change) between samples was accomplished using the $2^{-\Delta\Delta Ct}$ method. GAPDH (for gene expression) was used as the internal reference control.

Isolation of splenocytes

Single cell suspension of splenocytes was obtained by mashing the spleen through a 70μm cell strainer (Alkali Scientific Inc, Fort Lauderdale, FL) with a plunger. The isolated cells were washed and resuspended in RPMI media conditioned with 2.5% bovine calf serum (BCS) (VWR International, Radnor, PA) and 1% penicillin-streptomycin (Mediatech Inc, Manassas,VA).

Antibodies and flow cytometry

The antibodies used for immunofluorescence staining include CD4, CD11b, CD80, B220, DX5, TCR-β, and SiglecF (BD Biosciences, San Jose, CA). CD2, CD8, CD11c, CD28, CD40, CD48, CD86, CTLA-4, ICOS, IA/IE (MHCII), Ly6G, OXO-40 and 4-1BB were from Biolegend, San

Diego, CA. Fluorochrome conjugated CD1d-tetramer (CD1d-Tet) loaded with glycolipid antigen (PBS57), or unloaded controls were provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA). Data was collected on a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Statistics

Student's t-test with Welch's correction was used as indicated in the figure legends. Significance is shown as *($p<0.05$), **($p<0.01$), or ns for non-significant values. Statistical significance was determined using GraphPad Prism software (GraphPad, San Diego, CA).

Results and Discussion

CD2 is expressed in the lungs of human asthmatics. The ligand for CD2 in humans, CD58[93], was shown to be upregulated on the eosinophils from asthma patients[102]. In addition, the Th2-high endotype of asthma is the most common form of asthma in humans[3]. Therefore, we measured gene expression levels of CD2 and Th2 cytokines, IL-4 and IL-13, in the lungs of asthma patients. CD2 expression was significantly upregulated in the lungs of asthma patients in comparison to healthy donors (Figure 5). Similarly, Th2 cytokines, IL-4 and IL-13 gene expression was also significantly increased in the lungs of asthma patients (Figure 5). Together, these data suggest that CD2 is associated with the Th2-high endotype of asthma.

Generation of Cd2$^{-/-}$ mice in a Balb/c background. To investigate the role of CD2 in a mouse model of Th2-high asthma, we generated *Cd2*$^{-/-}$ mice in a Balb/c background (Balb/c.*Cd2*$^{-/-}$), due to the inherent Th2-biased phenotype of Balb/c mice[104]. We backcrossed *Cd2*$^{-/-}$ mice in a B6 background (B6.*Cd2*$^{-/-}$) with Balb/c mice for nine generations to obtain Balb/c.*Cd2*$^{-/-}$ mice (Figure 6A). The genotype of the Balb/c.*Cd2*$^{-/-}$ mice was confirmed by staining for H-2k^{d}, a marker for Balb/c background, on peripheral blood mononuclear cells (PBMCs) using flow cytometry (Figure 6B). Subsequently, we analyzed surface expression of CD2 on T cells, which showed negligible expression of CD2 in the generated Balb/c.*Cd2*$^{-/-}$ mice, similar to B6.*Cd2*$^{-/-}$ mice (Figure 6C).

Cd2$^{-/-}$ mice have normal immune cell distribution. After the mice were generated, we next sought to examine the immune cell compartment between the Balb/c (WT) and the Balb/c.*Cd2*$^{-/-}$ (*Cd2*$^{-/-}$) mice. Given that the lungs are the primary site of asthma pathogenesis, using the gating

strategy shown in Figure 7A, we analyzed frequency of lymphocytes such as B, NK, iNKT, T, CD4 T, and CD8 T cells in the lungs by flow cytometry. We subsequently analyzed frequency of innate immune cells such as macrophages, separated by low MHC expression (MHC^{lo}) or high MHC expression (MHC^{hi}), dendritic cells (DCs), eosinophils and neutrophils using the gating strategy shown in Figure 7B. The absolute number of B, NK, and iNKT cells in the lungs of the *Cd2*$^{-/-}$ mice were comparable to the WT mice (Figure 7C). Interestingly, the absolute number of T cells was significantly increased in the *Cd2*$^{-/-}$ mice in comparison to the WT mice (Figure 7C). However, T cell subsets such as CD4 and CD8 T cell numbers in the *Cd2*$^{-/-}$ mice were slightly increased in comparison to the WT mice but was not significant (Figure 7C). In the innate immune cell compartment, DC, eosinophil, and neutrophil absolute numbers in the *Cd2*$^{-/-}$ mice were comparable to the WT mice (Figure 7D). Strikingly, MHC^{lo} macrophages in the *Cd2*$^{-/-}$ mice were significantly reduced in comparison to the WT mice. Yet, MHC^{hi} macrophages were slightly reduced in the *Cd2*$^{-/-}$ mice but was not significant. This suggested that the *Cd2*$^{-/-}$ mice have comparable distribution of innate immune cells except overall alveolar macrophages, which they inherently have fewer numbers of.

Cd2$^{-/-}$ mice have normal expression of various costimulatory molecules. Costimulatory molecules are known to play a role in asthma pathogenesis[80] and can compensate immune cell function in the absence of another[100]. Hence, we next investigated the basal expression of different costimulatory molecules in the *Cd2*$^{-/-}$ mice. Costimulatory molecules that are part of the CD28:B7 family (CD28, CTLA-4, ICOS) and the tumor necrosis factor receptor (TNFR) superfamily (4-1BB and OX40) expressed on T cells from the lungs of *Cd2*$^{-/-}$ mice were comparable to the WT mice (Figure 8A). Correspondingly, the ligands for costimulatory molecules that are part of the

TNFR superfamily (CD40), the signaling lymphocyte activation molecule (SLAM) receptor family (CD48) and the CD28:B7 family (CD80 and CD86) expressed on B cells of *Cd2*$^{-/-}$ mice from the lungs were similar to WT mice (Figure 8B).

CD2 is expressed in the lungs of mice challenged with house dust mite extract (HDME). After observing increased CD2 expression in the lungs of asthma patients, we next sought to examine if CD2 also plays a role in a mouse model of Th2-high asthma, using HDME, a clinically relevant allergen. Balb/c (WT) mice, were intranasally challenged with HDME or PBS using the model shown in Figure 9A. One day after the last injection, mice were euthanized and lung CD2 and CD48 mRNA expression was measured by quantitative real-time PCR (qPCR). CD2 gene expression was increased in the WT mice challenged with HDME (WT HDME) in comparison to the WT mice treated with PBS (WT PBS) (Figure 9B). However, CD48 gene expression in WT HDME was comparable to the WT PBS mice (Figure 9B). Furthermore, CD48 gene expression in the lungs of *Cd2*$^{-/-}$ mice challenge with HDME (*Cd2*$^{-/-}$ HDME) was also comparable to *Cd2*$^{-/-}$ mice treated with PBS (*Cd2*$^{-/-}$ PBS) and WT HMDE mice (Figure 9B). These data suggested that CD48 is not upregulated in a mouse model of HDME-induced asthma, in the presence or absence of CD2.

Collectively, these data show that in a mouse model of acute challenge with HDME, CD2 is significantly increased, similar to the asthmatic humans. Several previous groups have studied the role of costimulatory molecules in the context of asthma. Costimulatory molecules part of the CD28:B7 family as well as the TNFR family have been extensively studied and have established roles in the pathogenesis of asthma[80]. However, given the heterogeneity of asthma, the roles of

costimulatory molecules in response to different allergens have not been classified yet. CD2 is one costimulatory molecule that has not been studied as extensively. A previous study has shown that $CD2^{high}$ monocytes that express higher levels of FcεRI in asthma patients correlated with higher serum IgE levels[103]. Although this study provides an association of CD2 with asthma, it does not provide evidence that CD2 plays a role in asthma, nor does is offer a mechanism by which CD2 could be playing a role. In the case of mouse models, a prior study observed that CD2 and CD48 expression is significantly increased in the lungs of mice sensitized and challenged with Ovalbumin (OVA)[105]. Similarly, we observed significantly increased CD2 expression in response to HMDE, but the CD48 expression was comparable to PBS treated mice. However, in the same study[105], CD2 expression was shown to be increased in the lungs of mice sensitized and challenged with Aspergillus fumigatus (Asp) but was not significant, whereas CD48 expression was significantly upregulated[105]. In complete contrast to the Asp-model of asthma from Munitz et al's study, we observed a significant increase in CD2 expression in the lungs of Balb/c mice induced with HDME, but not CD48[105]. The stark contrast in CD2 and CD48 expression in the different mouse models using different allergens indicates that asthma pathogenesis can also depend on the allergen used. The inconsistencies in results from different groups is perhaps due to this exact reason. Future studies in asthma need to be done using clinically relevant allergens and not experimental allergens such as OVA. This will further advance our knowledge of asthma pathogenesis and help us translate animal research to humans more accurately.

APPENDIX

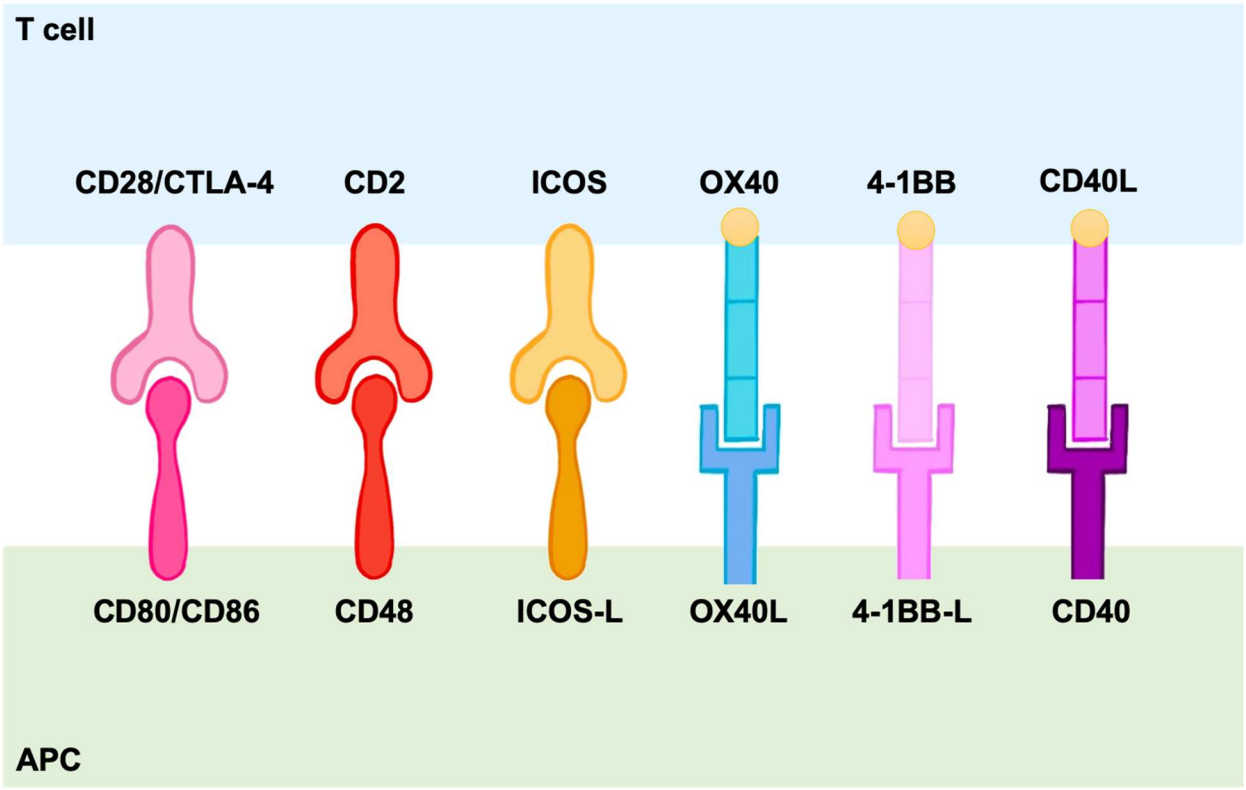


Figure 4. Costimulatory molecules and ligands on T cells and antigen presenting cells (APC). Costimulatory molecules on T cells include CD28, CD2, ICOS, OX40, 4-1BB, CD40L. Their ligands expressed on APCs include CD80/86, CD48 (in mice)/CD58 (in humans), ICOS-L, OX40L, 4-1BB-L, and CD40. An inhibitory molecule expressed on T cells that competes with CD28 to bind to CD80/86 is CTLA-4. All costimulatory molecules are part of the immunoglobulin superfamily but can be categorized into other sub-families. CD28, CTLA-4, CD80/86, ICOS and ICOS-L are part of the B7 family of receptors. OX40, OX40L, 4-1BB, 4-1BB-L, CD40 and CD40L are part of the tumor necrosis factor receptor (TNFR) superfamily. CD2 is part of the overall immunoglobulin superfamily of receptors and CD48 is part of the signaling lymphocyte activation molecules (SLAM) receptor family, which is also a sub-family of the immunoglobulin superfamily.

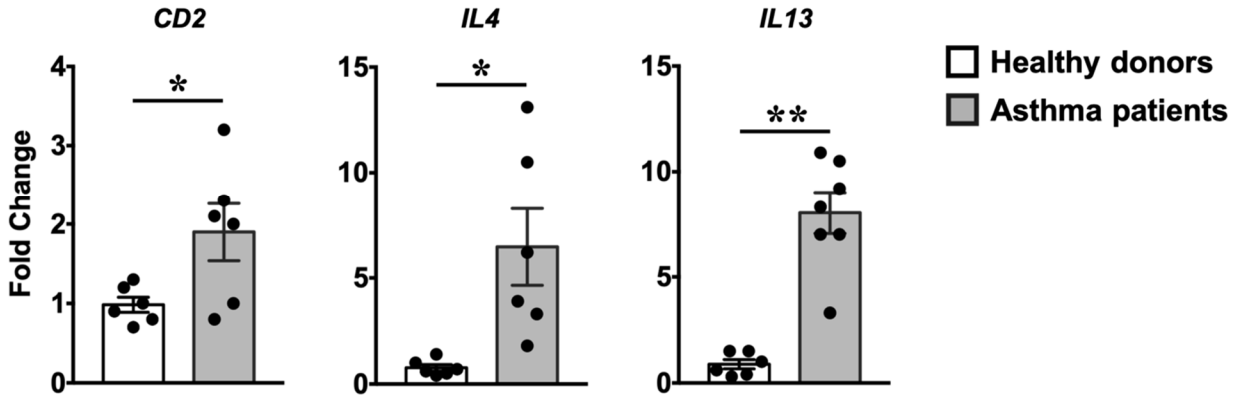


Figure 5. Increased gene expression of CD2 and Th2 cytokines in the lungs of human asthmatics. Whole lung tissue from healthy donors and asthma patients were analyzed for gene expression. mRNA levels of *CD2, IL-4* and *IL-13* are shown as mean fold change ± SEM with a total of 6-7 samples per cohort. Statistical significance was determined by Student's unpaired *t* test with Welch's correction. *p≤ 0.05, **p≤ 0.01.

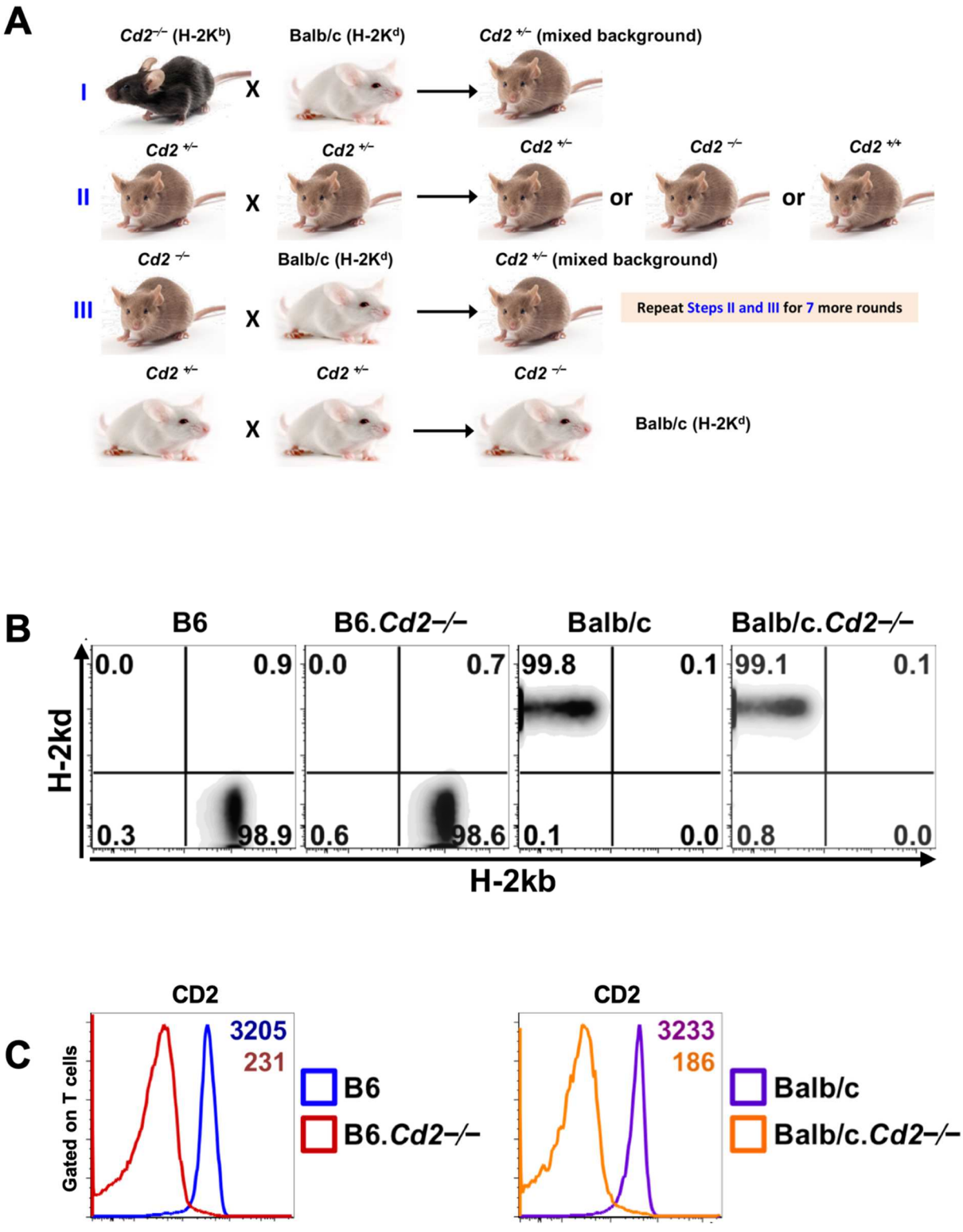


Figure 6. Generation of *Cd2*$^{-/-}$ mice.

Figure 6. (cont'd)

(A) Schematics of generation of *Cd2*$^{-/-}$ mice on a Balb/c background (Balb/c. *Cd2*$^{-/-}$). (I) *Cd2*$^{-/-}$ mice on a B6 background (B6. *Cd2*$^{-/-}$) were bred with Balb/c mice to obtain heterozygous mice with a mixed background. (II) The heterozygous mice with a mixed background were bred with each other to obtain *Cd2*$^{-/-}$ mice with a mixed background. (III) Finally, the *Cd2*$^{-/-}$ mice with a mixed background were bred with Balb/c mice. Steps II and III were repeated for 7 more rounds until heterozygous mice were in a Balb/c background. The heterozygous mice in a Balb/c background were then crossed to obtain *Cd2*$^{-/-}$ mice on a pure Balb/c background. (B) Peripheral blood mononuclear cells (PBMCs) from B6, B6. *Cd2*$^{-/-}$, Balb/c and Balb/c. *Cd2*$^{-/-}$ mice were analyzed for surface expression of $H2k^b$, $H2k^d$, CD2 and TCRβ by flow cytometry. Representative histograms (C) showing CD2 expression on T cells from B6 and B6. *Cd2*$^{-/-}$ mice (left) and Balb/c and Balb/c. *Cd2*$^{-/-}$ mice (right). Numbers in C indicate mean fluorescence intensity (MFI) for CD2 expression on T cells. Representative density plots shown are representative of 4 mice for each genotype.

