VIRAL-ALLERGEN INTERACTIONS

VIRAL-ALLERGEN INTERACTIONS:
INSIGHTS INTO THE ORIGINS OF ALLERGIC ASTHMA.

By

AMAL A. AL-GARAWI, M.A.

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TO MY PARENTS,

NORA & ALI

FOR THEIR UNCONDITIONAL LOVE, SUPPORT AND NEVER-ENDING PATIENCE.

WITH ALL MY LOVE!

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AUTHOR: Amal A. Al-Garawi, M.A. (Boston University)

SUPERVISOR: Dr. Manel Jordana

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ABSTRACT

Asthma is a chronic immune-inflammatory disease of the airways, characterized by reversible airflow obstruction and airway hyperresponsiveness (AHR), and is associated with the development of airway remodeling. While our understanding of the pathophysiology of allergic asthma has increased remarkably in the last few decades, the origins of the disease remain elusive. Indeed, studies indicate that the prevalence of allergic asthma, has increased dramatically over the last 30 years. Within this context, a number of environmental factors including respiratory viral infections have been associated with the onset of this disease but causal evidence is lacking. The work presented in this thesis examines the interactions between a respiratory viral infection, specifically influenza A, and the common aeroallergen house dust mite (HDM) in an experimental murine model. To this end, we investigated the impact of an acute influenza A infection on the exposure to a subclinical dose of HDM (Chapter 2) and addressed potential underlying immune mechanisms using a global, genomic approach (Chapter 3). Our data demonstrate an enhancement of immune inflammatory responses to HDM and reveals multiple immune pathways by which influenza A may enhance the response to subsequent allergen exposure. Collectively these immune pathways are capable of lowering the threshold of HDM responsiveness. Lastly, as allergic asthma develops in most instances during infancy, we investigated the impact of an influenza A infection on allergen responses in infant mice (Chapter 4). In this setting, acute influenza A infection subverts constitutive allergen hyporesponsiveness thus resulting in

sensitization, airway inflammation and, ultimately, structural and functional alterations persisting into adulthood.

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List of Abbreviations

AHR	airway hyperresponsiveness	IL	interleukin
Ab	antibody	Ig	immunoglobulin
Ag	antigen	N	neuraminidase
APC	antigen presenting cell	OVA	ovalbumin
CLR	C-type lectin receptors	PAMP	pathogen activated molecular patterns
DC	dendritic cells	PRR	pattern recognition receptors
DAMP	damage associated molecular pattern	p.i.	post infection
EC	epithelial cells	RSV	respiratory syncytial virus
HDM	house dust mite	hRV	human rhinovirus
FcεR1	high affinity IgE receptor	Th	T helper
GM-CSF	granulocyte-macrophage colony-stimulating factor	TLR	toll-like receptors
H	hemagglutinin	TSLP	thymic stromal lymphopoietin
INF	interferon	TNF-α	tumor necrosis factor-α

PREFACE

The research described in Chapters 2-4 of this "sandwich thesis" is presented as three independent but thematically related bodies of work that, as of April 2012, have all been published. The research performed in each manuscript required a collaborative effort with several colleagues, resulting in multiple authors.

CHAPTER 2 — Al-Garawi AA, Fattouh R, Walker TD, Jamula EB, Botelho F, Goncharova S, Reed J, Stampfli MR, O'Byrne PM, Coyle AJ, Jordana M. Acute, but not resolved, influenza A infection enhances susceptibility to house dust mite-induced allergic disease. *J Immunol. 2009 Mar 1; 182(5): 3095-104.*

This study was conducted over the period June 2006-August 2008. I, as the author of this doctoral thesis, designed and performed all the experiments, analyzed and interpreted the data and wrote the manuscript. Experimental assistance was provided by R. Fattouh (graduate student), T.D. Walker (technician), F. Botelho (post-doctoral fellow) and S. Goncharova (technician). E.B. Jamula was an undergraduate student who worked on this project under my supervision. J. Reed (MedImmune collaborator) kindly provided the influenza A viral strain PR8/38. Drs. M.R. Stampfli, P.M. O'Byrne and A.J. Coyle provided valuable scientific input.

CHAPTER 3 — Al-Garawi A*, Hussain M*, Ilieva D, Humbles AA, Kolbeck R, Stampfli MR, O'Byrne PM, Coyle AJ and Jordana M. Shifting of immune responsiveness to house dust mite by influenza A infection: Genomic insights. *J Immunol. 2012 Jan 15;188(2):832-43.*

This study was conducted from Dec 2008-February 2010. This manuscript was an equal contribution (*). I designed and performed all *in vivo* experiments, guided data analysis and wrote the manuscript. Mainul Hussain analyzed the data and also wrote the manuscript. Experimental assistance was provided by D. Ilieva (post-doctoral fellow). Drs. A. Humbles and R. Kolbeck (MedImmune collaborators), M. Stampfli, P.M. O'Byrne and A.J. Coyle critically appraised the manuscript.