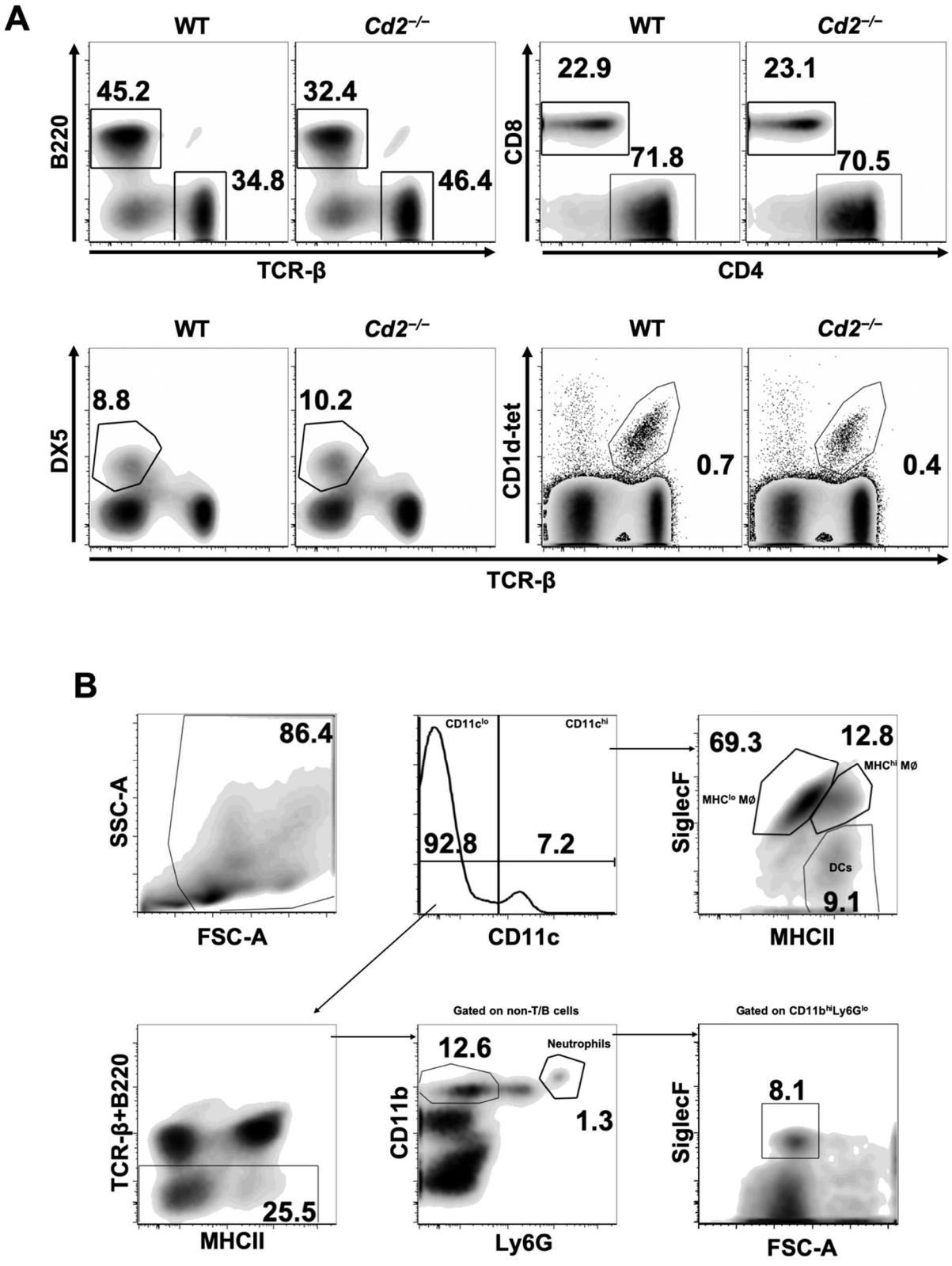
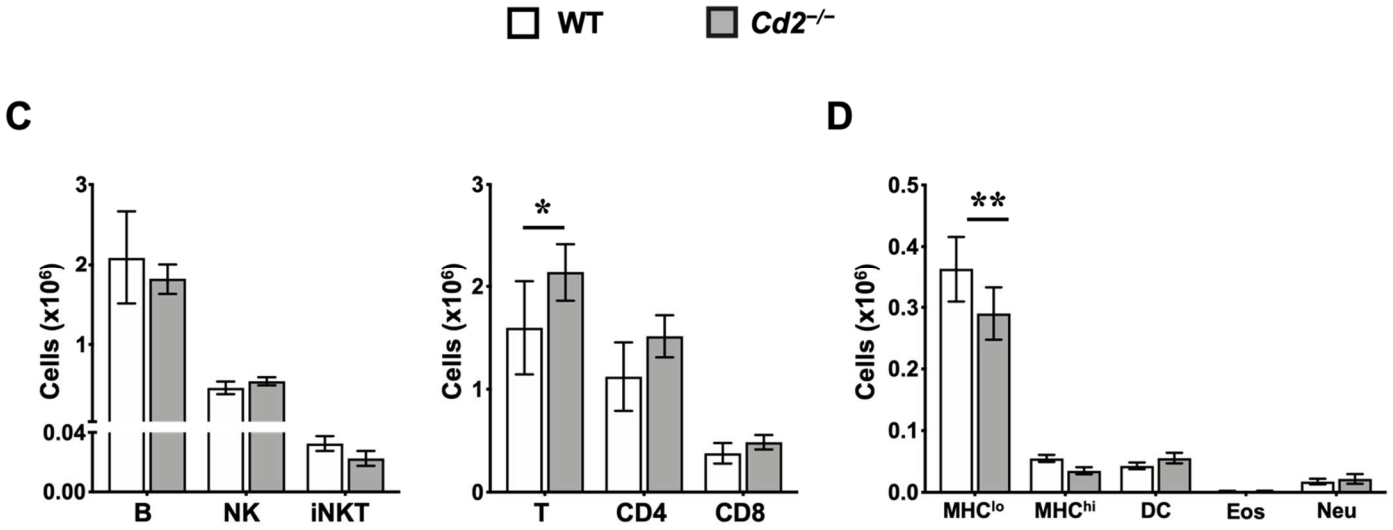


Figure 7. ***Cd2*** $^{-/-}$ **mice have normal immune cell distribution.**

Figure 7. (cont'd)



Representative flow cytometry analysis (A) showing lymphocyte (B, NK, iNKT, T, CD4 T, CD8 T cell) population in lungs of naïve WT and *Cd2*$^{-/-}$ mice. (B) Gating strategy of innate cells (MHC^{lo} and MHC^{hi} macrophages, dendritic cells, eosinophils, neutrophils) in lungs of WT mice. (C) Absolute numbers of lymphocytes (C) and innate cells (D) in lungs of naïve WT and *Cd2*$^{-/-}$ mice. Data shown is representative of 4 mice for each genotype. Statistical significance was determined by Student's unpaired *t* test with Welch's correction. *p≤ 0.05, **p≤ 0.01.

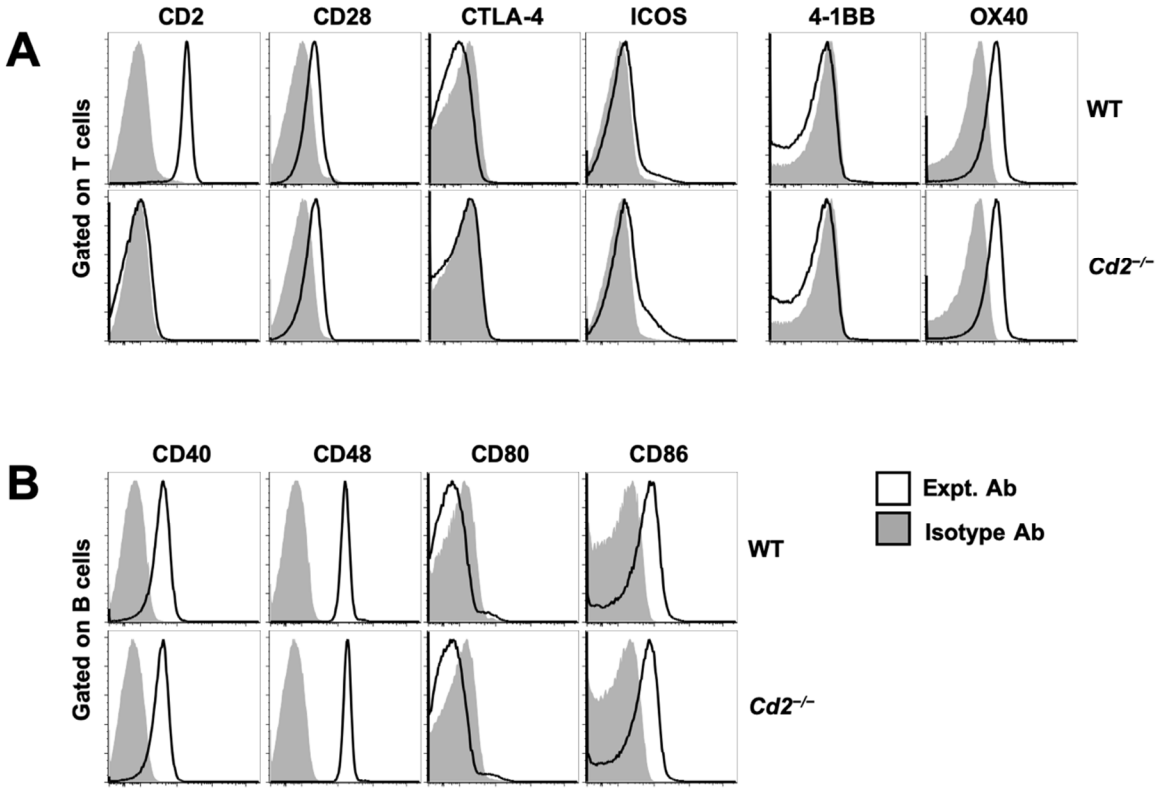


Figure 8. *Cd2*$^{-/-}$ mice have normal costimulatory molecule expression in comparison to the WT mice. Representative histograms (A) showing CD2 (left) CD28:B7 family of costimulatory molecules (middle) and TNFR superfamily receptor (right) expression on T cells. Representative histograms (B) showing the ligands for costimulatory molecules (CD40, CD48, CD80, CD86) expressed on B cells. Data in (A-B) is representative of 4 mice for each genotype.

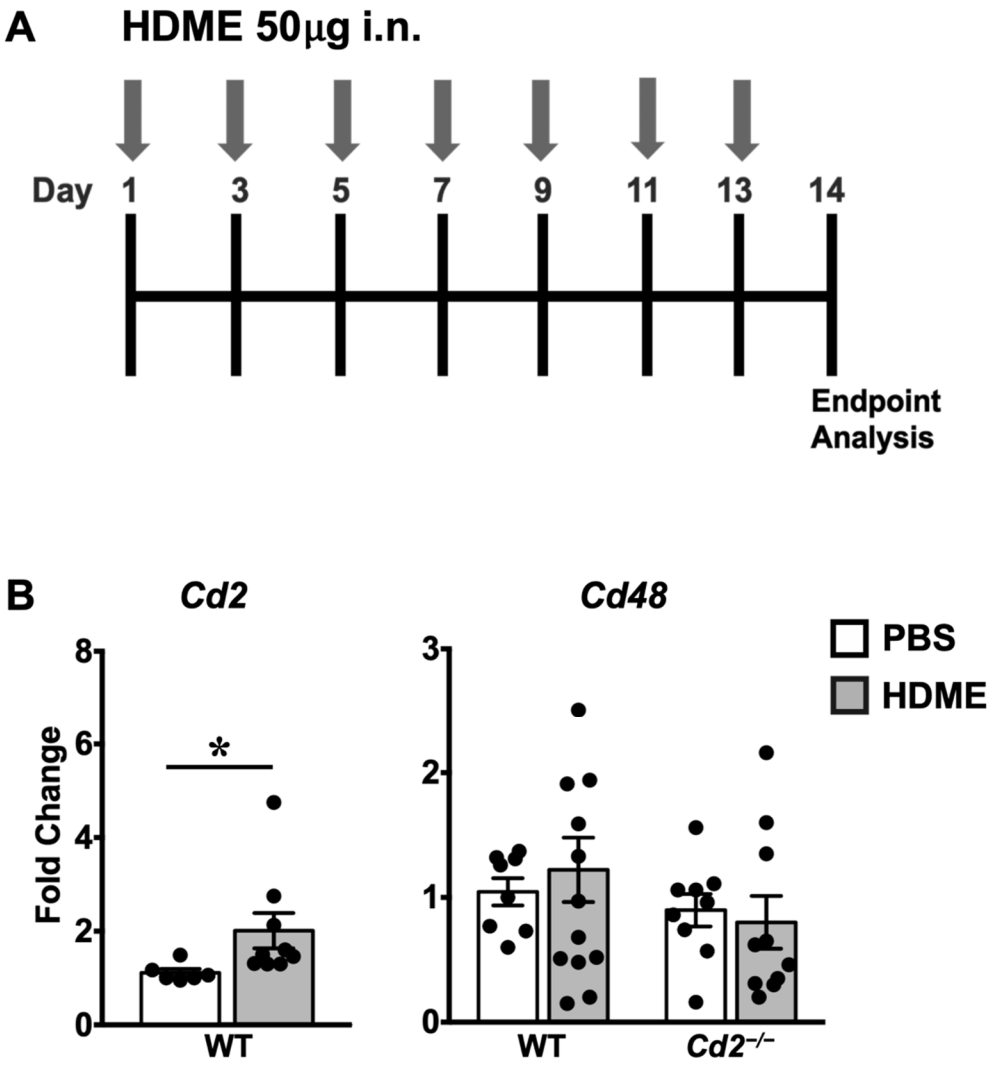


Figure 9. CD2 gene expression is upregulated in the lungs of HDME-induced asthmatic mice. (A) Schematics of the asthma model used in the study. Mice were challenged with HDME on alternate days for a total of 7 injections. Twenty-four hours after the last challenge, mice were analyzed for gene expression. (B) Lung tissue homogenates of WT mice injected with PBS or HMDE were analyzed for *Cd2* (left) by qPCR. Lung tissue homogenates of WT and *Cd2*$^{-/-}$ mice injected with PBS or HMDE were analyzed for *Cd48* (right) by qPCR. Data is presented as mean ± SEM and is pooled from 3 independent experiments with a total of 8-13 mice per cohort.

Figure 9. (cont'd)

Statistical significance was determined by using Student's unpaired *t* test with Welch's correction.

*$p \leq 0.05$.

CHAPTER 3

CD2 REGULATES AIRWAY HYPERRESPONSIVENESS AND LUNG INFLAMMATION IN AN HDME-INDUCED MOUSE MODEL OF ASTHMA

Introduction

Hallmark characteristics of asthma in humans include coughing, wheezing, shortness of breath due to airway hyperresponsiveness (AHR), inflammation, bronchoconstriction, goblet cell hyperplasia and airway remodeling[1]. However, in experimental mouse models of asthma, symptoms of asthma that are observed are AHR, inflammation, goblet cell hyperplasia and airway remodeling[106]. Evidence of AHR in mice in response to allergen has been investigated[107, 108] and methacholine challenge test has been used to measure AHR in mice[109].

Previous studies investigating the role of costimulatory molecules have all demonstrated their role in AHR, IgE production and inflammation[80], all of which are important characteristics of asthma pathogenesis. CD28 has been shown to play a role in lung inflammation, since CD28-deficient mice failed to develop lung inflammation in response to allergen[110]. ICOS has been shown to play a role in IgE and IgG1 production[82, 84] and was shown to prevent AHR in allergen-induced mice[83]. The administration of a CTLA-4 blocking antibody in mice with allergen induced asthma resulted in reduced production of allergen-specific IgE[86]. OX40 and OX40L has been shown to regulate Th2 cytokine production and lung inflammation[87, 88]. Lastly, 4-1BB has been shown to play a role in AHR, production of allergen-specific IgE and eosinophil infiltration into the lungs[91, 92].

Similarly, we investigated the role of CD2 in AHR, lung inflammation and cellular infiltration, as well as IgE and IgG1 production in response to HDME-induce asthma. Here, we challenged WT and *Cd2*$^{-/-}$ mice on a Balb/c background with HDME and show that AHR is significantly reduced in the *Cd2*$^{-/-}$ mice in comparison to the WT. Serum IgG1 levels are significantly decreased in the

Cd2$^{-/-}$ mice, however IgE levels are similar. We also show reduced total leukocytes in the bronchoalveolar lavage fluid (BALF) from *Cd2*$^{-/-}$ mice. Similarly, the number of eosinophils, monocytes and neutrophils are significantly reduced in the BALF of the *Cd2*$^{-/-}$ mice, but the number of lymphocytes remained comparable. Taken together, these data suggests that CD2 plays an important role in the development of AHR, IgG1 production, and lung inflammation and cellular infiltration, and is an attractive target for asthma therapy.

Materials and Methods

Mice

Mice were used as described in Chapter 2.

Mouse model of mild asthma

The same model was used as described in Chapter 2

AHR assessment

Mice were anesthetized with a cocktail of 100mg/ml Ketamine (Henry Schein Animal Health, Dublin, OH), 20mg/ml Xylazine (Akorn, Lake Forest, IL) and 10mg/ml Acepromazine (Henry Schein Animal Health, Dublin, OH) through intraperitoneal (i.p.) injection and tracheostomized. Airway mechanics was measured using forced oscillation technique using flexiVent, a small animal ventilator (SCIREQ®, Quebec, Canada). Parameters of AHR such as airway resistance (Rrs) and elastance (Ers) was assessed by a methacholine (MCh; Sigma-Aldrich, St. Louis, MO) challenge test with increasing doses of MCh.

Blood serum collection

Blood was collected from the superior mesenteric vein of the mouse and left at 4 °C overnight. Serum was collected the next day and analyzed for immunoglobulins such as IgE and IgG1 by ELISA (Invitrogen, Carlsbad, CA).

Assessment of lung inflammation

Left lobe of lungs was washed in PBS and fixed in 10% formalin. Samples were then embedded in paraffin, cut into 5-μm-thick sections and stained with hematoxylin and eosin (H&E). Digital images of sections at 10X magnification were obtained using a Nikon Eclipse 50i (Nikon, Minato, Tokyo, Japan) and INFINITY-3, using INFINITY ANALYZE 6.5.4 software (Lumenera Corporation, Ottawa, Ontario, Canada).

Total and differential leukocyte count from BALF

Whole lung was lavaged with 0.6 ml of sterile PBS three times to obtain BALF. Total BALF cells were counted, and 50,000 cells were cytospun onto a clean glass slide for differential counts using Giemsa staining (Sigma-Aldrich, St. Louis, MO). The slides were dried completely and stained in Giemsa for 3 minutes. The slides were next washed with distilled water two times and dried. The slides were then dipped in xylene (Avantor, Radnor Township, PA) for 2 seconds and a cover slip was placed immediately over the cells. For differential cell counting, a total of 200 cells were counted at 40X magnification from each sample slide; counted were monocytes, eosinophils, neutrophils, and lymphocytes.

Statistics

Statistical analysis was performed as described in Chapter 2.

Results and Discussion

Absence of CD2 attenuates airway hyperresponsiveness (AHR) in a murine model of allergic asthma. AHR is a hallmark symptom of asthma that both humans and mice, in experimental asthma, present with[1]. Hence, we assessed AHR parameters such as central airway resistance (Rrs) and elastic stiffness of airways or elastance (Ers) with increasing doses of methacholine (Mch) to observe if HDME sensitization has induced AHR in the WT and *Cd2*$^{-/-}$ mice using the same model shown in chapter 2 (Figure 10A). Rrs and Ers of the WT PBS mice increased with increasing doses of Mch. However, WT HDME mice showed significantly higher Rrs and Ers at increasing doses of Mch in comparison to the WT PBS mice, exhibiting increased AHR in response to HDME. On the other hand, in comparison to WT HDME mice, Rrs and Ers of *Cd2*$^{-/-}$ HDME mice were significantly lower at 50 and 100 mg/ml doses of MCh (Figure 10B), demonstrating that the absence of CD2 regulates AHR.

CD2 regulates immunoglobulin G1 levels (IgG1) in the serum in response to HDME. Another hallmark characteristic of allergic asthma is the increased presence of antibody in the serum of asthma patients[1]. Hence, we measured serum IgG1 and IgE levels in the mice. A significant decrease in serum IgG1 in *Cd2*$^{-/-}$ HDME mice in comparison to the WT HDME mice was observed (Figure11A), but not in serum IgE levels (Figure 11B). Additionally, we measured HDME-specific IgE levels in the mice and detected comparable levels in the *Cd2*$^{-/-}$ HDME and WT HDME mice (Figure 11C).