CHAPTER 4 Al-Garawi A, Fattouh R, Botelho F, Walker TD, Goncharova S, Moore CL, Mori M, Erjefalt JS, Chu DK, Humbles AA, Kolbeck R, Stampfli MR, O'Byrne PM, Coyle AJ, Jordana M. Influenza A facilitates sensitization to house dust mite in infant mice leading to an asthma phenotype in adulthood. *Mucosal Immunol. 2011 Nov; 4(6): 682-94.*

This study was conducted from May 2008-January 2011. I designed and performed all the experiments, analyzed and interpreted the data and wrote the manuscript. Experimental assistance was provided by R. Fattouh, F. Botelho, T.D. Walker, S. Goncharova and C.L. Moore (undergraduate student) and D. Chu (graduate student). M. Mori and Dr. J.S. Erjefalt (both Lund University collaborators) provided analysis on parenchymal remodeling and critically appraised the manuscript. Drs. A. Humbles and R. Kolbeck critically appraised the manuscript. M. Stampfli, P.M. O'Byrne and A.J. Coyle contributed valuable scientific input and critically appraised the manuscript.

Chapter 1

INTRODUCTION

PREFACE : ASTHMA AND ITS IMPACT ON SOCIETY

Asthma is a chronic immune-inflammatory disease of the airways, characterized by reversible airflow obstruction and airway hyperresponsiveness (AHR), and associated with the development of airway remodeling. Following exposure to a wide range of triggers, the airways of asthmatic individuals contract excessively resulting in breathlessness, wheezing, chest tightness and coughing. The disease can range from mild, occasional symptoms to severe, persistent symptoms with considerable morbidity and mortality rates. Worldwide, it is estimated that asthma affects over 300 million people and results in approximately 250,000 deaths (1, 2). Although asthma can develop at any age, it is most frequently diagnosed in young children and, in fact, is the most prevalent chronic disease during childhood (1). Once diagnosed, asthma is generally a lifelong affliction with a direct impact on the quality of life, affecting all aspects of daily activities and resulting in significant school and work absences; consequently, it places a considerable economic burden on both developed and developing countries (2). For example, in the United States alone, there were 1.75 million emergency visits and over 450,000 hospitalizations (3), resulting in asthma-related health care costs of $19.7 billion annually (4). Studies indicate that the prevalence of asthma, particularly in young children, has increased dramatically over the last 30 years, and the WHO estimates that the number of people with asthma will increase by more than 100 million by 2025. Much effort and investment has been placed on developing treatment options, however, there

is currently no cure for this disease (5). Given the rising prevalence of asthma especially among children, understanding the origins of this disease is more urgent than ever before. Hopefully, this knowledge will enable the development of effective prophylactic measures to help protect against the development of this disorder.

ASTHMA: A HISTORICAL PERSPECTIVE

Asthma has been recognized throughout the history of humankind, across different cultures and civilizations. The Greek term *asthmaino*, meaning "to exhale with open mouth, to pant", was used by Hippocrates to describe any type of disorders resulting in shortness of breath (6). It was not until the mid 18th century that asthma was distinguished from other respiratory illnesses as a separate entity (7). The most significant scientific insight into the pathophysiology of asthma came in the early 19th century when the British physician, and asthma sufferer, Henry Hyde Salter provided a detailed description of his own affliction describing it as a "paroxysmal dyspnea of a peculiar character, generally periodic, with intervals of healthy respiration between attacks", and referred to a "perverted nervous action" as the underlying cause (8). In his writings*: On Asthma: Its Pathology and Treatment,* Salter provided a description of the asthmatic airways noting with minute detail the presence of cellular components in sputum. Similar to physicians before him, Salter was convinced of a hereditary component. In addition, he recognized that asthma was influenced by precipitating factors such as sudden excitement, exercise and exposure to cold air, and observed that exposure to inhaled environmental factors such as emanations of horses, dogs, cats and animal dander (7-10) could also elicit asthmatic symptoms. These detailed observations, along with Paul Ehrlich's discovery of mast cells, basophils and eosinophils in the 1880's lay the foundations of what has become a major focus of modern day asthma research (9,

11).

THE EVOLUTION OF THE UNDERSTANDING OF ASTHMA AS AN ALLERGIC DISORDER

The term *allergie*, which stems from *allos* meaning "changed or altered state" and *ergon* meaning "reaction or reactivity", was coined in 1906 by the pediatrician Clemens von Pirquet working on serum sickness associated with vaccines against diphteria and scarlet fever (12). At the time, he (and others) observed that exposure to antitoxins could at once elicit both immunity (protection) as well as hypersensitivity after repeated injections. In his article, published in the *Münchener Medizinische Wochenschrift,* Pirquet asks, " How can this organism [that is protected against this disease] at the same time be supersensitive to the disease?" The apparent contradiction of the two phenomena led him to propose the term *allergy* and interestingly noted that the changed reactivity to the foreign substance involved "an interval of time [followed by]… the appearance of antibodies in the bloodstream which bring about the specific reactions with the causative foreign body". He proposed that the response was mediated by a "collision of antigen (Ag) and antibody (Ab)" thus, laying the foundations for an essential mechanism underlying the elicitation of the allergic phenomenon (12, 13). Although originally intended to refer to any generalized response against a foreign substance leading to disease, the term *allergy* soon became synonymous with *hyperreactivity or anaphylaxis* (13). In seemingly separate lines of research, experimental evidence from guinea pig models of anaphylaxis in 1906, proposed

that asthma, similar to anaphylaxis, was the result of sensitization to certain protein substances, rather than a nervous disorder (14). These early findings shifted the focus of asthma research towards the elucidation of mediators involved in the elicitation of asthma symptoms. For most of the early 20th century, however, the underlying question of how exposure to foreign protein substances (allergens) programs the allergic phenotype remained a mystery.

Pirquet's remarkable insights materialized decades later with the discovery of immunoglobulin E (IgE) in the 1960's, a major breakthrough (11). Subsequent studies showed that antigen cross-linking of IgE, bound to mast cells, led to the release of numerous biologically active mediators such as histamine and leukotrienes; chemical mediators responsible for the elicitation of allergic symptoms such as anaphylaxis (11). From a mechanistic perspective, the discovery of IgE and IgE-mediated responses served to differentiate asthma into two distinct types: allergic and non-allergic (15). While many clinical, biological and physiological features of asthma are shared, the single most distinguishing factor in allergic asthma is the production of specific IgE Abs directed against common aeroallergens. Today, it is estimated that at least 50% of all asthma cases are of the allergic type (16).

Following the identification of IgE, studies in the 1970's showed that the allergic cascade could be further dissociated into two phases: an early (humoral) phase and a late (cellular) response (17). The early phase is characterized by IgE-mediated triggering of mast cells and the rapid release of chemical mediators,

whereas the late phase consists of the influx of immune cells capable of producing numerous mediators (18). These advances set the stage for the next major paradigm in our understanding of the pathogenesis of asthma: the role of airway inflammation. This led to an explosion of information that uniquely helped to appreciate the complexity of the allergic diathesis.

THE IMMUNOPATHOLOGY OF ASTHMA

The notion that airway inflammation is an integral part of the pathogenesis of asthma was first noted by Henry Salter who remarked that "the inflammation or congestion of the mucous surface appears to be the stimulus, that through the nerves of the air-tube, excites the muscular walls to contract" (10). Studies in the early 20th century provided support for this hypothesis when histopathological analysis of lung tissue from patients who had died of asthma revealed the presence of an inflammatory infiltrate surrounding the bronchial tubes and within the lung parenchyma (19, 20). These studies revealed a complex cellular milieu characterized by the presence of various leukocyte populations, such as eosinophils, neutrophils, as well as large and small mononuclear cells. They also noted the presence of structural changes around the airways and blood vessels, including prominent hyperplasia of the mucosa and the active secretion of mucus, thickening of the bronchial walls as well as smooth muscle hyperplasia (19, 20). During the late 20th century much effort was placed on identifying the function of specific immune cells present in this milieu. In this regard, the eosinophil, which had long been presumed to have a protective role, emerged as a central

inflammatory cell type in the pathogenesis of asthma by virtue of its ability to secrete cytotoxic products (21). However, the role of eosinophils as the pre-eminent "asthmatogenic" cell type was eclipsed by the discovery of thymus-derived lymphocytes (T-cells) in the 1960's (22) when studies suggested that the recruitment of eosinophils could be attributed to the presence of these specialized immune cells via the release of various protein mediators such as chemokines and cytokines (23).