CD2 regulates leukocyte infiltration and overall inflammation in an HDME-induced asthma model. To examine if CD2 plays are role in lung inflammation, which is another major characteristic of asthma[1], we stained lung sections from WT and *Cd2*$^{-/-}$ HDME groups with H&E. Using representative images of the stained lung sections, we observed an overall decrease in inflammation in the *Cd2*$^{-/-}$ HDME mice in comparison to the WT mice (Figure 12A). We similarly observed a reduction in inflammation around bronchioles and blood vessels between the WT and *Cd2*$^{-/-}$ HDME groups (Figure 12B). In order to understand if the reduced inflammation was due to reduced leukocyte infiltration, we collected bronchoalveolar lavage fluid (BALF) from the mice and counted the total cell number. Total cell counts (TLC) were significantly reduced in *Cd2*$^{-/-}$ HDME mice in comparison to the WT HDME group (Figure 13A). We next stained the total cells from BALF to separate the immune cells through their morphology and counted monocytes, eosinophil, neutrophils and lymphocytes. BALF of PBS treated WT and *Cd2*$^{-/-}$ mice consisted primarily of monocytes (Figure 13B). In response to HDME, the WT mice had increased monocytes, but the *Cd2*$^{-/-}$ mice had comparable levels of monocytes to their PBS treated group (Figure 13B). Furthermore, the *Cd2*$^{-/-}$ HDME mice had significantly decreased monocytes in comparison to the WT HDME mice (Figure 13B). After HDME injection, the highest percentage of differential cells in the BALF was eosinophils (Figure 13C). Both eosinophils and neutrophils were significantly increased in the WT mice in response to HMDE (Figure 13C). However, they were both significantly reduced in the *Cd2*$^{-/-}$ HDME mice in comparison to the WT HDME mice (Figure 13C-D). The number of lymphocytes also increased significantly in response to HMDE in WT mice. Nonetheless, lymphocyte numbers in the BALF of *Cd2*$^{-/-}$ HDME mice were comparable to WT HDME mice (Figure 13E). These data suggest that reduced leukocyte infiltration, most

importantly the reduced number of eosinophils in the BALF contributes to the reduced overall inflammation seen in lung sections.

Taken together these data provide evidence that CD2 plays a pathogenic role in a HDME-induced mouse model of asthma. Multiple studies investigating different costimulatory molecules were previously shown to play a role in AHR, lung inflammation, and serum IgE production. ICOS and 4-1BB were both shown to play roles in regulating AHR[83, 91, 92], whereas CD28, OX40, OX40L and 4-1BB were shown to play roles in lung inflammation[87, 88, 91, 92, 110]. Furthermore, ICOS, CTLA-4 and 4-1BB were shown to regulate IgE production in different mouse models of asthma[82, 84, 86, 91, 92]. In comparison to these previous studies, CD2 plays a role in AHR, lung inflammation, and IgG1 production, but not IgE production in our HDME-induced mouse model of asthma. Costimulatory molecules each play different roles in regulating the different hallmark symptoms of asthma, and CD2 is no different. However, it is important to compare the role of CD2 in different models of asthma. The study by Munitz et al showed that injection of anti-CD2 monoclonal antibody in OVA challenged mice reduced inflammation and number of eosinophils in BALF[105], similar to our observations. In addition, they detected significant reduction in inflammation surrounding the bronchioles, which also agrees with our findings. However, they did not measure AHR or IgG1 and IgE production in their model, which leaves a gap in their interpretation of the role of CD2 as not being as important in comparison the CD48. Ongoing or emerging studies investigating the role of costimulatory molecules should include all hallmark characteristics of asthma when making conclusions about the specific role they play in the various characteristics.

APPENDIX

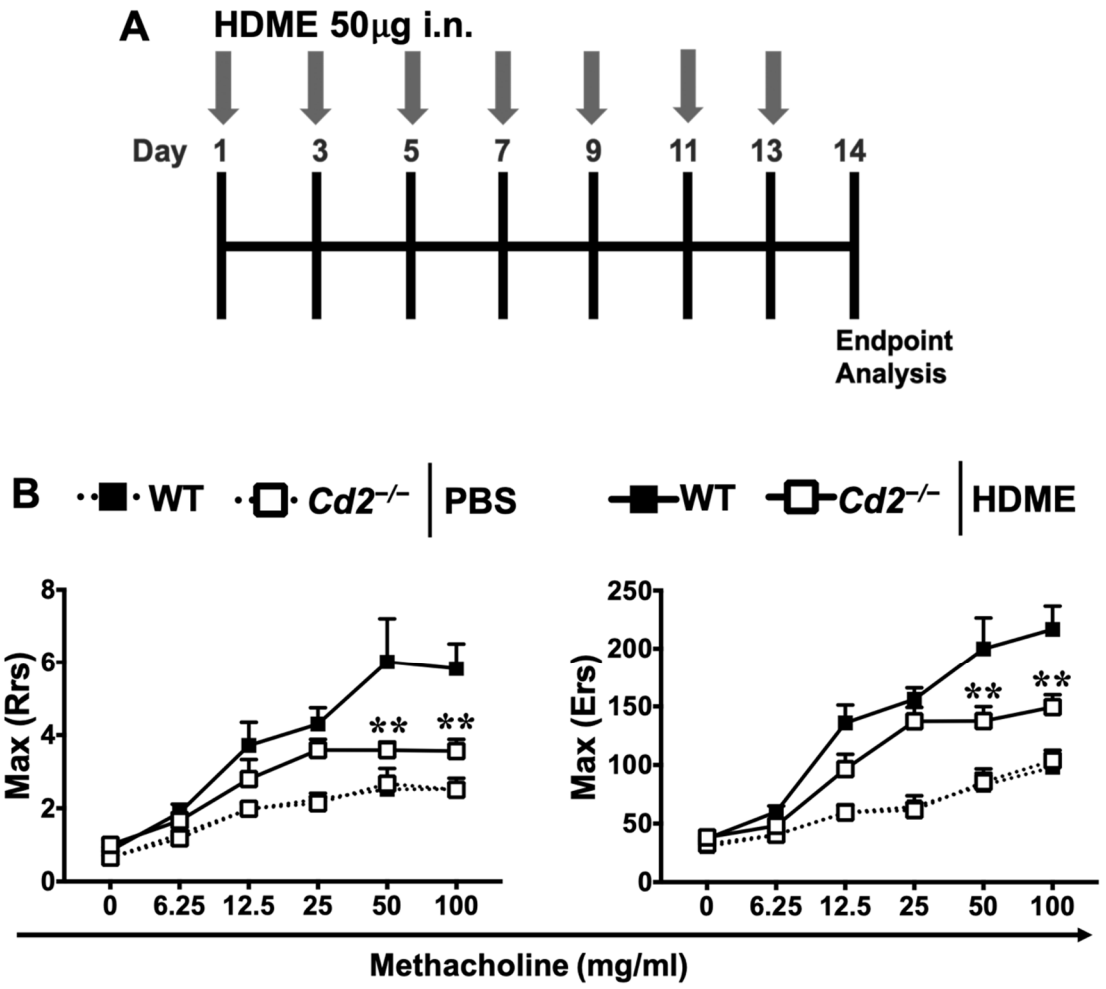


Figure 10. AHR is reduced in *Cd2*$^{-/-}$ mice challenged with HDME. (A) Schematics of the asthma model used in the study. Mice were challenged with HDME on alternate days for a total of 7 injections. Twenty-four hours after the last challenge, mice were analyzed for different endpoints. Assessment of lung constriction (B) (Resistance [Rrs]) and elastic stiffness of lungs, chest walls, and airways (Elastance [Ers]) after challenge with increasing doses of methacholine (Mch). Data is shown as mean ± SEM and pooled from 3-4 independent experiments with a total of 8-13 mice per cohort. Statistical significance was determined by two-way ANOVA with Tukey's test. **p≤ 0.01.

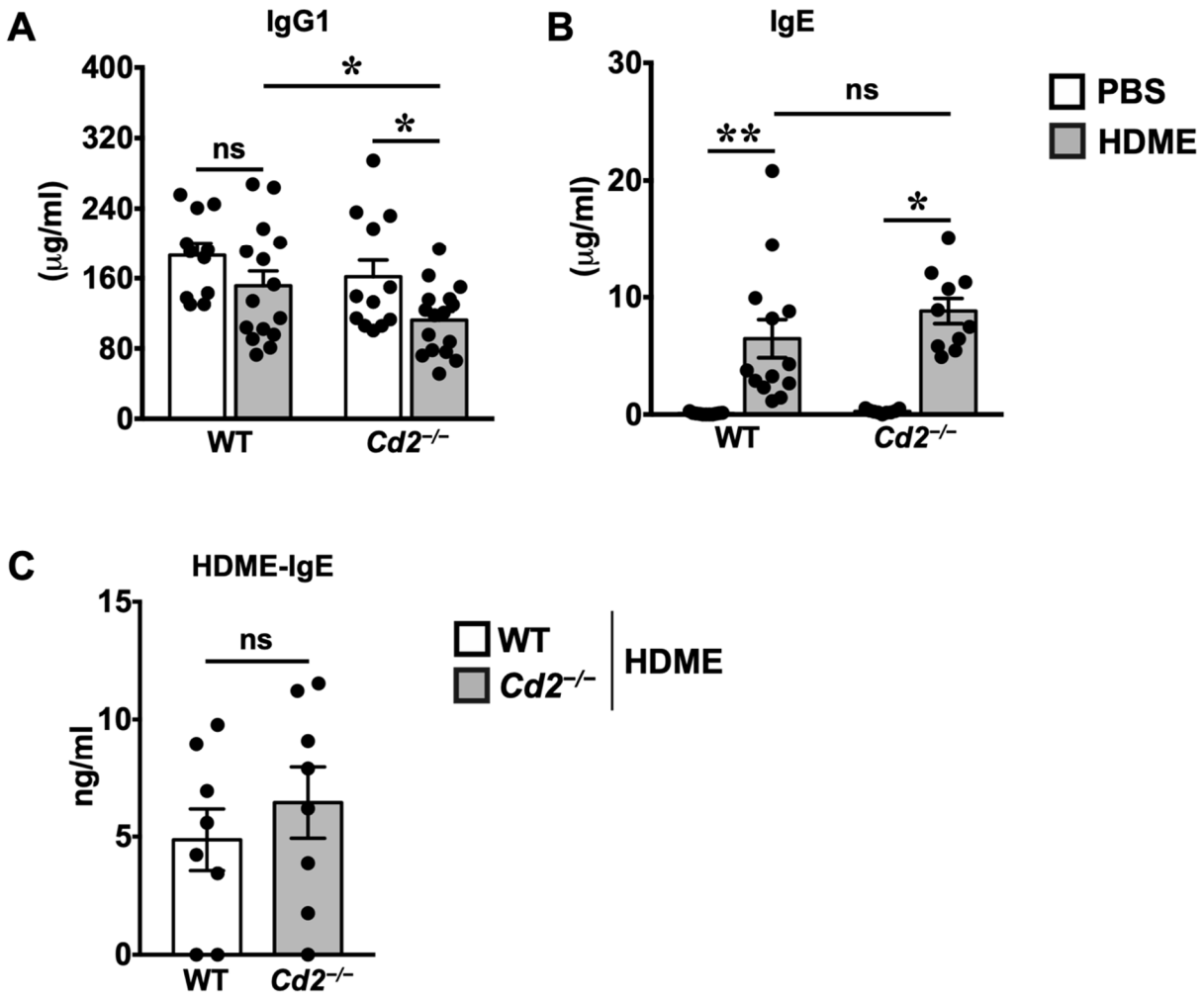


Figure 11. CD2 does not regulate IgE production in HDME-induced mouse model of asthma. WT and *Cd2*$^{-/-}$ mice that were either injected with PBS or HDME were anesthetized 24 h after the last HDME challenge and analyzed for different endpoints. Levels of serum IgG1(A) and IgE (B) in WT and *Cd2*$^{-/-}$ mice treated with PBS or challenged with HMDE are shown. Level of HDME-IgE (C) in WT and *Cd2*$^{-/-}$ mice challenge with HDME is shown. Data are shown as mean ± SEM and pooled from 4 independent experiments with a total of 11-17 mice per cohort. Statistical significance was determined by Student's unpaired *t* test with Welch's correction (B-D). **p≤ 0.01, *p≤ 0.05.

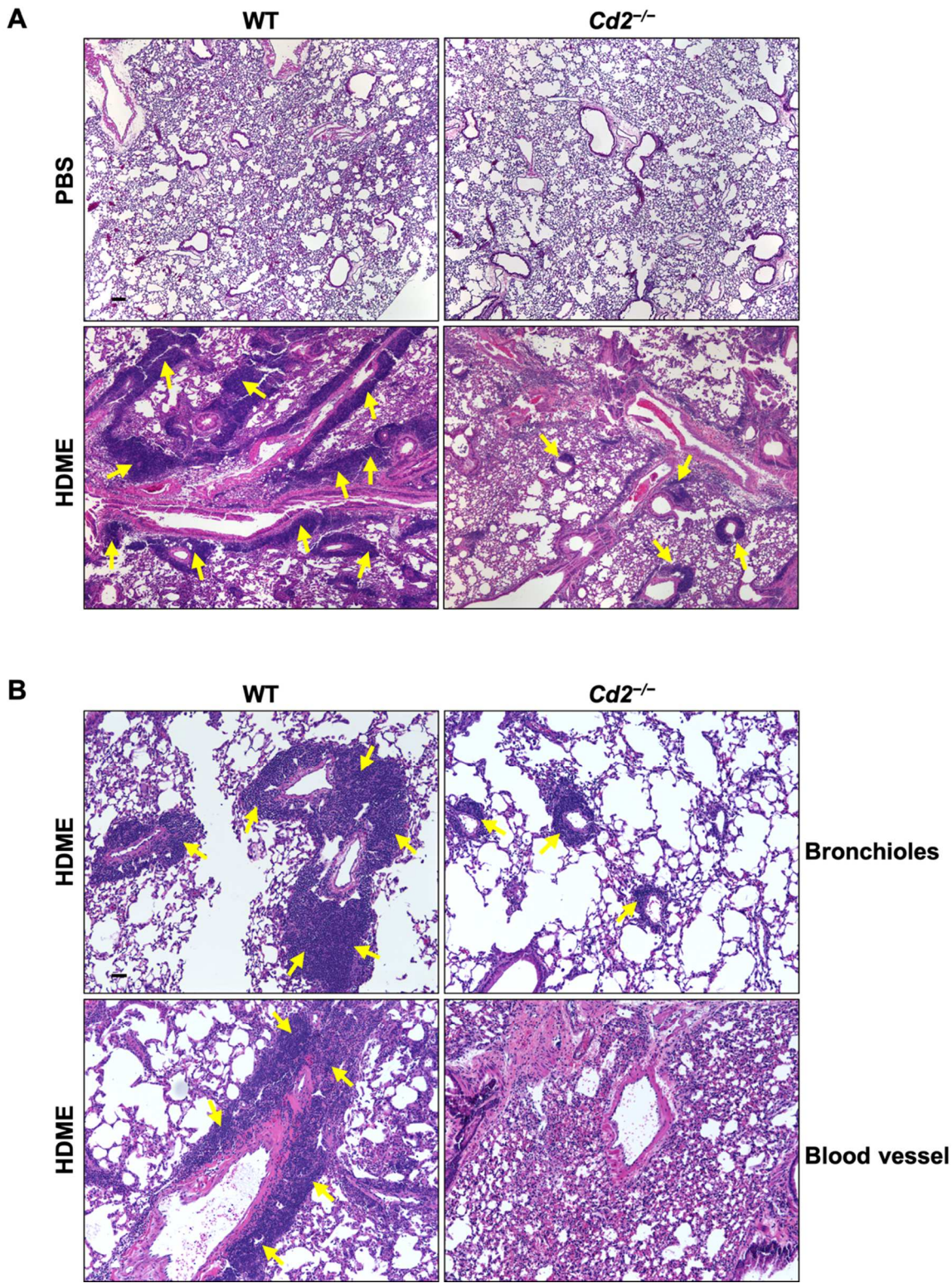


Figure 12. Reduced inflammation in *Cd2*$^{-/-}$ mice challenged with HDME.

Figure 12. (cont'd)

(A) Hematoxylin and eosin (H&E) staining of lung section of WT and *Cd2*$^{-/-}$ mice injected with PBS or challenged with HMDE. Representative micrographs at 4X magnification are shown. (B) Representative micrographs of bronchioles and blood vessels from WT and *Cd2*$^{-/-}$ mice challenged with HDME at 10X magnification are shown. Yellow arrows indicate cellular infiltration (inflammation). Data is representative of 3 independent experiments with a total of 8-13 mice per cohort. Scale bar: 100μm.

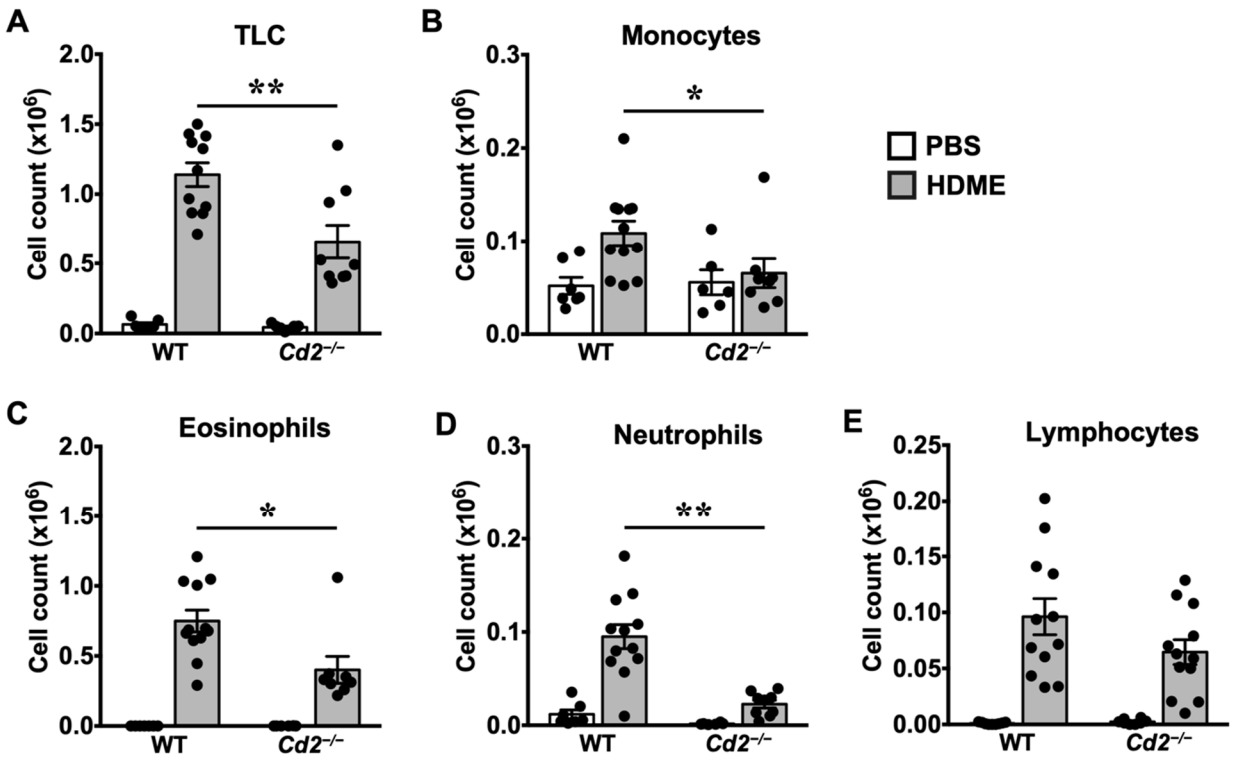


Figure 13. Reduced eosinophil, monocyte and neutrophil infiltration in *Cd2*$^{-/-}$ mice challenged with HDME. BALF from WT and *Cd2*$^{-/-}$ mice injected with PBS or challenged with HMDE were analyzed for total (A) and differential (B-E) cell counts. BALF cytospins showing total monocytes (B) eosinophils (C) neutrophils (D) and lymphocytes (E). Data are shown as mean ± SEM and pooled from 3 independent experiments with a total of 8-13 mice per cohort. Statistical significance was determined by Student's unpaired *t* test with Welch's correction. *p≤ 0.05, **p≤ 0.01.

CHAPTER 4

CD2 REGULATES IL-13 SIGNALING AND GOBLET CELL HYPERPLASIA IN A MOUSE MODEL OF HDME-INDUCED ASTHMA

Introduction

The heterogeneity of asthma is mostly contributed by the different cytokine profiles of the different endotypes, and each cytokine secreted in response to different allergens or irritants have distinct roles in the pathogenesis of asthma[111]. Th1 cytokines that play a major role in asthma are IFNγ and TNFα. IFNγ has been found to be highly expressed in severe asthmatics and in exacerbated asthma and is produced predominantly by Th1 cells and CD8 T cells[112]. TNFα is produced by multiple cells including T cells, macrophages, mast cells, and epithelial cells. TNFα has been shown to be produced in asthmatics after allergen exposure and can stimulate epithelial cells to produce chemokines such as CCL5 and cytokines such as IL-8 and GM-CSF[113, 114, 115]. Proinflammatory Th1 cytokines such as IL-1α and IL-1β has also been reported to be increased in BAL of asthmatics. IL-1α induces fever and IL-1β can activate macrophages to secrete inflammatory cytokines[116].