Major advances in cellular immunology led to the discovery of $CD4^+$ and $CD8^+$ T cells and the notion that the immune system consists of both an innate (antigen non-specific) and adaptive (antigen specific) arm (24, 25). Importantly, the identification of dendritic cells (DCs) in 1973 provided a missing link, bridging innate and adaptive immune responses (26). By 1986, $CD4^+$ T cells were further subdivided into two distinct subsets T-helper (Th)1 and Th2 based on their unique cytokine profiles (27), with Th1 cells predominantly producing interferon (IFN)-γ and interleukin (IL)-2, while Th2 cells are major producers of IL-4, IL-5 and IL-13. With regards to asthma, specific Th2 cytokines were shown to promote IgE class switching, mast cell recruitment, eosinophilia and mucus production (28).

There has been an outstanding progress in the understanding of the regulation of inflammation, immunity and immunopathology in the lung in the last 30 years. Numerous cell types, cytokines and chemokines have been identified and their roles in the allergic cascade have been recognized. In addition

to the identification of various hematopoietic cells and their role in immunity, it has become clear that structural cells, such as bronchial epithelial cells (EC), also play important immunoregulatory functions (29). Historically viewed simply as a structural cell with absorptive and barrier functions, EC represent the body's first line of defense protecting against invading pathogens. Furthermore, it is now fully appreciated that EC can respond to a variety of internal and external stimuli and release a host of factors that can both activate immune cells and restore homeostasis (30). Moreover, it is increasingly recognized that epithelial-derived factors provide an important link between innate and adaptive immune responses (29).

Based on these advances, it is now thought that allergic asthma is a complex immune-inflammatory disease largely characterized by a Th2-polarized immune response with the ensuing production of cytokines such as IL-4, IL-5 and IL-13, eosinophilic inflammation as well as IgE-mediated mast cell activation and the release of chemical mediators such as histamine and leukotrienes (28). From a pathological perspective, advances in bronchoscopy have confirmed and expanded upon the structural changes first observed almost a century ago. Currently, remodeling of the airways is characterized by the loss of epithelial integrity, thickening of basement membrane, development of subepithelial fibrosis, goblet cell hyperplasia and mucus production as well as increased smooth muscle mass and angiogenesis (31, 32).

HOUSE DUST MITE AS ALLERGEN

Allergic asthma is thought to arise as a result of aberrant immune responsiveness to otherwise harmless environmental proteins, also known as allergens. A defining characteristic of allergens is their ability to act as potent inducers of IgE synthesis (33); a process commonly referred to as "sensitization". Environmental allergens that can elicit allergic asthma comprise both indoor and outdoor allergens and include substances derived from animal dander (dogs and cats), arthropods (such as mites and cockroaches), seasonal pollens and certain molds. Interest in (house dust) mite-related allergic disease was initiated by the physician Storm van Leeuwan in the 1920's while working on mite-related allergies in farmers exposed to wheat and oats (34). At the time, he noted that exposure to house dust collected from damp surfaces elicited allergic responses, and speculated that mites contained within house dust were the culprits eliciting these responses. Attempts to link the presence of mites to house dust, however, remained fruitless and, as World War II approached, all mite-related research efforts were abandoned (34).

A renewed effort to resolve the question of the "mysterious house dust" occurred several decades later, when the physician Reindert Voorhorst similarly noted an association between exposure to house dust and allergic disease identifying the mite *Dermatophagoides pteronyssinus (Dp)* as the source of the long sought "house dust allergen" in 1967 (35, 36). *Dp*, more commonly known

as house dust mite (HDM), is the most pervasive aeroallergen worldwide. HDM thrive in humid environments and because of their small size (20-320μm) mite populations are difficult to control, thus ensuring constant, ubiquitous exposure (37). Today, it is estimated that 15-20% of the population in industrialized nations are sensitized to HDM (38).

It has been proposed that the allergenicity of allergens is largely accounted for by the ability of various molecular constituents to activate innate defense mechanisms at mucosal surfaces (33, 39). In this regard, HDM consists of a complex mixture of many protein and non-protein components of various sizes and function. In fact, mites produce over 3000 proteins of which 5% are thought to be allergens (37). So far, over 21 different groups of dust mite allergens have been identified, based on different functional categories (40). Many of these include cystein and serine proteases, chitinases, binding proteins as well as structural homologues of innate molecules (33, 41).

Surprisingly, a large body of experimental research on allergic asthma has utilized until recently the innocuous protein antigen chicken egg ovalbumin (OVA)**.** However, the stark differences between the complex biochemical nature of HDM with the simple molecular structure of OVA raises doubts as to whether the immunologic pathways elicited by these two vastly different entities are the same. Moreover, animal models based on the OVA system require the introduction of antigen into the peritoneal cavity along with chemical adjuvants (aluminum hydroxide) to elicit productive immunity, thus, circumventing the

natural process of mucosal sensitization. Hence, much less is know at this time about the precise mechanisms by which naturally occurring aeroallergens interact with the immune system, and are able to elicit harmful immune responses.

THE ORIGINS OF ALLERGIC ASTHMA

That asthma occurs in susceptible individuals implies a genetic pre-disposition. Hence, an extraordinary effort has been dedicated to identify asthma susceptibility genes (42). Indeed, more than 100 asthma-related genes have been identified to date but, individually, each has a very low attributable risk and poor replication across different populations (43, 44). An important clue that would contribute to our understanding regarding the origins of asthma emerged with the advent of epidemiological studies at the turn of the century. These studies began estimating the prevalence of asthma in the population and, over time, revealed a slow but steady trend towards an increased prevalence of asthma in the post WWII period (45). Since then, a dramatic rise in prevalence has been recorded over the last 30 years (46). According to the International Study of Asthma and Allergies in Childhood (ISAAC), the current prevalence of asthma in children under the age of 14 has reached close to 40% in some industrialized countries (47). This "asthma epidemic" argues against a population-wide genetic shift as a pre-eminent factor underlying the increase in prevalence (46). Indeed, the lack of a clear association between disease manifestation and asthma susceptibility genes has brought about a paradigm shift, away from a static, linear gene-disease relationship to one that favors an integrated multi-factorial approach that takes

into account not only the genetic background but also the effects of additional factors such as environmental exposures to entities other than allergens (e.g. microbes or pollution) and developmental influences (43).

ENVIRONMENTAL INFLUENCES

The notion that exposure to certain environmental factors can influence the expression of allergic disease had been noted centuries ago (7). In his treatise of asthma, the 17th century physician, Sir John Floyer, described cold air, air pollution, infection and tobacco smoke as exacerbating factors of asthma (48). In addition to exacerbating existing disease, it is now fully appreciated that exposure to tobacco smoke, maternal smoking, occupational chemical and outdoor pollutants (diesel exhaust particles, particulate matter) and certain diet/lifestyle factors are risk factors for the development of asthma (49). Similarly, respiratory infections are increasingly thought to play important roles in the pathogenesis of allergic asthma.

THE HYGIENE HYPOTHESIS

That exposure to infections may influence the expression of allergic disease was first proposed in 1976. Gerrard *et al*., observed a virtual absence of allergic disease in a community of native Canadians living in Saskatchewan. This observation led these investigators to hypothesize that frequent infections to helminths, bacteria and viruses may have imparted a protective effect against the development of allergic disease (50). Furthermore, it was speculated that improved hygienic conditions as a result of better housing and improved

healthcare would increase the susceptibility to develop allergic disease (50). Thus, this study planted for the first time the notion that exposure to "clean" (no infections) *versus* "dirty" (infections) environments, in early life could influence the expression of allergic disease. Subsequently, David Strachan in 1989, proposed the "*Hygiene Hypothesis*" after examining environmental, socioeconomic and perinatal factors to explain the increased prevalence of hay fever in a cohort of British children (51). He proposed that "the rise in allergic disease could be prevented by infection in early childhood transmitted by unhygienic contact with older siblings or acquired prenatally …" (51). The notion that family size and birth order could impart a protective effect was based on the observation that the development of hay fever was inversely associated with family size and the number of older siblings. As a result, the *Hygiene Hypothesis* unleashed an era of epidemiological inquiries into the effects of environmental exposures (such as microbial and viral infections) on the emergence of allergic disease. It gained popularity throughout the next decades, and a plethora of studies proposed an immunologic mechanism with the underlying tenet that exposure to microbial infections induced protective Th1 immunity that, in turn, would limit the development of Th2-responses. Thus, it was argued that early life infections would invest protection, through the production of IFN-γ and IL-12, against the generation of Th2-mediated allergic disease (52) .

Since the framing of the *Hygiene Hypothesis*, however, there has been a considerable amount of controversy regarding the impact of infections on the

development of allergic asthma. Numerous studies citing evidence supporting a protective effect of family size and birth order, microbial infections and vaccinations, are inconclusive or plagued by inconsistencies (53). In addition, studies have emerged showing that certain bacterial infections in early life may, in fact, promote, the development of asthma (54). Similarly, there is a considerable amount of controversy regarding the impact of viral infections on the development of asthma. While some epidemiological studies report protective effects (55-57), there is now a growing number of studies that suggest that certain respiratory infections in infancy may promote the development of asthma during childhood (58-60).