Hallmark Th2 cytokines include IL-4, IL-5, and IL-13. IL-4 is a cytokine primarily secreted by Th2 cells and ILC2s and plays a major role in the maturation of B cells into plasma cells that produce IgE[34]. IL-5 is a known recruiter of eosinophils into the site of inflammation[117]. IL-5 increases expression on adhesion molecules in surrounding endothelial cells thus enabling circulating eosinophils to extravasate into the inflammatory site[117]. IL-13 is a major effector cytokine in asthma and has multiple roles in various hallmarks of asthma. IL-13 is known to induce smooth muscle contraction, goblet cell hyperplasia resulting in mucus hypersecretion, as well as fibrosis. It can induce B cells to produce IgE and also plays a role in airway hyperresponsiveness[118]. Th17 cytokines such as IL-17A and IL-17F have been reported by many

groups to be highly expressed in asthmatics with neutrophilic asthma[119]. IL-17A is a known recruiter of neutrophils and is secreted by Th17 cells and ILC3s[47]. IL-17F has been proposed to play a role in airway hyperresponsiveness, however, the mechanism is not well understood[120]. Lastly, epithelial cell derived cytokines such as IL-25 and IL-33 were reported to play an important role in the initiation of Th2-high asthma by inducing production of IL-5 and IL-13 by ILC2s[3].

Immune cell recruitment into the airway microenvironment is key in the progression of asthma and chemokines play a major role in innate and adaptive immune cell recruitment. CCL3, CCL4 and CCL5 are all chemokines that are expressed highly in the BALF of asthmatics[121, 122]. CCL3. also known as MIP-1α is a known recruiter of eosinophils during initial eosinophil localization and CCL11 plays a role in secondary eosinophil infiltration and degranulation[123]. CCL4, also known as MIP-1β, has been also shown to recruit eosinophils into the inflammation site[122]. Chemokine receptors such as CCR8 is expressed by Th2 cells[124] and has been demonstrated to play a role in Th2-high asthma[125].

Here, we show that the lungs of *Cd2*$^{-/-}$ mice challenged with HDME have significantly reduced gene expression of CCL3, CCL4, and CCR8 in comparison to the WT. The gene expression of Th1 cytokines TNFα, IL-1α and IL-1β were also significantly reduced in the *Cd2*$^{-/-}$ mice, while IFNγ was not. Amongst the gene expression of Th2 cytokines IL-13 gene expression was significantly reduced in the *Cd2*$^{-/-}$ mice, while IL-4, IL-5, and IL-10 were not. Similarly, amongst the gene expression of alarmins such as IL-33 and IL-25, only IL-25 gene expression was significantly reduced in *Cd2*$^{-/-}$ mice. Amongst receptors that regulate IL-13 cytokine expression such as IL13Rα1 and IL13Rα2, only IL13Rα1 was significantly reduced in *Cd2*$^{-/-}$ mice. BALF

cytokine levels of IL-4 was comparable but IL-13 was significantly reduced in the *Cd2*$^{-/-}$ mice. Lastly, PAS+ staining of lung sections from *Cd2*$^{-/-}$ mice showed reduced PAS+ area and cells as well as reduced gene expression of polymeric mucins such as Muc5ac and Muc5b in the lungs. Taken together, these data show that CD2 regulates IL-13 cytokine gene expression and production which also effects the downstream targets of IL-13 such as mucin production. This data suggests that CD2 is a novel target that specifically regulates IL-13 which is the most important effector cytokine is Th2-high asthma and has the potential to be a therapy for all IL-13 mediated disease symptoms.

Materials and Methods

Mice

Mice were used as described in Chapter 2.

Mouse model of mild asthma

The same mouse model was used as described in Chapter 2.

Assessment of goblet cell hyperplasia

Lung samples were washed in PBS and fixed in 10% formalin. Samples were then embedded in paraffin, cut into 5-µm-thick sections and stained with Periodic acid-schiff (PAS). Digital images of sections were obtained at 10X magnification using a Nikon Eclipse 50i (Nikon, Minato, Tokyo, Japan) and INFINITY-3, using INFINITY ANALYZE 6.5.4 software (Lumenera Corporation, Ottawa, Ontario, Canada).

A total of 9 pictures of each lung section were obtained from each sample at 10X magnification. Selecting only the PAS positive portions of the picture, PAS positive cells as well as the percent area of PAS positive cells of each picture was calculated by measuring intensity of PAS staining using ImageJ software (NIH, Bethesda, MD).

Quantitative real-time PCR

Quantitative real-time PCR was performed as described in Chapter 2.

BALF cytokine analysis

BALF was obtained as described in Chapter 3. BALF was analyzed for IL-4 (BD Biosciences, San Diego, CA) and IL-13 (Invitrogen, Carlsbad, CA) cytokines by ELISA.

Statistics

Statistical analysis was performed as described in Chapter 2.

Results and Discussion

CD2 regulates monocyte, eosinophil and neutrophil infiltration by regulating various chemokines in HDME-induced asthma. We analyzed key chemokines that are responsible for immune cell recruitment into the airways to understand the mechanism by which *Cd2*$^{-/-}$ HDME mice have reduced immune cell infiltration. CCL3 and CCL4 are chemokines that are known to recruit monocytes, neutrophils, and eosinophils. They play a role in recruitment of eosinophils, specifically during initial localization in asthma[123]. In response to HDME, WT mice showed a dramatic increase in CCL3 and CCL4 gene expression. However, gene expression of both CCL3 and CCL4 was significantly reduced in *Cd2*$^{-/-}$ HDME mice in comparison to the WT group. Additionally, CCL5, which is also a known recruiter of eosinophils[125], was analyzed for gene expression. In response to HDME, CCL5 gene expression was significantly reduced in the WT and *Cd2*$^{-/-}$ mice in comparison to their respective PBS groups (Figure 14). However, *Cd2*$^{-/-}$ HDME mice showed comparable levels of CCL5 gene expression to the WT HDME mice. CCL11, also known as eotaxin-1, has been shown to play a role in late stage eosinophil recruitment and degranulation in an experimental asthma model[123]. In response to HDME, WT mice showed a significant increase in CCL11 gene expression in the WT and *Cd2*$^{-/-}$ mice in comparison to their respective PBS groups. However, *Cd2*$^{-/-}$ HDME mice had comparable levels of CCL11 gene expression to the WT mice, suggesting that only initial recruitment of eosinophils is defective in the *Cd2*$^{-/-}$ mice (Figure 14). Lastly, we investigated lymphocyte recruitment by analyzing the chemokine receptor CCR8, which is expressed on Th2 cells[124]. CCR8 gene expression in response to HDME in WT and *Cd2*$^{-/-}$ mice were significantly increased. Conversely, CCL11 gene

expression was significantly reduced in the *Cd2*$^{-/-}$ HDME mice in comparison to the WT HDME mice (Figure 14).

CD2 specifically regulates IL-13 gene expression and protein production by regulating IL-13Rα1 and IL-13Rα2 expression. Traditionally, Th1 cytokines have been shown to counter regulate Th2 expression and vice versa[126, 127]. Hence, we analyzed Th1 cytokines to observe if they were upregulated as a form of protection in the HDME-induced Th2-high model of asthma. Th1 cytokines such as IFNγ, IL-1α, IL-1β, and TNF-α gene expression was measured by quantitative real-time PCR. All Th1 cytokines in WT HDME mice showed comparable gene expression to WT PBS mice. However, in *Cd2*$^{-/-}$ HDME mice, except IFNγ, the other Th1 cytokines IL-1α, IL-1β, and TNF-α gene expression was significantly reduced compared to WT HDME mice and *Cd2*$^{-/-}$ PBS mice (Figure 15).

Since house dust mite models have been shown to be of a Th2-high endotype[78], we next analyzed major Th2 cytokines such as IL-4, IL-5, IL-13 and IL-10 mRNA expression in the lungs. Except IL-10, all the other Th2 cytokines in WT HDME mice had dramatically increased gene expression in comparison to WT PBS mice (Figure 16). IL-4, IL5 and IL-10 gene expression in *Cd2*$^{-/-}$ HDME mice, however, were comparable to the WT HDME group. Strikingly, we observed that IL-13 gene expression was significantly reduced in the *Cd2*$^{-/-}$ HDME mice compared to WT HDME group (Figure 16), suggesting CD2 specifically regulates IL-13 and not IL-4, IL-5 and IL-10.

Growing evidence from recent studies in humans have shown that in addition to Th2 cytokines, Th17 cytokines are also increased in samples from asthma patients[128]. Furthermore, epithelial cell

derived cytokines, known as alarmins, also play a prominent role in Th2-high endotypes of asthma[129]. Consequently, we next analyzed Th17 cytokines IL17A and IL-17F, and alarmins IL-17E and IL-33 gene expression in the lungs of WT and *Cd2*$^{-/-}$ HDME treated mice. IL-17A and IL-33 gene expression increased in response to HDME in WT mice, but IL17E and IL-17F were reduced. When comparing *Cd2*$^{-/-}$ HDME mice with WT HDME mice, we found a significant decrease only in IL-17E gene expression (Figure 17), and not IL-17A, IL-17F or IL-33, which were comparable to the WT HDME group. Next, to test if the gene expression of the Th2 cytokines observed in Figure 16 correlated with protein expression, we analyzed IL-4 and IL-13 protein levels in the BALF of WT and *Cd2*$^{-/-}$ HDME mice and observed a significant reduction only in IL-13 levels in the *Cd2*$^{-/-}$ HDME mice when compared to WT HDME mice (Figure 18A & 18B). This observation on specific suppression of IL-13 gene and protein expression in *Cd2*$^{-/-}$ mice was striking and most intriguing since the other major Th2 cytokines were not affected.

To understand why IL-13 gene expression and cytokine production was specifically targeted and not IL-4 or IL-5, we investigated the cytokine receptors that regulate IL-13: IL-13Rα1 and IL-13Rα2. Previously, IL-13Rα1 was show shown to be the primary receptor involved in IL-13 signaling[130]. However, recent studies have provided evidence for membrane bound IL-13Rα2 to play a role in signaling as well[131, 132, 133, 134], even though it was previously deemed a decoy receptor that neutralized IL-13[135]. We hence analyzed IL-13Rα1 and IL-13Rα2 gene expression in the lungs of WT and *Cd2*$^{-/-}$ mice challenged with HDME. IL-13Rα1 gene expression was slightly reduced but not significant, in response to HDME in WT mice. Similarly, IL-13Rα1 gene expression in *Cd2*$^{-/-}$ HDME in comparison to the WT HDME group was also comparable. However, IL-13Rα1 gene expression was significantly reduced in *Cd2*$^{-/-}$ HDME mice in

comparison to *Cd2*$^{-/-}$ PBS mice. This suggested that in response to HMDE, the *Cd2*$^{-/-}$ mice downregulated IL-13Rα1 gene expression more significantly than the WT mice. In contrast, membrane IL-13Rα2 gene expression was significantly increased in WT mice in response to HDME but decreased in *Cd2*$^{-/-}$ HDME mice in comparison to the WT HDME mice (Figure 19). This suggested that both IL-13Rα1 and IL-13Rα2 play a role in regulating IL-13 gene expression and protein production in the absence of CD2.

Absence of CD2 results in downregulation of mucus staining and polymeric mucin production in HDME-induced model of asthma. Till this point, our most intriguing finding was the reduction in IL-13 gene and protein expression. The direct downstream target of IL-13 is the production of mucus by goblet cells in the airways[118]. Hence, we analyzed goblet cell hyperplasia through PAS staining of lung sections from WT and *Cd2*$^{-/-}$ HDME treated mice and saw a significant reduction in PAS staining shown in representative images (Figure 20A). Quantification of percent positive cells and percent area of PAS positive staining in the lung sections was also significantly reduced in the *Cd2*$^{-/-}$ HDME mice in comparison to the WT HDME group (Figure 20B). Lastly, we analyzed for polymeric mucins found most abundantly in the lungs, Muc5ac and Muc5b[136], gene expression in the lungs of WT and *Cd2*$^{-/-}$ HDME mice. Both Muc5ac and Muc5b expression was increased in response to HDME in the WT mice. However, they were both significantly reduced in the *Cd2*$^{-/-}$ HDME mice in comparison to the WT HMDE group (Figure 21), suggesting CD2 indirectly regulates downstream IL-13 targets by regulating IL-13 signaling.

Here we show that CD2 strikingly regulates the chemokines responsible for immune cell infiltration as well as major effector Th2 cytokine, IL-13, and in turn regulates downstream IL-13

targets such as goblet cell hyperplasia and polymeric mucin production. Chemokines are known to play an imperative role in immune cell recruitment. In terms of eosinophil recruitment, CCL3, CCL4, CCL5 and CCL11 were all shown to play a role in experimental asthma[123]. More specifically, CCL3 was shown to induce early phase eosinophil recruitment, whereas CCL11 was shown to play a role in late phase recruitment[123]. Here we have shown that in response to HDME, gene expression of CCL3, CCL4 and CCL11 increase significantly, but not CCL5, suggesting they are the primary chemokines responsible for eosinophil recruitment in a HDME-induced model of asthma. In addition, *Cd2*$^{-/-}$ HDME mice exhibited significantly lower gene expression of both CCL3 and CCL4, but not CCL11. This suggests that the reduction in eosinophil levels seen in BALF from the *Cd2*$^{-/-}$ HDME mice were a result of decreased CCL3 in the early phase of recruitment and late phase recruitment by CCL11 was not enough to overcome the initial defect.

Th1 cytokines were previously shown to counter regulate Th2 mediated inflammation[126, 127]. We have shown that IL-1α, IL-1β, and TNF-α were significantly reduced in the *Cd2*$^{-/-}$ HDME mice. This reduction in IL-1α, IL-1β, and TNF-α perhaps can be attributed to the reduction in monocyte infiltration, since monocytes that differentiate into macrophages are known to produce these cytokines[137]. In addition to Th1 cytokines, we have analyzed Th17 cytokines as well as epithelial-derived cytokines, known as alarmins. We have observed a significant reduction only in IL-17E gene expression in the *Cd2*$^{-/-}$ HDME mice. Since IL-17E is not just produced by epithelial cells, but also eosinophils and macrophages[138], the reduction of both of these cell types in the BALF could contribute to the reduced gene expression of IL-17E.

Previously, IL-4 and IL-13 were known to play redundant roles in Th2 mediated infections[139]. However, investigators later isolated distinct functions of IL-4 and IL-13 using mice deficient in both cytokines. For example, IL-13 was shown to play a more predominant role in driving lung pathology in asthma by inducing robust AHR, goblet cell hyperplasia, mucus production and smooth muscle contraction[140, 141]. Similarly, IL-13 was also shown to play a role in eosinophil infiltration from blood into the lungs[142]. Although IL-4 was also shown to be sufficient in inducing AHR and lung inflammation in the absence of IL-13[140, 141, 143], it was shown to play a more prominent role in humoral immunity responses such as IgE production[140]. Here we show that IL-13 gene expression and protein levels in BALF are significantly reduced in the *Cd2*$^{-/-}$ HDME mice. In agreement to this finding, we also observed decreased gene expression of both IL-13Rα1 and IL-13Rα2, which were both shown by multiple previous studies to play a role in IL-13 mediated AHR and goblet cell hyperplasia[118]. Similarly, we also show reduced gene expression of polymeric mucins, Muc5ac and Muc5b, and reduced PAS+ staining from lung sections of *Cd2*$^{-/-}$ HDME mice. These data provide evidence that there is reduced IL-13 mediated signaling and in turn reduced IL-13 mediated hallmark of asthma such as AHR, goblet cell hyperplasia and mucus production. However, the data does not provide insight into how IL-13 gene expression is reduced. Th2 cells are prominent producers of IL-13 in asthma[111] and do not express IL-13Rα1[144]. There is also no evidence in literature of T cells expressing membrane IL-13Rα2, which suggests that IL-13 production by T cells is not regulated by IL-13Rα1 or membrane IL-13Rα2. However, mice express a soluble form of IL-13Rα2 that is produced by alternate splicing[145], which was shown to be able to neutralize IL-13 with higher affinity than membrane bound IL-13Rα2[146]. Since we observed reduced cytokine levels in the BALF of the *Cd2*$^{-/-}$ HDME mice, it is possible that these mice have higher gene expression of soluble IL-13Rα2, which in turn neutralizes the IL-13 that is

being produced. The reduced IL-13 in turn perhaps leads to decreased IL-13Rα1 or membrane IL-13Rα2, since IL-13 levels were shown to regulate expression of both of these receptors[146]. These data however highlight the interesting role CD2 plays in regulating IL-13 and its receptors and provides new details on IL-13 signaling, of which not much is known.

APPENDIX

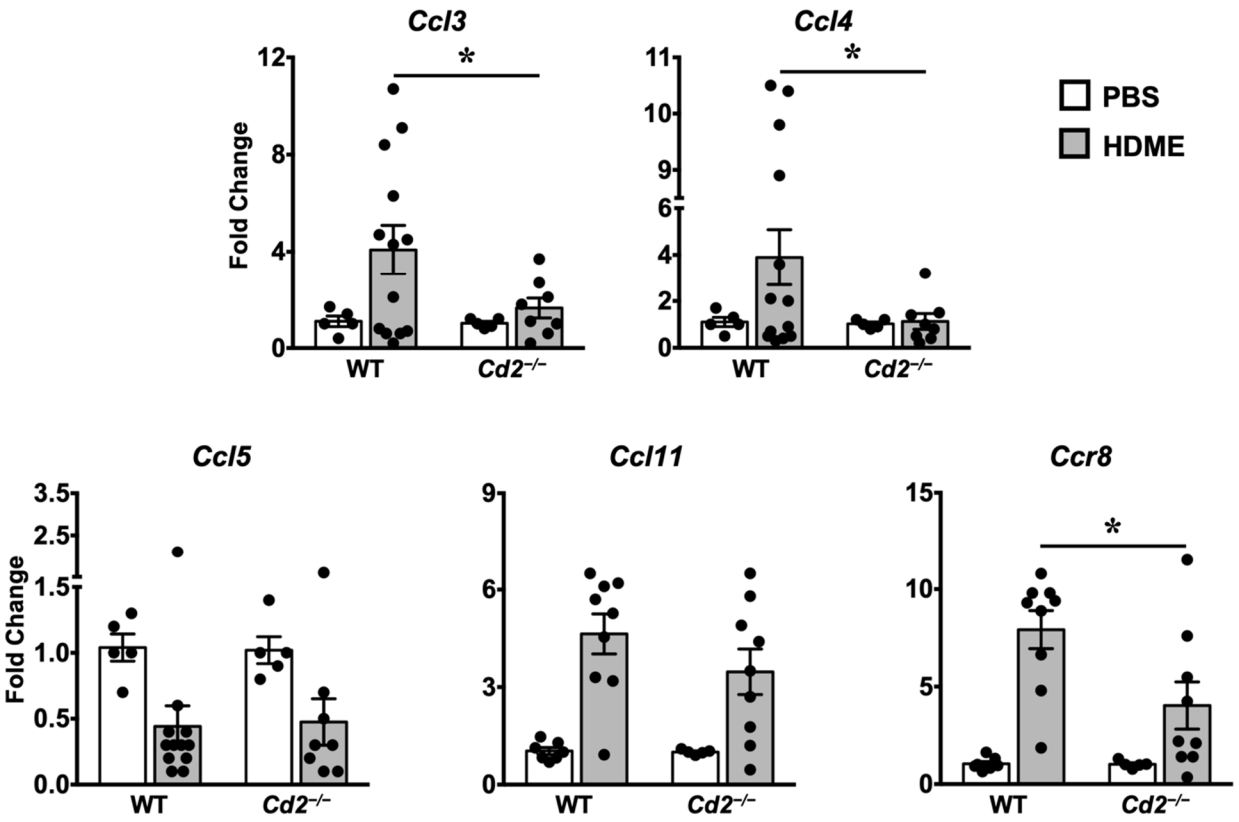


Figure 14. Reduced chemokine gene expression in *Cd2*$^{-/-}$ mice challenged with HDME. Lung tissue homogenates of WT and *Cd2*$^{-/-}$ mice injected with PBS or HMDE were analyzed for the mRNA levels of various chemokines including *Ccl3, Ccl4*, *Ccl5, Ccl11* and *Ccr8* by qPCR. Data is shown as mean fold change ± SEM and pooled from 3 independent experiments with a total of 8-13 mice per cohort. Statistical significance was determined by Student's unpaired *t* test with Welch's correction. *p≤ 0.05.