Inconsistencies of the *Hygiene Hypothesis* are further exemplified by arguments that it fails to account for a concurrent increase in Th1-mediated diseases, such as autoimmunity, or the increasing rise in allergy in inner-city areas and the developing world (61). Thus, while the *Hygiene Hypothesis* is the most coherent theory to emerge in the 21st century to propose an explanation for the increased prevalence of asthma, its simplistic and dichotomous approach has raised doubts over its global applicability to a wide array of microbial organisms. Indeed, as Armann and von Mutious state, "the relationship between the microbial world and the development of asthma is not well understood" (62) and, as Strachan himself concedes, "further work is needed… to identify the nature of the protective agent and timing of its effect on atopic sensitization" (53).

RESPIRATORY VIRUSES AND ASTHMA

The notion that infections, like the common cold, play an important role in the manifestation of asthma symptoms has been recognized for centuries. Moses Maimonides, a 12th century physician-philosopher, described the cause of his patients asthmatic attack as "a dripping from the brain that most commonly occurs during the rainy season" (63). Modern day epidemiologic studies show that acute asthma exacerbations most frequently occur during winter and fall seasons (64, 65). Of these exacerbations, over 85% are associated with exposure to respiratory viruses (66, 67). Importantly, in addition to exacerbating current symptoms of asthma, recent epidemiological studies investigating the natural history of asthma have suggested that certain respiratory viral infections may promote the onset of allergic disease.

Although asthma may develop at any age, it is most frequently diagnosed during childhood (68). Wheezing, a classic characteristic of asthma has long been used as an early indicator for the development of allergic airway disease. The Tucson Children's Respiratory Study, a landmark prospective birth cohort study, initially described distinct wheezing phenotypes and showed that while most children who wheezed during infancy did not develop asthma symptoms, approximately 30% of those who exhibited *persistent* wheezing in the pre-school years progressed to develop asthma during childhood (69, 70). Similarly, the timing of allergen exposure appears to be critical within the context of persistent

wheezing, as shown by a multicenter birth cohort study in which early *allergen sensitization* to perennial allergens, before the age of 3, but not later in childhood, was found to be associated with a decrease in lung function and the presence of allergic airway disease (69-71). These studies have helped identify the early years as a critical period during which immune responses to aeroallergens may be permanently programmed. While transient wheezing episodes are a common occurrence during infancy, the factors influencing the development of persistent wheezing in young children remain unclear. Acute respiratory illnesses due to respiratory viral infections are a major health threat in early life. Indeed, epidemiologic studies have shown that by 2 years of age, most children will have been exposed to at least one respiratory virus (60, 72). Therefore, viral infections are a legitimate candidate for facilitating sensitization to allergens in early life.

Sigurs *et al* first showed that respiratory syncitial virus (RSV)-induced severe viral bronchiolitis early in life increases the risk of allergic sensitization, wheezing and asthma diagnosis in school-aged children (59, 60). Prospective birth cohorts involving healthy infants (of atopic parents) showed that viral-induced wheezing during infancy was strongly associated with the onset of persistent wheezing by 3 years of age and the development of asthma by 6 years of age, in particular when allergen sensitization occurred early rather than later during childhood (58, 73, 74). Contrary to the outcome predicted by the *Hygiene Hypothesis*, namely, that early life respiratory infections impart a protective

effect, these studies suggest that lower respiratory viral infections increase the susceptibility of the developing lung to respond to aeroallergen exposure.

Many human studies have focused on RSV and, more recently, human rhinovirus infections (hRV), as these are two of the most common respiratory viruses in children. However, recent advances in viral detection methods have identified additional viral types as etiological agents of acute lower respiratory illness and wheezing in infants. These include, coronavirus, para-influenza virus, human metapneumovirus, adenovirus and influenza A (58).

Influenza A, a central component in this thesis work, is an important human pathogen resulting in yearly seasonal epidemics that affect approximately 5-15% of the population in the northern hemisphere (75). Since its identification in 1933, there has been much interest in understanding the viral structure, its genome, the infectious cycle and release of new virions, as well as the immune responses elicited against the virus (76, 77). Influenza is a segmented, negative stranded RNA virus belonging to the Orthomyxoviridae family of viruses. Influenza viruses are further classified into three types: A, B or C. Of these, type A is responsible for most clinical infections in humans (78). The viral genome consists of 8 segmented strands of RNA encoding 11 viral proteins, of which two are envelope glycoprotein, hemagglutinin (H) and neuraminidase (N). These are involved in the attachment to target cells, (such as bronchial epithelial cells) and the release of progeny virions from host cell surfaces; in addition, they also

function as major antigenic sites for the production of neutralizing Abs (79). Influenza A is further subdivided based on the antigenic properties of the H and N proteins, thus resulting in many different subtypes. To date, 16 H and 9 N variants have been described (80) generating mild to highly virulent strains (81). Depending on the virulence of the specific viral strain involved, the resulting airway inflammation can range from a mild accumulation of immune cells to severe immunopathology of the lung, manifested as bronchiolitis and pneumonia in susceptible individuals, particularly the elderly and the very young (81). Indeed, influenza A infections, while less prevalent than RSV and hRV, are a significant cause of severe lower respiratory illness in young children frequently leading to hospitalization (82, 83). In preschool children, the rate of influenza infections is estimated to be up to 40% annually, with the most severe infections occurring in infants younger than 6 months of age (84). However, the role influenza A infections may play in the development of asthma in young children has not been established.

MODELING VIRUS-ALLERGEN INTERACTIONS

While prospective cohort studies in humans are essential to identify potential risk factors, these studies have also some limitations. For example, they can rarely ever permit to establish causality, and often produce associations that are relatively weak and have limited predictive value (85). In addition, they cannot precisely decipher the immunological history of the person and, of course,

are unsuited to investigate mechanisms. Indeed, whether certain respiratory viral infections cause asthma or are merely "biomarkers" targeting individuals genetically at risk is a subject of current debate (42). In this context, animal models of allergic asthma are invaluable tools in which to comprehensively model viral-allergen interactions.

The impact that respiratory viral infections, specifically influenza A, have on the development of allergic airway disease in adult mice has been examined by a number of studies, largely using OVA as a surrogate allergen (86-91). These studies have produced diverse outcomes (92, 93) that seem to depend on the specific experimental setting, including the timing of allergen exposure as well as the specific type of virus used. However, the controversial and, in some instances, seemingly contradictory nature of these data also intimate the limitations of OVA models of allergic asthma as discussed earlier. Perhaps the most notable drawback of these models is that they are precluded from directly investigating airway *mucosal* sensitization. Thus, in order to overcome these limitations, the studies described in this thesis are based on models of mucosal sensitization utilizing the common aeroallergen HDM previously established in our laboratory (37, 94). Importantly, these models elicit allergic sensitization, an immune inflammatory response characterized by Th2-mediated eosinophilic inflammation, as well as hallmarks of structural remodeling and lung dysfunction.

RATIONALE AND GOALS OF THIS THESIS WORK

The immune system primary role is to protect the host against harmful intruders such as bacteria, viruses and other microbial organisms. As the mucosal surfaces of the lung and airways are constantly exposed to an onslaught of various microbial and non microbial entities, the decision of whether to mount inflammatory responses is tightly regulated (68). Despite ubiquitous allergen exposure, the fact that the majority of the world's population is free from allergic disease suggests that tolerance, a state of homeostatic responsiveness, rather than inflammation, is the natural response to aeroallergens. Indeed, aeroallergens are, in principle, harmless proteins and, consequently, do not warrant the launching of an immune-inflammatory response against them. However, the context in which the immune system encounters foreign material may critically determine the immunologic outcome of this interaction (95). In this regard, this thesis aims to investigate conditions that facilitate the emergence of allergic disease. The hypothesis that viral infections may alter the immune milieu in such a way as to privilege non-homeostatic immunological responsiveness to aeroallergens and initiate processes that may lead to allergic disease will be examined by three connected studies.

First, the study described in Chapter 2 will examine whether a prior influenza A infection alters the immune response to a subclinical dose of a naturally occurring aeroallergen, HDM, and furnish insight as to whether the

"timing" of allergen exposure impacts the allergic outcome. From a larger perspective, this study aims to establish whether exposure to an environmental factor, such as a severe respiratory infection, can either promote or protect against the emergence of the allergic phenotype .

To gain a better appreciation of the influenza A-induced microenvironment from a global perspective, Chapter 3 describes the use of whole genome microarray to examine gene expression in whole lung at the time of allergen exposure. In particular, the study aims to investigate how the flu-induced environment may contribute to altering mucosal responsiveness to HDM.