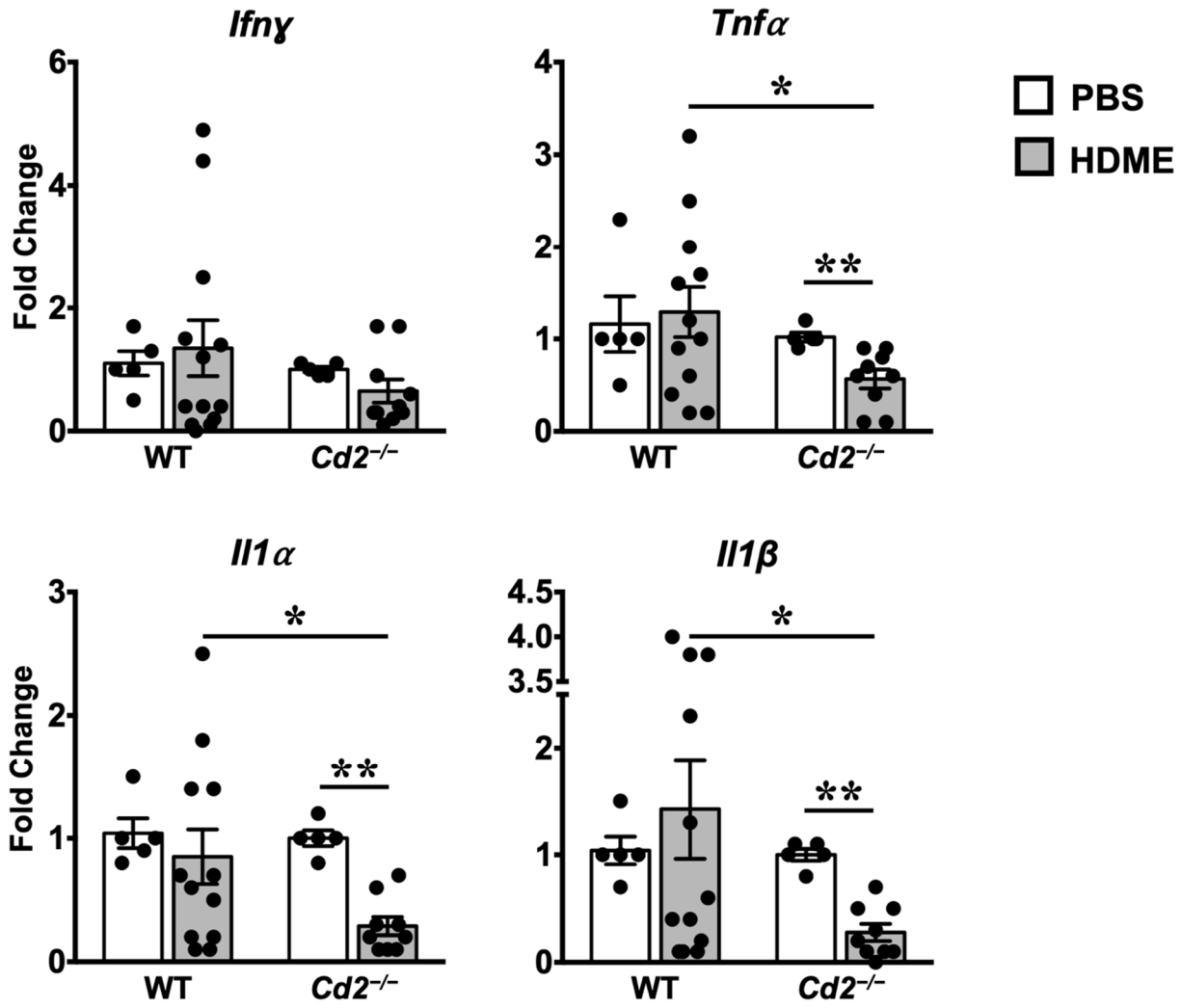


Figure 15. Th1 cytokine gene expression are significantly reduced in the lungs of HDME-challenged *Cd2*$^{-/-}$ mice. Lungs of WT and *Cd2*$^{-/-}$ mice injected with PBS or HMDE were analyzed for gene expression of *Ifnγ, Tnfα, Il1α* and *Il1β* . Data is presented as mean fold change ± SEM and is pooled from 3 independent experiments with a total of 8-13 mice per cohort. Statistical significance was determined by using Student's unpaired *t* test with Welch's correction. *p≤ 0.05, **p≤ 0.01.

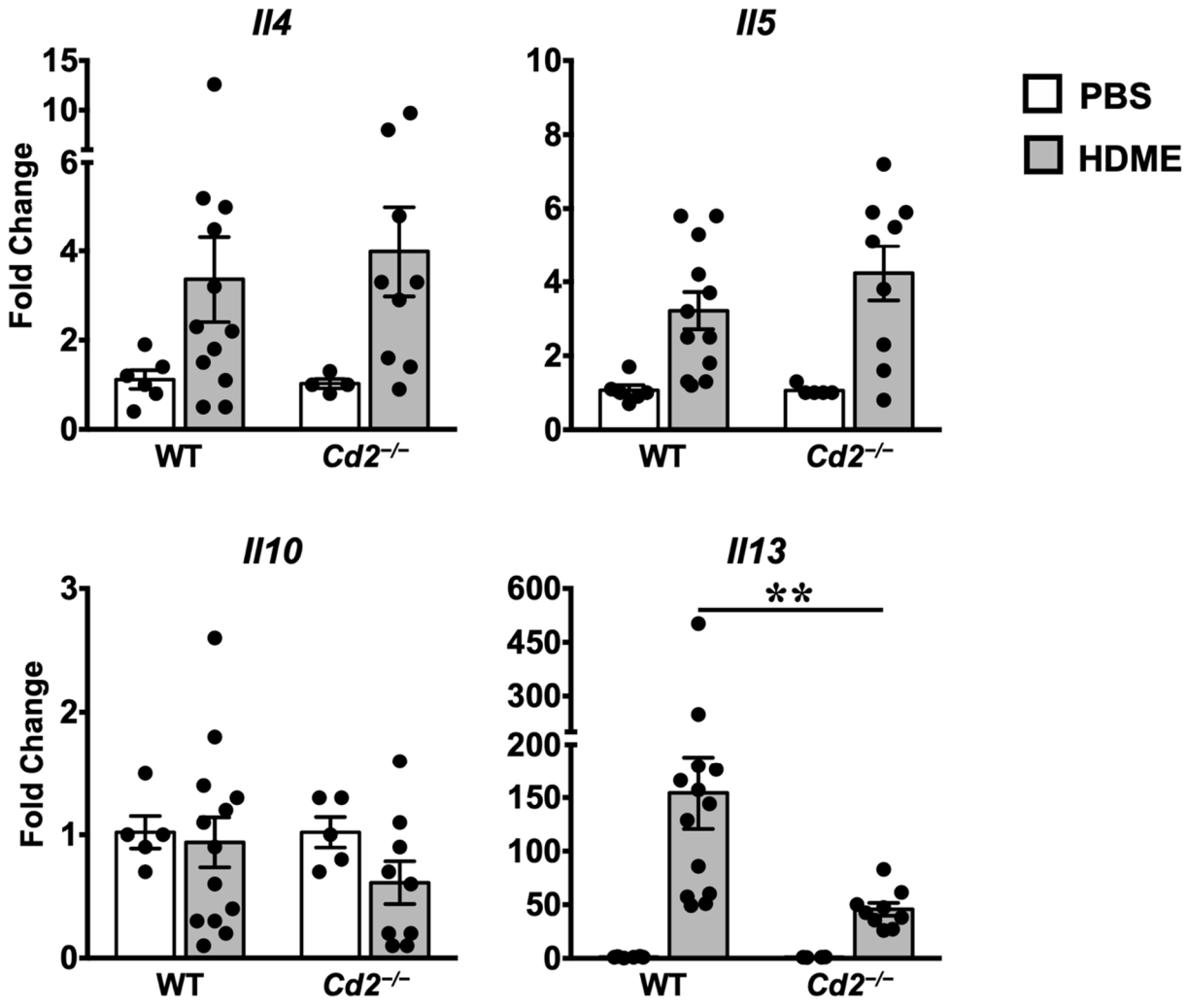


Figure 16. IL-13 gene expression is significantly reduced in the lungs of HDME-challenged *Cd2*$^{-/-}$ mice. Lungs of WT and *Cd2*$^{-/-}$ mice injected with PBS or HMDE were analyzed for gene expression of *Il4, Il5, Il10* and *Il13*. Data is presented as mean fold change ± SEM and is pooled from 3 independent experiments with a total of 8-13 mice per cohort. Statistical significance was determined by using Student's unpaired *t* test with Welch's correction. **p≤ 0.01.

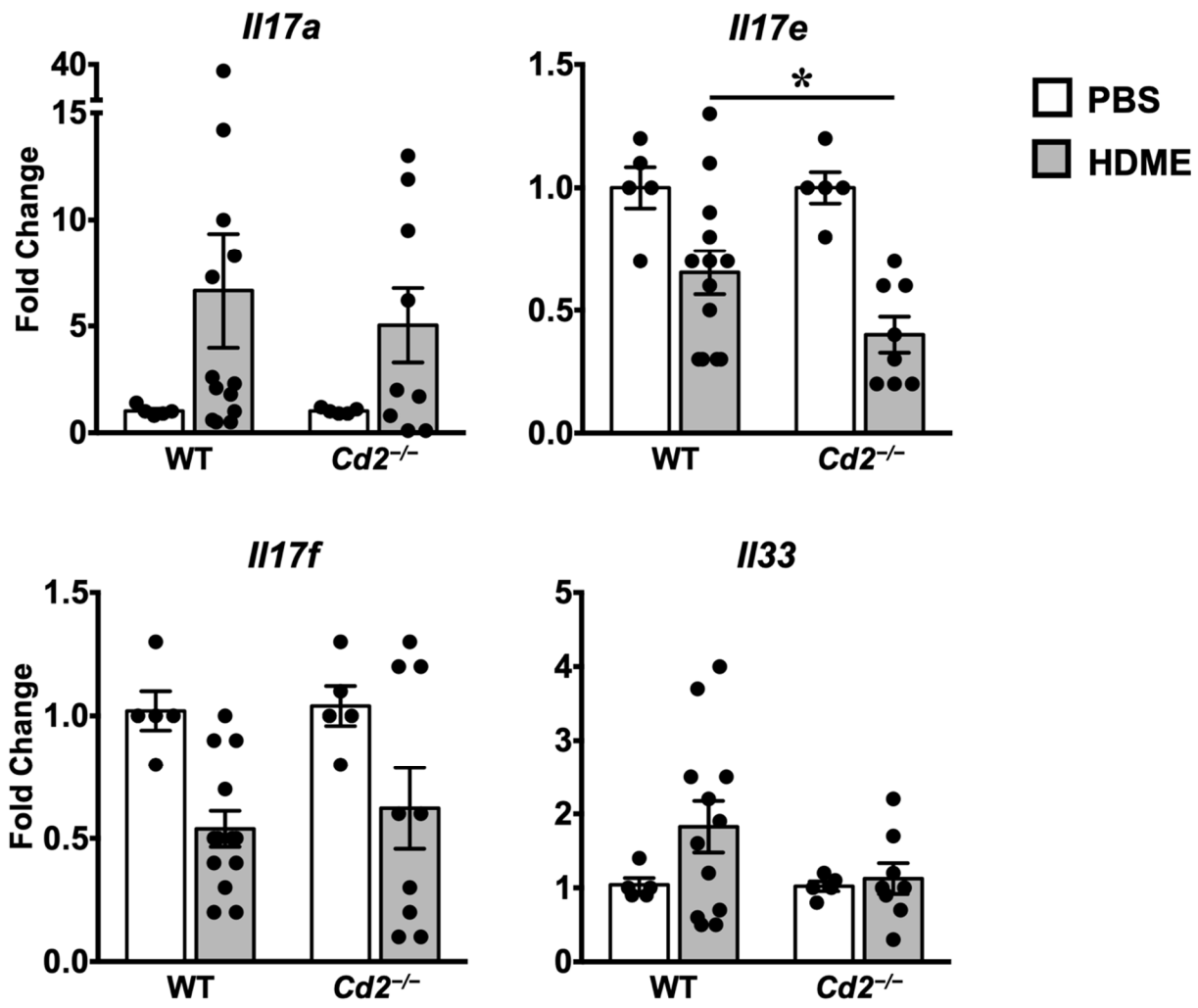


Figure 17. IL-17E gene expression is significantly reduced in the lungs of HDME-challenged *Cd2*$^{-/-}$ mice. Lungs of WT and *Cd2*$^{-/-}$ mice injected with PBS or HMDE were analyzed for gene expression of *Il17a, Il17e, Il17f* and *Il33*. Data is presented as mean fold change ± SEM and is pooled from 3 independent experiments with a total of 8-13 mice per cohort. Statistical significance was determined by using Student's unpaired *t* test with Welch's correction. *p≤ 0.05.

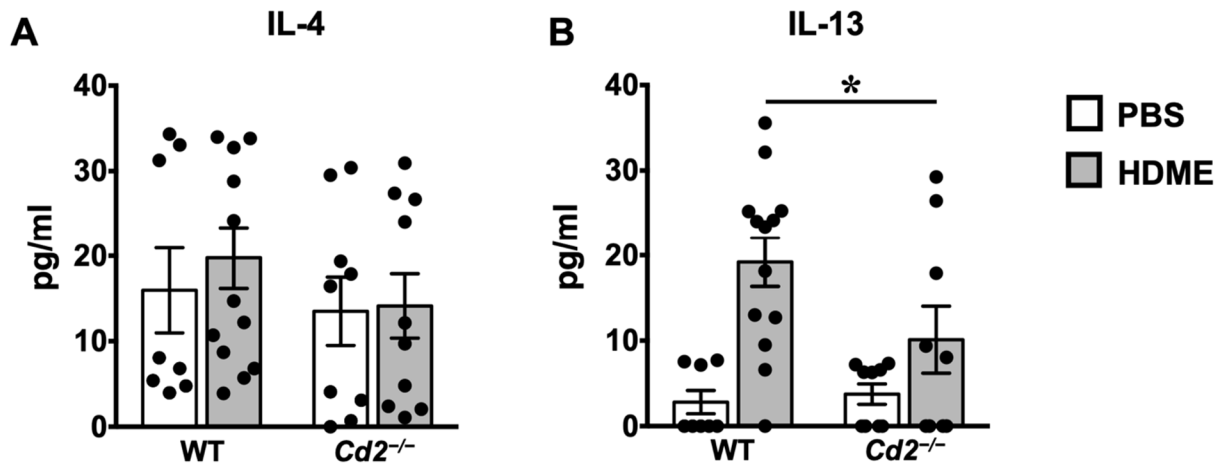


Figure 18. IL-13 protein levels are significantly reduced in the BALF of HDME-challenged *Cd2*$^{-/-}$ mice. Lungs of WT and *Cd2*$^{-/-}$ mice injected with PBS or HMDE were analyzed for various cytokines. (A) IL-4 and (B) IL-13 cytokine levels in the BALF supernatant of WT and *Cd2*$^{-/-}$ mice challenged or not with HMDE. Data is presented as mean ± SEM and is pooled from 3 independent experiments with a total of 8-13 mice per cohort. Statistical significance was determined by using Student's unpaired *t* test with Welch's correction. *$p \leq 0.05$.

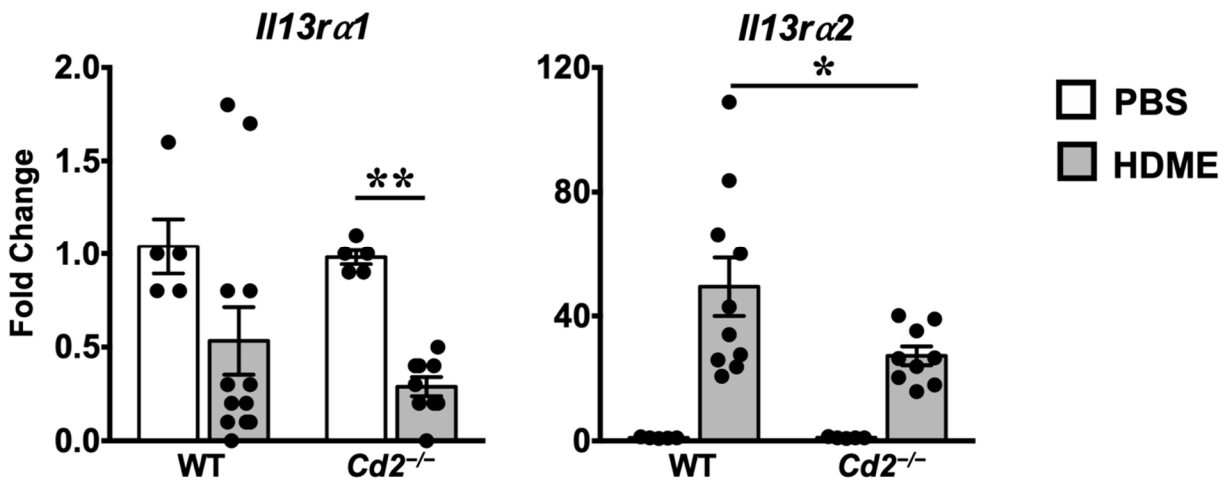


Figure 19. IL-13Rα1 gene expression is reduced in the lungs of HDME-challenged *Cd2*$^{-/-}$ mice. Lungs of WT and *Cd2*$^{-/-}$ mice injected with PBS or HMDE were analyzed for gene expression of *Il13rα1* and *Il13rα2*. Data is presented as mean fold change ± SEM and is pooled from 3 independent experiments with a total of 8-13 mice per cohort. Statistical significance was determined by using Student's unpaired *t* test with Welch's correction. **p≤ 0.01.

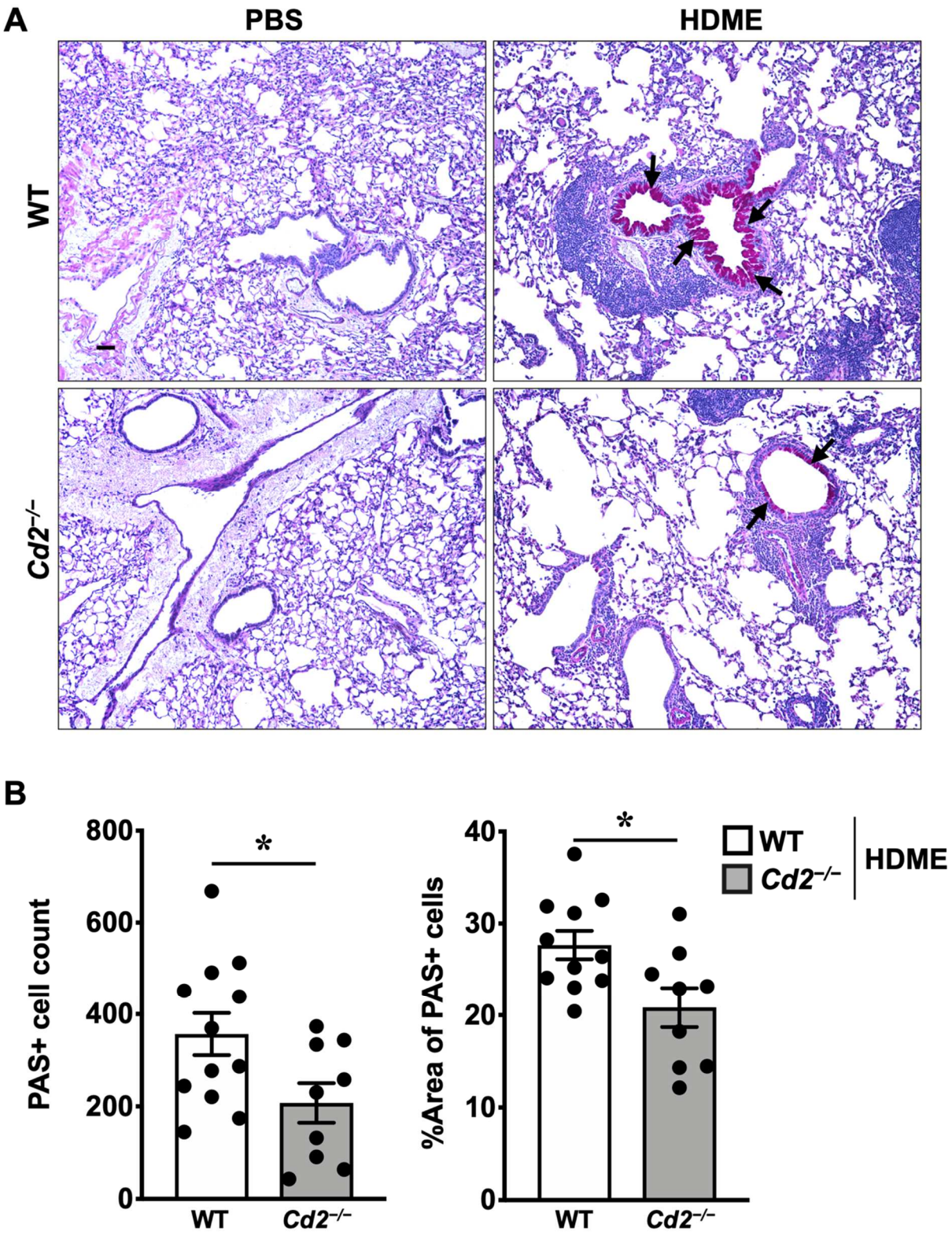


Figure 20. Reduced goblet cell hyperplasia in the lungs of HDME-challenged *Cd2*$^{-/-}$ mice.

Figure 20. (cont'd)

WT and *Cd2*$^{-/-}$ mice were challenged with or without HMDE. Twenty-four hours after the last injection of HDME, mice were sacrificed, and lung tissues were analyzed for PAS staining. (A) Representative lung images at 10X magnification and (B) PAS positive cell counts and % area of PAS positive cells is shown. Scale bar: 100μm. Data in B are presented as mean ± SEM and are pooled from 3 independent experiments with a total of 10-12 mice per cohort. Statistical significance was determined using Student's unpaired *t* test with Welch's correction. *$p \leq 0.05$.

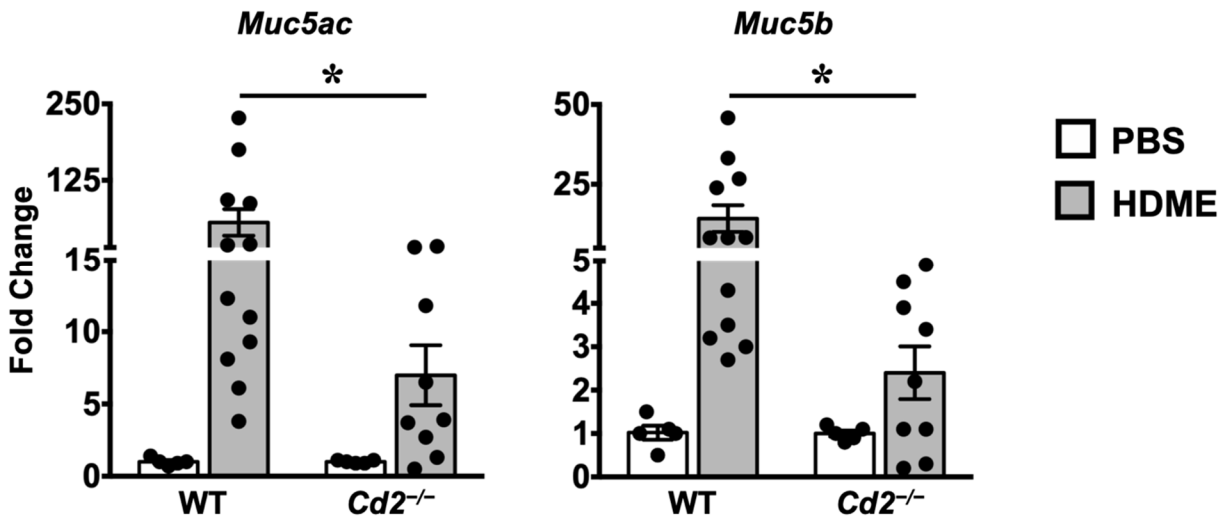


Figure 21. Reduced mucin production in the lungs of HDME-challenged *Cd2*$^{-/-}$ mice. WT and *Cd2*$^{-/-}$ mice were challenged with or without HMDE. Twenty-four hours after the last injection of HDME, mice were sacrificed, and lung tissues were analyzed for gene expression of polymeric mucins *Mus5ac* and *Muc5b*. Data are presented as mean fold change ± SEM and are pooled from 3 independent experiments with a total of 8-13 mice per cohort. Statistical significance was determined using Student's unpaired *t* test with Welch's correction. *$p \leq 0.05$.

Table 2. List of Taqman primers used for mRNA expression.

Target gene	Assay ID	Catalog number*
IL-4	Mm00445259_m1	4331182
IL-5	Mm00439646_m1	4331182
IL-10	Mm01288386_m1	4331182
IL-13	Mm00434204_m1	4331182
IL-13Rα1	Mm01302068_m1	4331182
IL-13Rα2	Mm00515166_m1	4331182
IL-17A	Mm00439618_m1	4331182
IL-17E	Mm00499822_m1	4331182
IL-17F	Mm00521423_m1	4331182
IL-33	Mm00505403_m1	4331182
IFNγ	Mm01168134_m1	4331182
TNFα	Mm00443258_m1	4331182
IL-1α	Mm00439620_m1	4331182
IL-1β	Mm00434228_m1	4331182
CCL3	Mm00441259_g1	4331182
CCL4	Mm00443111_m1	4331182
CCL5	Mm01302427_m1	4331182
CCL11	Mm00441238_m1	4331182
CCR8	MM99999115_s1	4331182
MUC5AC	Mm01276718_m1	4331182
MUC5B	Mm00466391_m1	4331182
CD2	Mm00488928_m1	4331182
CD48	Mm00455932_m1	4331182

*All the primers were purchased from Applied Biosystems (Foster City, CA)

CHAPTER 5

CD2 REGULATES EXPRESSION OF VARIOUS MICRO-RNAS IN A MOUSE MODEL OF HDME-INDUCED ASTHMA

Introduction

Recent studies have suggested that miRNAs play a major role in asthma. Micro RNAs are a small non-coding RNAs that are highly conserved and are about 18-22 nucleotides in length[147]. They initiate posttranscriptional or translational suppression processes by binding to the 3′□untranslated region (3′□UTR) of their target mRNAs[148, 149]. They can also suppress gene expression through mRNA degradation. Multiple miRNAs can regulate one gene, or a single miRNA can regulate multiple genes[148, 149]. Micro RNAs can play a role in multiple aspects of asthma pathogenesis. They can regulate epithelial cell, smooth muscle cell, or immune cell functions.

In epithelial cells of asthma patients, miR-19a was found to be upregulated. The target of miR-19a was found to be TGF-βR2 which activates other transcription factors related to cell proliferation[150]. MiR-181-5p was also shown to be decreased in airway epithelia of patients with high sputum and bronchial submucosal eosinophilia[151]. A few more miRNAs that were associated with airway smooth muscle include miR-10, miR-142-3p, miR-25, and miR-133[152, 153, 154, 155]. MiR-10 and miR-142-3p has been shown to be associated with airway smooth muscle hypertrophy and hyperplasia by targeting PI3 kinase pathway[152, 153]. MiR-25 has been shown to target secretion of chemokines such as eotaxin and RANTES and miR-133 has been associated with contraction of airway smooth muscles by regulating IL-13[154, 155]. Multiple miRNAs have been associated with the Th2 high endotype of asthma. These include miR-155, miR-181a, miR-21, miR-19a, miR-221-3p, miR-1248, miR-146a, and the let-7 family. MiR-155 has been shown to target IL-4, IL-5, IL-13, IL-17a, and CTLA-4. It has been associated with the regulating the activation and proliferation of T cells as well as mucus production and enhanced inflammation in asthma[156, 157]. MiR-181a has

been associated with increasing the sensitivity of T cells to peptide antigens[158] and miR-21 and miR-19a has been shown to promote T cell differentiation towards a Th2 phenotype[158, 159, 160, 161]. MiR-221-3p was shown to upregulate IL-4[162] and miR-1248 was shown to upregulate IL-5[163, 164]. In contrast, miR-146a has been shown to inhibit IL-5 and finally, IL-13 downregulation is associated with the let-7 family of miRNAs[165, 166]. Several other miRNAs that have been associated with neutrophilic asthma which includes miR-199a-5p, miR-223-3p, miR-142-3p and miR-629-3p, all of which was found in human induced sputum[167]. Bronchial epithelial cells express miR-629-3p and macrophages, neutrophils and monocytes express miR-223-3p and miR-142-3p[167]. Reduced pulmonary function was shown to be associated with miR-199a-5p[167]. The mechanistic link between airway neutrophilia in severe asthma patients and higher miR-629-3p expression is IL-8. MiR-629-3p induces epithelial cells to upregulate IL-8 which in turn recruit neutrophils into the airways[168]. Taken together, current literature has shed light onto miRNAs and the important mechanistic roles they play in the pathogenesis of the different endotypes of asthma.

Here we have screened for miRNAs and show that several miRNAs are upregulated in the lungs of *Cd2*$^{-/-}$ mice challenged with HDME. We have divided them into three categories according to their function: airway smooth muscle cell (ASMC) proliferation, goblet cell hyperplasia and inflammation, as well as IL-13 regulation. MiR-19b-3p, miR-23b-3p, miR-142-3p, miR-150-3p, miR-384-5p and miR-708-5p, all of which has been shown to play a role in ASMC proliferation, were significantly upregulated in the *Cd2*$^{-/-}$ mice. MiR-155-5p, miR-221-3p and miR-223-5p, which were shown to play a role in inflammation, and miR-145-5p, which was shown to play a role in the production of Muc5ac and Muc5b, were all significantly reduced in the absence of CD2. Lastly, we most strikingly show that the Let-7 family of miRNAs, which have been shown to

directly target IL-13 transcripts, are significantly reduced in the absence of CD2. Collectively, these data suggest that CD2 indirectly regulates AHR and IL-13 levels by specifically regulating miRNAs and reveals a novel mechanism that strengthens the position of CD2 as a potential target for therapy.

Materials and Methods

Mice

Mice were used as described in Chapter 2.

Mouse model of mild asthma

The same model was used as described in Chapter 2.

Quantitative real-time PCR

Lungs were dissociated in TRIzol solution (Invitrogen) using a high-speed homogenizer (Fisher Scientific, Hampton, NH) and total RNA was extracted as per manufacturer's protocol. RNA (2 μg) was reverse transcribed into cDNA using the Taqman Advanced miRNA cDNA synthesis kit according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Real-time quantitative PCR was performed using Quant Studio™ 3 system (Applied Biosystems) with validated Taqman primers and Fast Advanced Master Mix according to manufacturer's instructions. Relative gene expression data (fold change) between samples was accomplished using the $2^{-\Delta\Delta Ct}$ method. 18S was used as the internal reference control.

Statistics

Statistical analysis was performed as described in Chapter 2.

Results and Discussion

Protective miRNAs are upregulated in the absence of CD2. Several micro RNAs (miRNA) have been shown to play either protective or proinflammatory roles in asthma[149]. Hence, we screened for various miRNAs to detect changes, and have divided them into groups according to their function known in the literature. In comparison to WT PBS mice, *Cd2*$^{-/-}$ PBS mice showed comparable levels of all miRNAs analyzed (Figure 22A) indicating that the absence of CD2 did not alter miRNA expression in the lungs. Out of the miRNAs analyzed, miR-155-5p, miR-145-5p, miR-708-5p, miR-223-5p, miR-150-5p, miR-142-3p, miR-19b-3p, miR-221-3p, miR-150-3p and miR-221-5p expression was significantly increased in WT HDME mice in comparison to WT PBS mice with the exception of miR-23b-3p which was significantly reduced (Figure 22B). Except miR-206 and miR-221-5p, which showed comparable levels of gene expression, all the remaining miRNA expression was significantly higher in *Cd2*$^{-/-}$ HDME mice in comparison to the WT HDME group (Figure 22B). In previous literature, increased expression of miR-19b-3p, miR-23b-3p, miR-142-3p, miR-150-3p, miR-384-5p and miR-708-5p were shown to inhibit airway smooth muscle proliferation or AHR through different mechanistic pathways[169, 170, 171, 172, 173, 174]. For example, miR-19b-3p, miR23b-3p and miR-142-3p were all shown to inhibit TGF-β dependent airway smooth muscle cell proliferation[169, 170, 171]. MiR-150-3p was shown to inhibit ASMC proliferation by downregulating BCYRN1[172] and miR-384-5p was shown to reduce AHR by reducing autophagy of ASMCs[173]. Lastly, miR-708-5p was shown to inhibit ASMC proliferation by reducing CD38 expression on ASMCs[174]. In agreement with the previous literature, expression of miR-19b-3p, miR-23b-3p, miR-142-3p, miR-150-3p, miR-384-5p and miR-708-5p was significantly increased in the *Cd2*$^{-/-}$ HDME mice in comparison to the WT (Figure 23). MiR-145-

5p, known to regulate polymeric mucin expression by downregulating epithelial growth factor (EGFR)[175], was significantly upregulated in the lungs of *Cd2*$^{-/-}$ HDME mice as well as certain miRNAs that regulate inflammation such as miR-155-5p, miR-221-3p and miR-223-5p[156, 157, 176, 177] (Figure 24). Lastly, we analyzed the expression of the Let-7 family of miRNAs, of which Let-7d, Let-7f, Let-g and Let-7i have been shown to directly bind to the 3' UTR of IL-13 mRNA and degrade it[166]. With the exception of Let-7c, all the Let-7 family of miRNAs was significantly increased in the *Cd2*$^{-/-}$ HDME mice in comparison to the WT (Figure 25) suggesting that the absence of CD2 IL-13 gene expression is suppressed through the Let-7 family of miRNAs.

Taken together, these data shows that CD2 can regulate the expression of various miRNAs that enhance characteristics of asthma including AHR, inflammation and IL-13 production. Here we have identified several miRNAs playing protective roles in the absence of CD2. An increase in miR-145-5p was previously shown to reduce MUC5AC and MUC5B expression by targeting epidermal growth factor receptor (EGFR) in an OVA-induce model of asthma[175]. Similarly, we show a significant increase in miR-145-5p in the *Cd2*$^{-/-}$ mice resulting in a protective role in this model. We also observed a significant decrease in Muc5ac and Muc5b, indicating that miR-145-5p perhaps plays the same role in this model by targeting EGFR and reducing mucin expression in turn. An increase in miR-19b-3p, miR-23b-3p, miR-142-3p, miR-150-3p, miR-150-5p, miR-384-5p and miR-708-5p was previously shown to inhibit airway smooth muscle cell proliferation[169, 170, 171, 172, 173, 174]. Airway smooth muscle cell proliferation also is a known contributor of AHR and an increase in these 7 miRNAs correlates to the decreased AHR we observe in the *Cd2*$^{-/-}$ mice. A mouse deficient in miR-223 was shown to have highly proliferative eosinophil progenitors[175]. Similarly, we show an increase in miR-223-5p that perhaps results in

less eosinophils in the *Cd2*$^{-/-}$ mice. Likewise, another study showed that miR-223 deficient mice have more enhanced proliferation of neutrophil progenitors[176]. In agreement to the previous studies, we observed a decrease in neutrophils as well, in the *Cd2*$^{-/-}$ mice. This data suggests that in response to HDME, *Cd2*$^{-/-}$ mice produce more miR-223-5p which in turn results in reduced proliferation of both eosinophil and neutrophil progenitors. Another important miRNA in asthma, miR-155-5p, has been implicated to play a role in many different cell types with distinct roles in each in the context of asthma[156]. An interesting study previously showed that miR-155-5p can directly target IL-13Rα1 mRNA preventing translation into protein in macrophages and reduce IL-13 induced differentiation into M2 phenotype[177]. We observed reduced IL-13Rα1 gene expression in the lungs, which could be due to the increased miR-155-5p. Lastly, the Let-7 family of miRNAs has been shown to directly target IL-13 mRNA transcripts and intranasal delivery of Let-7 miRNA resulted in alleviated asthma feature in OVA-induced mice[166]. We have shown that except, Let-7c, all the Let-7 family of miRNAs are increased in the *Cd2*$^{-/-}$ mice challenge with HDME. This suggests that the increase in the Let-7 family of miRNAs also contributed to amelioration of asthma symptoms, perhaps by targeting IL-13 mRNA transcripts, in turn reducing IL-13 gene expression in the *Cd2*$^{-/-}$ mice. Collectively, these data show that not only does the absence of CD2 specifically target IL-13, but it also results in the increase of various protective miRNAs, providing protection of asthma symptoms through multiple different pathways.

APPENDIX

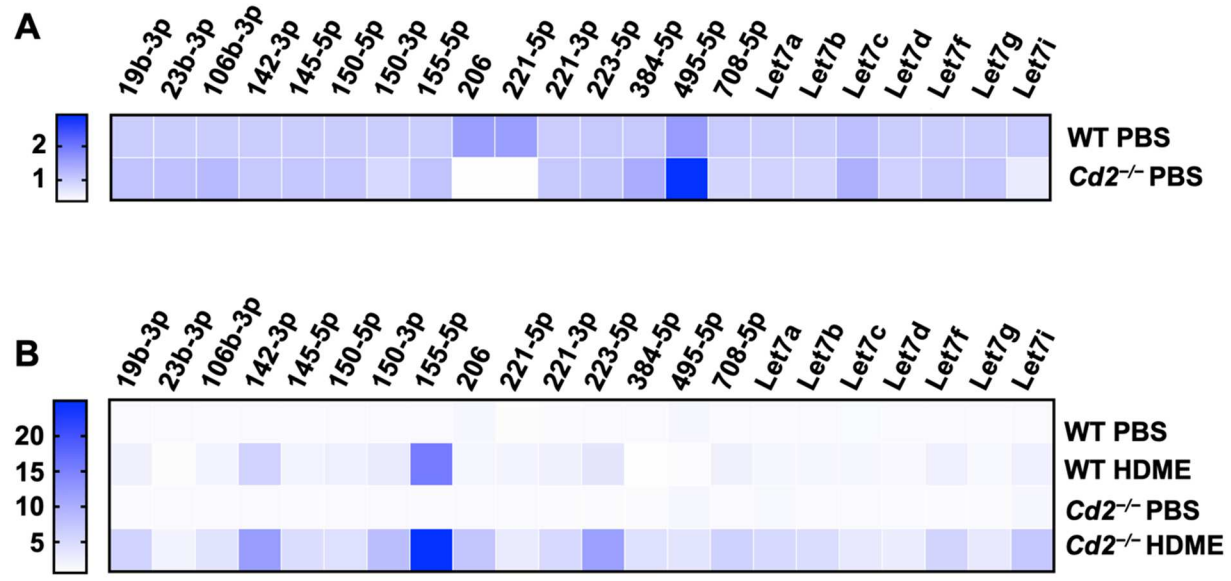


Figure 22. Increased expression of various miRNAs in the lungs of *Cd2*$^{-/-}$ mice challenged with HDME. WT and *Cd2*$^{-/-}$ mice were challenged with or without HMDE. Twenty-four hours after the last injection of HDME, mice were sacrificed, and lung tissues were analyzed for gene expression. (A) Heat map of gene expression of various miRNAs in lungs of PBS-treated *Cd2*$^{-/-}$ mice normalized to similarly treated WT animals. (B) Heat map of gene expression of various miRNAs in the lungs of WT and *Cd2*$^{-/-}$ mice injected with HDME, normalized to respective PBS control mice. Data is presented as mean ± SEM and are pooled from 2 independent experiments with a total of 7-8 mice per cohort.

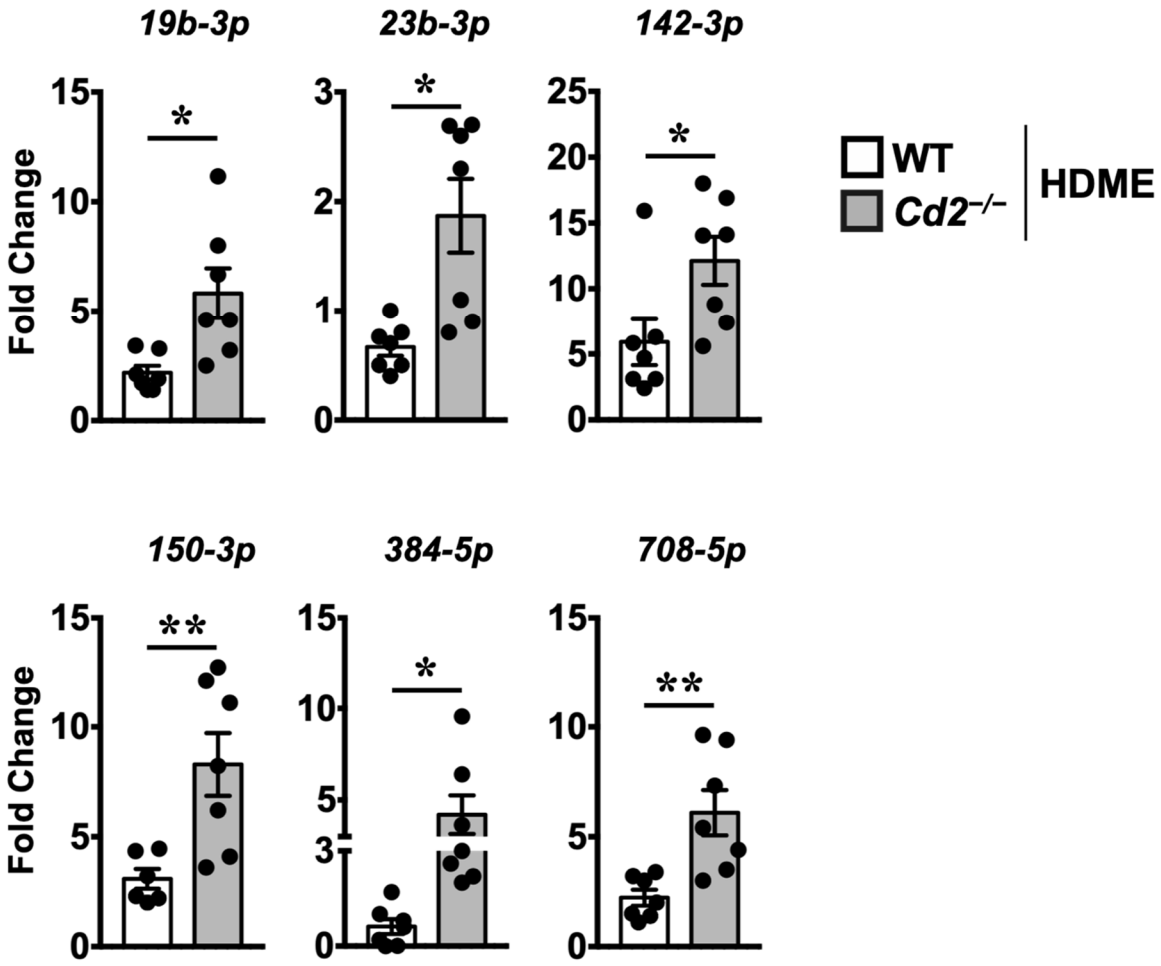


Figure 23. Increased expression of miRNAs associated with regulation of airway smooth muscle proliferation in the lungs of *Cd2*$^{-/-}$ mice challenged with HDME. WT and *Cd2*$^{-/-}$ mice were challenged with or without HMDE. Twenty-four hours after the last injection of HDME, mice were sacrificed, and lung tissues were analyzed for miRNA expression as indicated. Data is presented as mean ± SEM and are pooled from 2 independent experiments with a total of 7-8 mice per cohort. Statistical significance was determined using Student's unpaired *t* test with Welch's correction. *p≤ 0.05 or **p≤ 0.01.

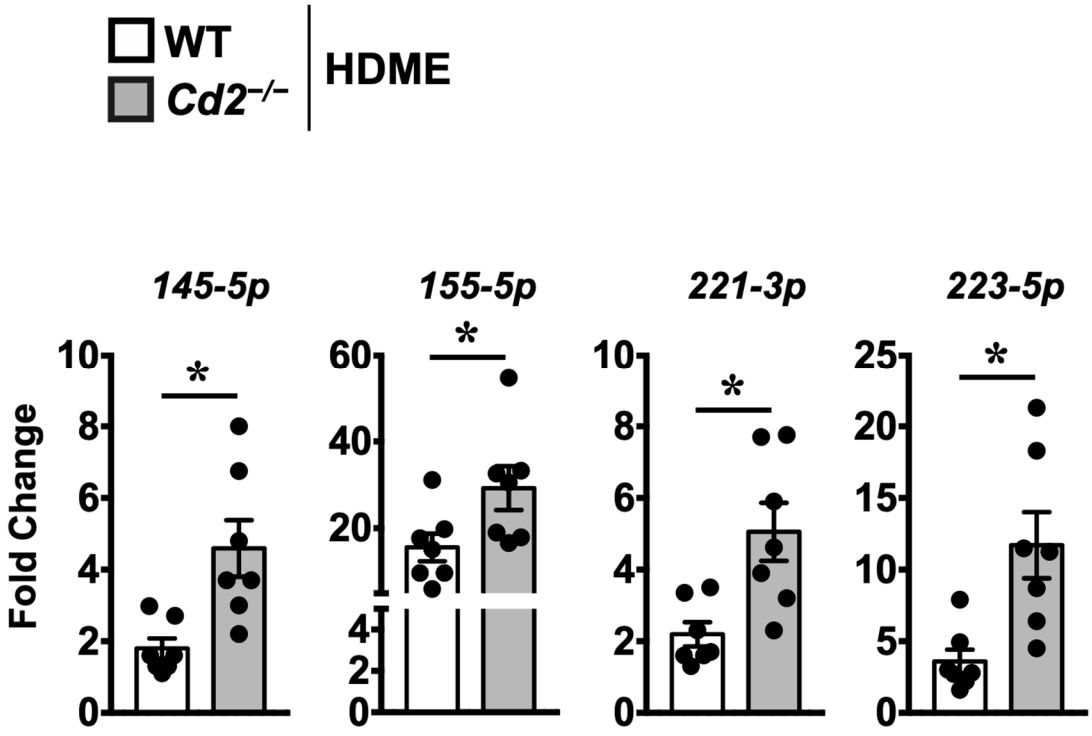


Figure 24. Increased expression of miRNAs associated with regulation of goblet cell hyperplasia and inflammation in the lungs of *Cd2*$^{-/-}$ HDME mice. WT and *Cd2*$^{-/-}$ mice were challenged with or without HMDE. Twenty-four hours after the last injection of HDME, mice were sacrificed, and lung tissues were analyzed for miRNA expression as indicated. Data is presented as mean ± SEM and are pooled from 2 independent experiments with a total of 7-8 mice per cohort. Statistical significance was determined using Student's unpaired *t* test with Welch's correction. *p≤ 0.05.

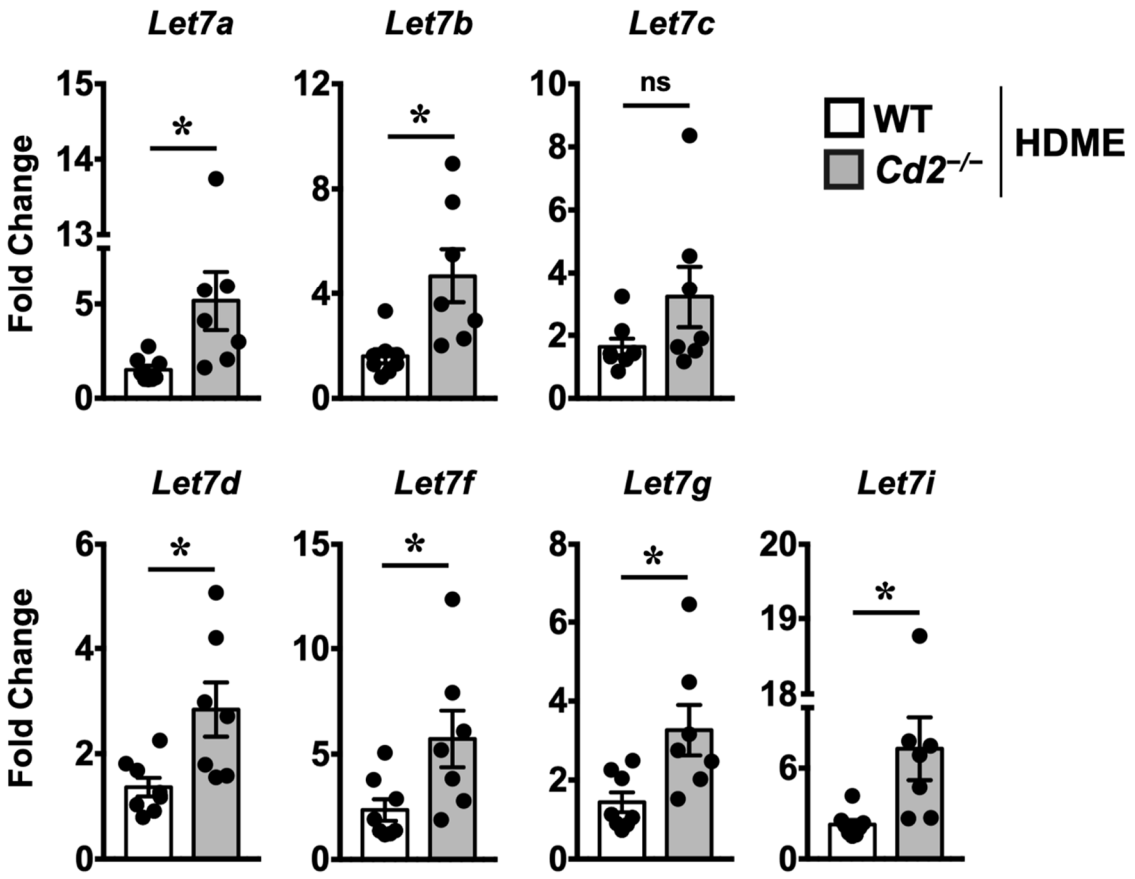


Figure 25. Increased expression of Let7 family of miRNA in the lungs of *Cd2*$^{-/-}$ mice challenged with HDME. WT and *Cd2*$^{-/-}$ mice were challenged with or without HMDE. Twenty-four hours after the last injection of HDME, mice were sacrificed, and lung tissues were analyzed for gene expression of Let-7 family of miRNAs as indicated. Data is presented as mean ± SEM and are pooled from 2 independent experiments with a total of 7-8 mice per cohort. Statistical significance was determined using Student's unpaired *t* test with Welch's correction. *$p \leq 0.05$.

Table 3. List of Taqman primers used for miRNA qPCR

miRbase ID	Assay ID	Catalog number*
miR-19b-3p	mmu478264	A25576
miR-23b-3p	mmu478602	A25576
miR-106b-3p	mmu478412	A25576
miR-142-3p	477910	A25576
miR-145-5p	477916	A25576
miR-150-5p	477918	A25576
miR-150-3p	mmu481552	A25576
miR-155-5p	mmu480953	A25576
miR-206	477968	A25576
miR-221-5p	mmu481659	A25576
miR-221-3p	mmu481005	A25576
miR-223-5p	rno481008	A25576
miR-384-5p	mmu481153	A25576
miR-495-5p	mmu482778	A25576
miR-708-5p	mmu481258	A25576
miR-Let-7a	478575	A25576
miR-Let-7b	478576	A25576
miR-Let-7c	478577	A25576
miR-Let-7d	mmu478439	A25576
miR-Let-7f	478578	A25576
miR-Let-7g	478580	A25576
miR-Let-7i	478375	A25576

*All the primers were purchased from Applied Biosystems (Foster City, CA)

CHAPTER 6

CD2 REGULATES AHR IN A TH2-LOW MURINE MODEL OF NEUTROPHILIC ASTHMA

Introduction

Poorly controlled asthma, in which disease symptoms are not responsive to corticosteroid treatment, is known as severe asthma[4, 178, 179]. In the US and Europe, up to 50% of the healthcare costs is spent on severe asthma patients, even though about only 5-10% of overall asthma patients are severe asthmatics[180, 181]. IL-13 has been linked to steroid resistant asthma, since it has been found to be highly expressed in steroid resistant asthma patients[182, 183]. Recently, a clinically relevant mouse model has been established that has recapitulated clinical representation of severe asthma[184]. This model of asthma was shown to be neutrophilic and initiated by a Th1 and Th17 response through IFNγ and IL-17A cytokines. Additionally, in this model of steroid resistant severe asthma, IL-13 is highly expressed in the lungs. We have previously shown in a Th2-high model of acute asthma, that CD2 specifically regulates the production of IL-13 levels and in turn ameliorates disease symptoms such as AHR and lung inflammation.

Hence, to further investigate the role of CD2 in a Th2-low endotype of asthma, we have established the severe asthma model in our lab. Here we show that the *Cd2*$^{-/-}$ mice induced with severe asthma exhibit significantly reduced AHR. *Cd2*$^{-/-}$ mice also produce significantly increased IgE, but not IgG1. Most strikingly, we show that the *Cd2*$^{-/-}$ mice induced with severe asthma display significantly reduced gene expression of IL-13 and IL-17 cytokine, but not IFNγ. Collectively, these data reveal that the role of CD2 in the regulation of IL-13 cytokine is intact, even in a severe model of asthma that has a sensitization and re-challenge phase. Most surprisingly, CD2 is also shown to regulate the production of IL-17A and not IFNγ. Taken together, these data suggest that CD2 regulates IL-13 cytokine in different models of asthma and suggests a role in steroid resistant

asthma through the regulation of both IL-13 and IL-17A. Given the fact the both IL-13 and IL-17A are huge players in steroid resistant severe asthma, CD2 once again reveals itself to be a multifaceted molecule, and a novel target for the therapy for asthma.

Materials and Methods

Mice

Mice were used as described in Chapter 2.

Mouse model of severe asthma

A previously described mouse model of severe asthma was used (1). Balb/c (wild-type) and *Cd2*$^{-/-}$ age-matched female mice were sensitized intranasally (i.n) with 25µl of 1µg/µl house dust mite extract (HDME) (28750 EU/vial; Stallergenes Greer, UK) mixed with 5µl of 1µg/µl c-di-GMP (Invivogen, San Diego, CA) on alternate days for a total of 3 injections. After 5 days of rest, mice were then challenged with HDME (25µg) and a lower dose of 0.5µl of 1µg/µl c-di-GMP on day 11 and with HDME alone on days 12 and 13. Using this low dose c-di-GMP, mice were challenged two more times on days 18, 19, 20 and 25, 26, 27. Twenty-four hours after the final HDME challenge, mice were anesthetized for measurement of airway hyperresponsiveness (AHR) and then sacrificed for collection of blood and lung tissue for various endpoint analysis.

AHR assessment

Mice were prepared for AHR assessments as described in Chapter 3. Parameters of AHR such as Newtonian airway resistance (Rn) was assessed by a methacholine (MCh; Sigma-Aldrich, St. Louis, MO) challenge test with increasing doses of MCh.

Blood serum collection

Blood serum collection was performed as described in Chapter 3.

Quantitative real-time PCR

Quantitative real-time PCR was performed as described in Chapter 2.

Statistics

Statistical analysis was performed as described in Chapter 2.

Results and Discussion

Absence of CD2 attenuates airway hyperresponsiveness (AHR) in a murine model of severe asthma. To analyze the role of CD2 in a Th2-low neutrophilic model of asthma, using an established model by a previous study[184], we sensitized Balb/c (WT) and *Cd2*$^{-/-}$ mice intranasally with a high dose of c-di-GMP and 25µg HDME (H+G) and subsequently challenged them with a low dose c-di-GMP and 25µg HDME (H+G), followed by two doses of 25µg HDME alone (Figure 26A). Twenty-four hours after the last challenge, mice were anesthetized and assessed for AHR parameter such as resistance to airflow in central airways (Rn) with increasing doses of methacholine (Mch) to observe if H+G sensitization and challenge has induced AHR in the WT and *Cd2*$^{-/-}$ mice (Figure 26B). Rn of the WT PBS mice increased with increasing doses of Mch. However, WT H+G mice showed significantly higher Rn at increasing doses of Mch in comparison to the WT PBS mice, exhibiting increased AHR in response to H+G. On the other hand, in comparison to WT H+G mice, Rn of *Cd2*$^{-/-}$ H+G mice were significantly lower at 50 and 100 mg/ml doses of MCh (Figure 26B), demonstrating that the absence of CD2 results in ameliorated AHR.

Since we used an allergen for sensitization, we measured serum immunoglobulin G1 (IgG1) and immunoglobulin E (IgE) levels in the mice. No significant difference in serum IgG1 in *Cd2*$^{-/-}$ H+G mice in comparison to the WT H+G mice was observed (Figure 27A), but we did detect a significant increase in serum IgE levels (Figure 27B). We noticed, however, that the *Cd2*$^{-/-}$ PBS mice had inherently higher levels of IgE in the serum (Figure 27B). Hence, to examine IgG1 and IgE production in response to H+G injection, we calculated fold change from respective PBS

groups. There was no significant difference in IgG1 fold change, but IgE fold change was significantly decreased in the *Cd2*$^{-/-}$ H+G mice, suggesting the *Cd2*$^{-/-}$ mice make less IgE in response to H+G injection.

CD2 regulates IL-13 and IL-17 gene expression in a mouse model of severe asthma. In this established model of severe asthma, Th1 cytokine, IFNγ and Th17 cytokine, IL-17 were shown to play specific roles in asthma pathogenesis. IFNγ was shown to play a role in AHR whereas IL-17A was shown to play a role in airway neutrophilia[184]. Additionally, IL-13 was also upregulated in this model of severe asthma[184], and since we have shown that CD2 specifically regulates IL-13 gene expression in our Th2-high endotype of asthma, we analyzed for gene expression of all the three cytokines mentioned above. IFNγ, IL-17A and IL-13 gene expression was significantly increased in WT mice challenged with H+G in comparison to WT PBS mice. However, IFNγ gene expression was comparable in the *Cd2*$^{-/-}$ H+G mice in comparison to the WT H+G group (Figure 28) whereas both IL-17A and IL-13 gene expression were significantly reduced (Figure 28). This data suggests that in addition to targeting IL-13, IL-17A is also specifically regulated by CD2, shining a light on CD2 as a potential target for therapy of all endotypes of asthma.

Here we have shown that CD2 not only plays a role in a Th2-high asthma, but also in a Th2-low endotype of asthma, that is predominantly of a Th1 and Th17 phenotype. Th2-low endotype of asthma is more common in the elderly and the obese but can also results from suppression of Th2-high asthma with the use of medication, by microbial infection, or by irritants[13, 16, 17, 18, 19, 20, 21]. We show a significant reduction in AHR in the *Cd2*$^{-/-}$ mice along with reduced IgE production. In this model of Th2-low asthma, it was shown that IFNγ plays a role in AHR but not IL-17A[184].

However, in contrast, we observed a significant reduction in only gene expression of IL-17A and not IFNγ in the *Cd2*$^{-/-}$ mice. This data suggests that IFNγ perhaps does not play a role in AHR and IL-17A does, which has been shown previously by another group[185]. Although our data is in contrast with Raundhal et al's[184] results, we also observed a significant reduction in IL-13 gene expression in the *Cd2*$^{-/-}$ mice, which could be the reason for ameliorated AHR. The reduction in IL-13 gene expression even in a Th2-low asthma model demonstrates the specificity of CD2 in regulating IL-13. In addition, although this Th2-low model is not an acute challenge model but a sensitization and challenge model, IL-13 is still reduced in the *Cd2*$^{-/-}$ mice, maintaining the role of CD2 in regulating IL-13 in any model of HDME-induced asthma. Given the increasing evidence of Th17 producing cells to be the cause of steroid resistant asthma[3], CD2 can be used in conjunction with corticosteroids to ameliorate hard to control steroid resistant Th2-low asthma as well.

APPENDIX

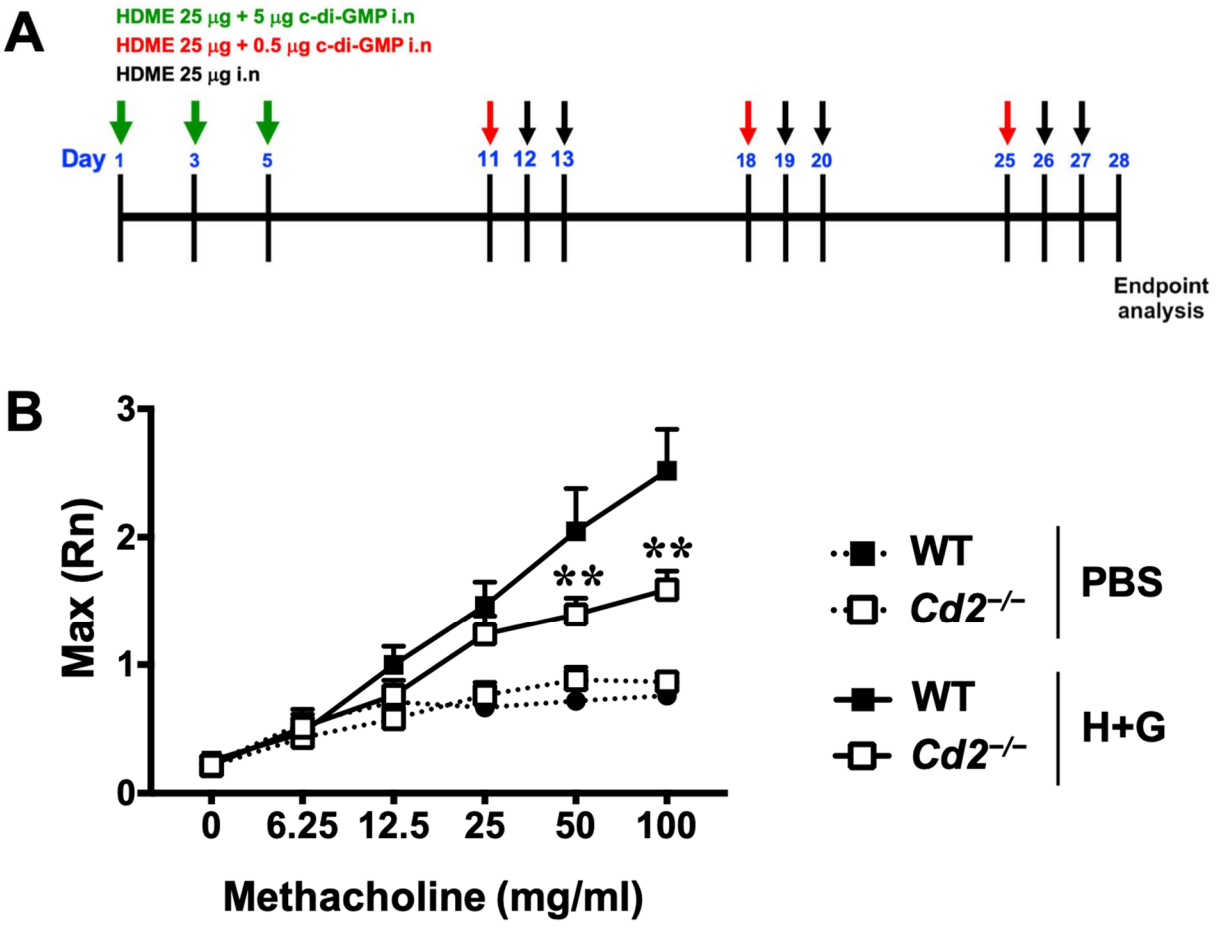


Figure 26. CD2 regulates AHR in a mouse model of severe asthma. (A) Schematics of the asthma model used in the study. Mice were sensitized and challenged with HDME+c-di-GMP (H+G) or treated with PBS as described in the methods section of chapter 6. Twenty-four hours after the last challenge, mice were analyzed for resistance to airflow in central airways (B) (Newtonian resistance [Rn]) after challenge with increasing doses of methacholine. Data is shown as mean ± SEM and pooled from 3 independent experiments with a total of 10-12 mice per cohort. Statistical significance was determined by two-way ANOVA with Tukey's test. **p≤ 0.01.

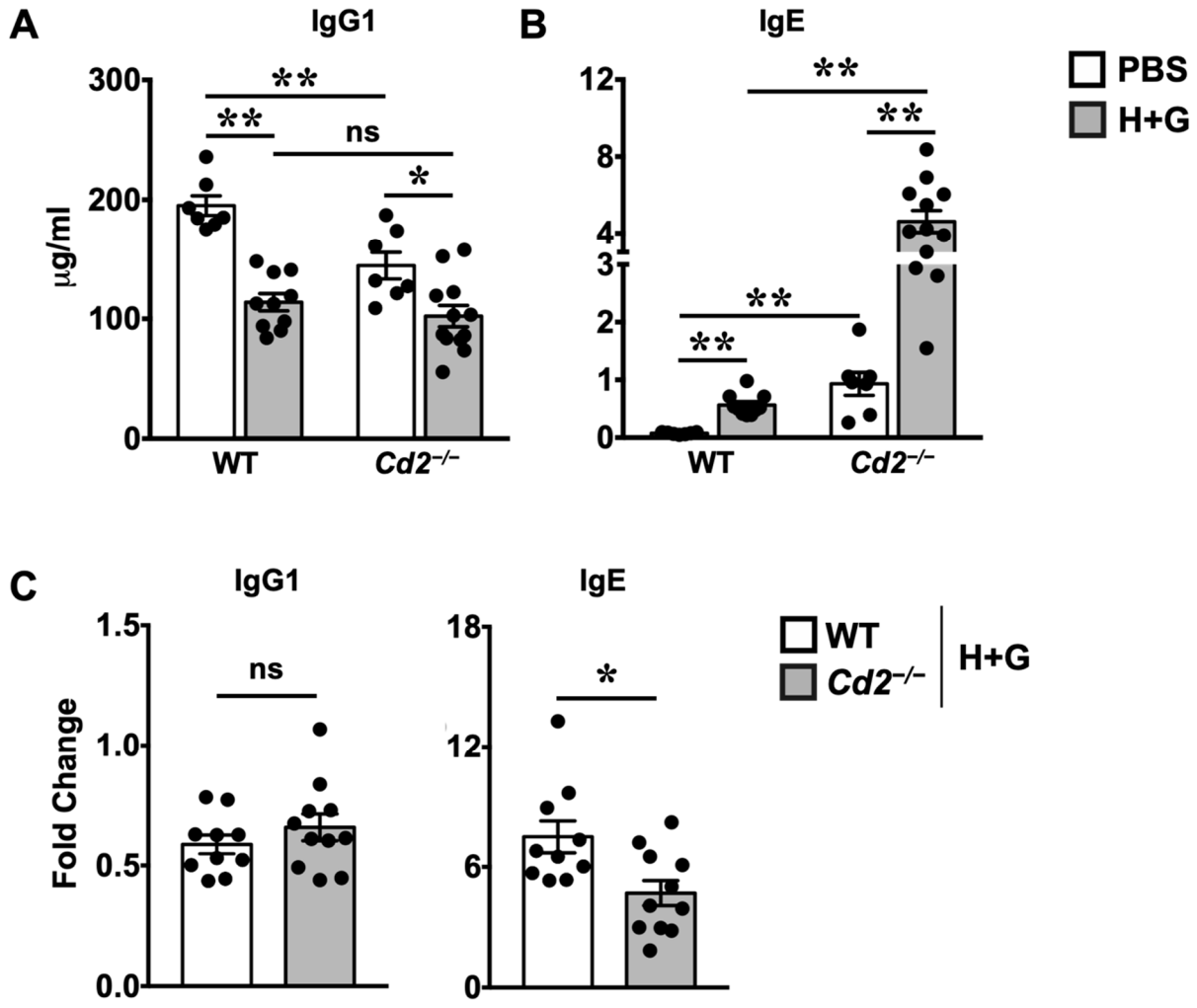


Figure 27. CD2 regulates immunoglobulin production in a mouse model of severe asthma. WT and *Cd2*$^{-/-}$ mice that were sensitized and challenged with H+G or treated with PBS were euthanized twenty-four hours after the last H+G challenge and analyzed for levels of serum (A) IgG1and (B) IgE. (C) Fold change in IgG1 and IgE levels in H+G-challenged mice as compared to PBS treated mice. Data is shown as mean fold change ± SEM and pooled from 3 independent experiments with a total of 11-12 mice per cohort. Statistical significance was determined by Student's unpaired *t* test with Welch's correction. **p≤ 0.01, *p≤ 0.05.

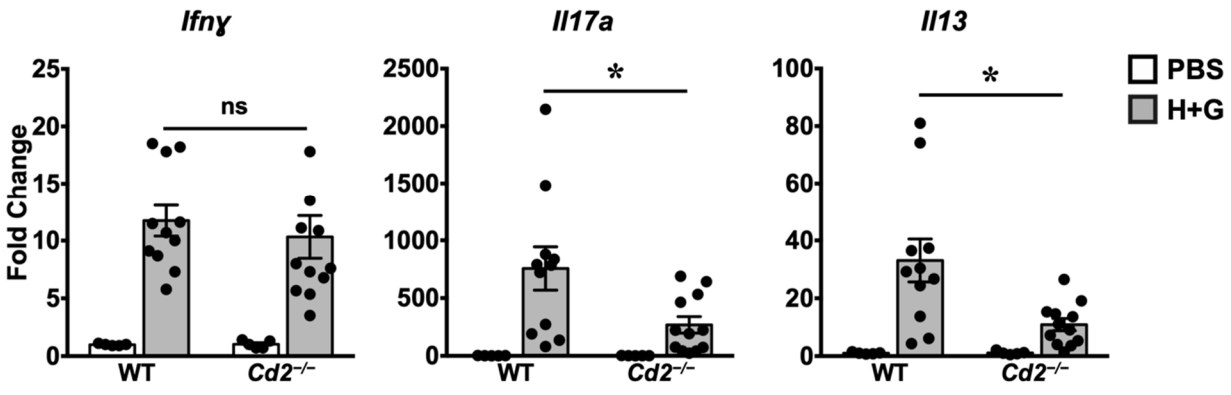


Figure 28. IL-17A and IL-13 gene expression is significantly reduced in the lungs of H+G challenged *Cd2*$^{-/-}$ mice. Lungs of WT and *Cd2*$^{-/-}$ mice treated with PBS or H+G were analyzed for gene expression of *Ifnγ, Il17a* and *Il13*. Data is presented as mean fold change ± SEM and is pooled from 3 independent experiments with a total of 11-12 mice per cohort. Statistical significance was determined by using Student's unpaired *t* test with Welch's correction. *p≤ 0.05.

CHAPTER 7

OVERALL DISCUSSION

Here we have used a clinically relevant allergen, HDME, and have shown that CD2 specifically regulates IL-13 mediated AHR, mucin production and goblet cell hyperplasia in a Th2-high endotype of experimental asthma. In addition, we have also shown that CD2 regulates AHR, IL-13 and IL-17A gene expression in a Th2-low endotype of experimental asthma. In this study, CD2 deficient mice injected with HDME exhibited ameliorated AHR. CD2 deficiency also resulted in significantly lower levels of chemokines CCL3 and CCL4, which are responsible for recruitment of eosinophils, monocytes and neutrophils into the site of inflammation. In agreement with this observation, eosinophils, monocytes and neutrophil numbers were decreased in the BALF of CD2 deficient mice as well as overall inflammation. Most strikingly, CD2 deficiency caused a marked reduction in IL-13 gene expression in the lungs and cytokine levels in the BALF, but not the other hallmark Th2 cytokines IL-4 and IL-5. We also observed reduced gene expression of IL13Rα1 and membrane IL-13Rα2 in the absence of CD2. Since IL-13 is a known inducer of goblet cell hyperplasia and mucus production, consistent with our observation of reduced IL-13 gene expression and protein levels, CD2 deficiency also caused significantly lower gene expression of polymeric mucins such as Muc5ac and Muc5b, along with lower PAS positive staining in lung sections. Lastly, the expression of various miRNAs that have been established to play protective roles in asthma, were significantly increased in CD2 deficient mice, providing insight into the potential mechanism of how CD2 regulates IL-13 mediated AHR in an HDME-induced mouse model of asthma shown in Figure 29.

Several previous groups have studied the role of costimulatory molecules in the context of asthma. Costimulatory molecules part of the CD28:B7 family and the TNFR family have been extensively studied and have established roles in the pathogenesis of asthma[80]. However, given the

heterogeneity of asthma, the roles of costimulatory molecules in response to different allergens have not been classified yet. CD2 is one costimulatory molecule that has not been studied as extensively. In comparison to previous studies investigating the roles of other costimulatory molecules, CD2 plays a role in AHR, lung inflammation, goblet cell hyperplasia and mucus production but not IgE production in an HDME-induced mouse model of asthma. In agreement with these observations, we found significantly reduced CCL3 and CCL4 gene expression, responsible for eosinophil recruitment, correlating to the reduced eosinophil numbers in BALF as well as overall inflammation observed in the *Cd2*$^{-/-}$ mice. We also found no difference in IL-4 gene expression, which is consistent with our observation of similar IgE levels between the WT and *Cd2*$^{-/-}$ mice induced with HDME. Lastly, our observation of IL-13, IL-13Rα1 and IL-13Rα2 gene expression reduced in the *Cd2*$^{-/-}$ mice were consistent with the decreased expression of polymeric mucins, Muc5ac and Muc5b, along with reduced PAS+ staining in lung sections. Since IL-13 was also shown to play a role in eosinophil recruitment by increasing vascular cell adhesion molecule 1 (VCAM1)[186], the reduction in IL-13 could have also contributed to the reduced eosinophils. This finding of reduced IL-13 is intriguing due to the specific regulation of IL-13, and not IL-4, by CD2.

IL-13 was shown to have important functions on nonhematopoietic cells such as smooth muscle cells, fibroblasts, and epithelial cells. It was previously shown to enhance smooth muscle contraction and proliferation in vitro[187] and induce collagen synthesis in human fibroblasts[188]. In epithelial cells, IL-13 can induce chemokine expression[189] and induce goblet cell metaplasia[140, 141, 190]. Taken together, these functions of IL-13 promote inflammation in allergic disorders and contribute to changes in the airway resulting in AHR and airway remodeling. Since IL-13 has been

reported to have no effects on T cells[191], unlike IL-4, IL-13 does not play an important role in the differentiation of naïve T cells to a Th2 phenotype. It rather plays an important role in the effector phase of allergic disorders. This effector role has been further proved by many observations in vivo, including administration of IL-13[140, 141] and overexpression of IL-13 in the lungs of transgenic mice[190] resulting in increased inflammation and IL-13 blockade in allergic inflammation[140, 141], which resulted in reduced inflammation. Additional evidence that IL-13 is a critical effector molecule was provided by a study in which inducible expression of IL-13 resulted in potent stimulation of matrix metalloproteinases and cathepsin proteases in the lung. Furthermore, overexpression of IL-13 in the lungs resulted in symptoms of emphysema and goblet cell hyperplasia[192]. These studies provide evidence that IL-13 is an important effector cytokine both in chronic obstructive pulmonary diseases and asthma. These studies also highlight the importance to target IL-13 in asthma, which has been attempted multiple times, without success in clinical trials. Recently, two anti-IL-13 clinical trials were not successful in controlling severe asthma and were discontinued and a current one in phase I of clinical trial[71, 72, 73]. This is perhaps due to the gap in knowledge of IL-13 signaling via its receptors. IL-13 has two receptors, IL-13Rα1 and IL-13Rα2[193, 194, 195, 196, 197, 198]. IL-13Rα2 can be expressed as a membrane bound form or a soluble form produced by alternate splicing[145]. Previously, it was thought that the membrane bound IL-13Rα2 was a decoy receptor and is not involve in signaling[135] and only IL-13Rα1 plays a role in signaling[130]. However, a recent study has provided evidence of IL-13Rα2 playing a role in experimental asthma[133] in addition to multiples other studies, which provided evidence of IL-13Rα2 involved in signaling[131, 132, 134]. Upon transgenic expression of specifically the membrane IL-13Rα2 in the absence of the soluble form, asthma features are enhanced[133]. These studies suggest that IL-13Rα1 and IL-13Rα2 both have important roles in downstream functions mediated

by IL-13. However, previous studies have also suggested that IL-13 level is responsible for the expression of both of these receptors in the lungs[146]. In that case, it is possible that increased IL-13 levels result in the increased expression of its receptors and creates a positive feedback loop, which when targeting the receptors, will only result in increased IL-13 production to compensate for the blocked IL-13Rα1 and IL-13Rα2. These data suggest that not enough is known about IL-13 signaling via its receptors and perhaps provides a reason to why targeting IL-13 has not been successful yet. We have shown that CD2 regulates IL-13 and its receptors which highlights its potential in being a novel target in the treatment of Th2-high asthma. The absence of CD2 leads to reduced IL-13, IL-13Rα1 and IL-13Rα2 gene expression that in turn results in reduced IL-13 protein levels in the BALF. In this study, we have not been able to show the order of events that leads to reduced IL-13 cytokine. Perhaps the soluble form of IL-13Rα2 is increased resulting in decreased IL-13 protein, which in turn reduces the gene expression of IL-13Rα1 and membrane IL-13Rα2. However, that is yet to be determined. In a mouse model of OVA-induced asthma, a previous study did show that administration of monoclonal antibody against CD2 resulted in reduced inflammation and number of eosinophils in the BALF, but not PAS+ staining[105]. We similarly observed a reduction in inflammation and eosinophil recruitment, but in contrast to Munitz et al's study[105], we show reduced polymeric mucin expression and goblet cell hyperplasia. Perhaps these inconsistencies are due to the different allergens used and different models of sensitization and challenge.

In addition to the specific targeting of IL-13 by CD2, we show reduced expression of Th1 cytokines such as IL-1α, IL-1β, and TNF-α as well as reduced IL17E expression. The reduction in these Th1 cytokines is perhaps a contribution of the reduced monocyte infiltration. Infiltrating

monocytes differentiate into macrophages in target tissues and are known to produce IL-1α, IL-1β, and TNF-α in large amounts[3]. In addition, the reduction in these Th1 cytokines could also be attributed to the inherently low numbers of macrophages observed in the CD2-deficient mice. Given that asthma patients were shown to express a $CD2^{high}$ monocyte population, perhaps blocking CD2 could inhibit function of these monocytes that produce IL-1α, IL-1β, and TNF-α[3]. Similarly, IL-17E is reduced in the absence of CD2, demonstrating yet another proinflammatory cytokine that is a target of CD2. Since IL-17E has an established role as an alarmin in the Th2-high endotype of asthma[138], the reduction in the absence of CD2 adds to the advantages of blocking CD2 as potential therapy. We also show increase in various miRNAs known to play protective roles in asthma. The fact that the Let-7 family of miRNAs is increased in the absence of CD2 highlights blocking CD2 as a multifaceted approach to therapy. We also show that the absence of CD2 in a Th2-low model of asthma results in reduced AHR and IL-13 and IL-17 gene expression. Given that monoclonal antibodies targeting one cytokine has been effective only in a subset of patients demonstrates the need of novel therapeutic targets that could result in enhancing multiple pathways of protection rather than just one. Additionally, given that steroid resistant asthma is a huge burden on public health worldwide, therapies that can help treat steroid resistance is of utmost importance. Steroid resistant asthma is primarily associated with Th17 cell[3] and we show that IL-17 is reduced in the absence of CD2. This suggests that CD2 can be targeted in combination with steroids to ameliorate steroid resistant asthma in addition to its potential as a target in Th2-high asthma.

Taken together, our data shows that CD2 plays a role in enhancing IL-13 mediated asthma characteristics such as AHR, mucus production, and goblet cell hyperplasia and presents itself as

an attractive target for asthma therapy, given that IL-13 targeted therapy has been unsuccessful overall[71, 72, 73]. Other than AHR, goblet cell hyperplasia and increased mucus production, IL-13 has also been shown to be a key mediator of airway fibrosis[199]. Perhaps targeting CD2 in asthma patients sensitized to house dust mite can help ameliorate AHR and prevent development of fibrosis in patients with asthma is early stages such as children. Hence, more studies need to be done to understand the mechanism by which CD2 regulates IL-13 and IL-17A in Th2-high and Th2-low asthma respectively, in order to enhance the translational value of CD2 as a potential therapeutic target for house dust mite induced asthma. Experiments that can tease apart the exact types of immune cells that are affected by the absence of CD2 can provide insight into the mechanism of protection shown in this study.

APPENDIX

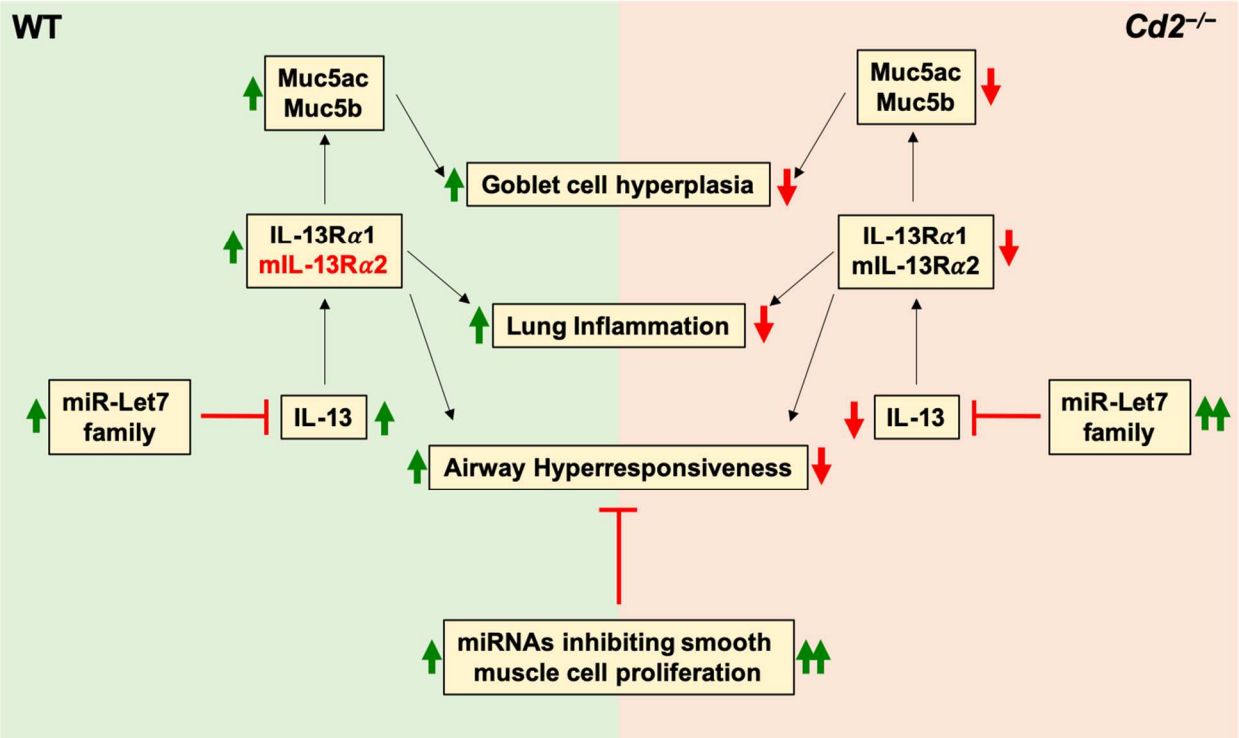


Figure 29. Absence of CD2 ameliorates IL-13 mediated asthma features. In Balb/c (WT) mice, HDME challenge causes airway hyperresponsiveness (AHR), lung inflammation and goblet cell hyperplasia. IL-13 increases expression of IL-13Rα2 which in turn induces production of polymeric mucins *Muc5ac* and *Muc5b.* Increased mucin production in turn causes goblet cell hyperplasia. In response to these asthma features, expression of Let-7 family of miRNAs and miRNAs that inhibit airway smooth muscle cell (ASMC) proliferation increases, as a protective mechanism. In the absence of CD2, IL-13 is reduced which leads to reduced IL-13Rα1 and IL-13Rα2. This results in reduced asthma features. In addition, expression of Let-7 family of miRNAs and miRNAs that inhibit airway smooth muscle cell (ASMC) proliferation increases more significantly in comparison to the WT, resulting in further protection.

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