That allergic asthma commonly develops in children underscores the importance of environmental influences in early life. Thus, modeling viral-allergen interactions during the neonatal period is particularly important as it represents a time frame of rapid lung development, encompassing not only structural but also immunological changes (96-98). There are currently no experimental data available that provide insight into mucosal aeroallergen exposure during acute respiratory viral infection in early life. In this respect, the study described in Chapter 4 aims to: 1) investigate the impact of an acute flu infection on HDM responsiveness in early life, 2) delineate the basic immunology underlying these effects, and 3) determine the structural (remodeling) and functional (lung mechanics) consequences of the influenza-HDM interaction.

Collectively, the three studies outlined contribute important insights on how immune perturbations of the lung microenvironment, in the form of a severe viral respiratory infection, may influence the development of asthma. From a larger perspective, they lay out a platform upon which to explore effective primary intervention strategies.

Chapter 2

ACUTE, BUT NOT RESOLVED, INFLUENZA A INFECTION ENHANCES SUSCEPTIBILITY TO HOUSE DUST MITE INDUCED ALLERGIC DISEASE

Amal A. Al-Garawi[*], Ramzi Fattouh[*], Tina D. Walker[*], Erin B. Jamula[*], Fernando Botelho[*], Susanna Goncharova[*], Jennifer Reed[‡], Martin R. Stampfli[*], Paul M. O'Byrne[†], Anthony J. Coyle[*,‡] and Manel Jordana[§].

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In this study we have examined the consequences of exposure to a low dose of the common aeroallergen HDM during the course of a influenza A infection. We chose a low dose of allergen (5μg) that, by itself, elicits only mild inflammatory responses with no lung dysfunction. The study delineates the nature and kinetics of the local and systemic immune-inflammatory response in the lung of mice following intranasal infection with influenza A. It demonstrates that exposure to HDM during the peak immune-inflammatory response, at day 7 post infection (p.i.), significantly enhanced eosinophilic inflammation and HDM-specific IgE and IgG_1 responses, and increased mucous production over that observed in mice exposed to HDM alone. Importantly, in the context of this acute influenza A infection, these immunologic and structural changes lead to marked lung dysfunction. In contrast, the inflammatory response elicited by HDM was reduced when exposure occurred during the resolution phase (day 40 p.i.). Our study reveals that the pro-inflammatory environment established during an acute influenza A infection enhances Th2-polarized immunity to a low dose of HDM, and precipitates marked lung dysfunction. Thus, allergen exposure under these conditions might increase the susceptibility to manifest allergic disease.

The Journal of Immunology

Acute, but Not Resolved, Influenza A Infection Enhances Susceptibility to House Dust Mite-Induced Allergic Disease[1]

Amal A. Al-Garawi,* Ramzi Fattouh,* Tina D. Walker,* Erin B. Jamula,* Fernando Botelho,* Susanna Goncharova,* Jennifer Reed,‡ Martin R. Stampfli,* Paul M. O'Byrne,† Anthony J. Coyle,*‡ and Manel Jordana*[2]

The impact of respiratory viral infections on the emergence of the asthmatic phenotype is a subject of intense investigation. Most experimental studies addressing this issue have used the inert Ag OVA with controversial results. We examined the consequences of exposure to a low dose of the common aeroallergen house dust mite (HDM) during the course of an influenza A infection. First, we delineated the kinetics of the immune-inflammatory response in the lung of mice following intranasal infection with influenza A/PR8/34. Our data demonstrate a peak response during the first 10 days, with considerable albeit not complete resolution at day 39 postinfection (p.i.). At day 7 p.i., mice were exposed, intranasally, to HDM for 10 consecutive days. We observed significantly enhanced eosinophilic inflammation, an expansion in Th2 cells, enhanced HDM-specific IgE and IgG1 responses and increased mucous production. Furthermore, lung mononuclear cells produced enhanced IFN-γ and IL-5, unchanged IL-13, and reduced IL-4. These immunologic and structural changes lead to marked lung dysfunction. This allergic phenotype occurs at a time when there is a preferential increase in plasmacytoid dendritic cells over myeloid dendritic cells, activated CD8$^+$ T cells, and increased IFN-γ production, all of which have been proposed to inhibit allergic responses. In contrast, the inflammatory response elicited by HDM was reduced when exposure occurred during the resolution phase (day 40 p.i.). Interestingly, this was not associated with a reduction in sensitization. Thus, the proinflammatory environment established during an acute influenza A infection enhances Th2-polarized immunity to a low dose of HDM and precipitates marked lung dysfunction. *The Journal of Immunology,* **2009, 182: 3095–3104.**

Allergic asthma is a chronic inflammatory disease mediated by a Th2-polarized immune response involving eosinophilic inflammation, mucous overproduction, bronchial hyperreactivity, and, eventually, airway remodeling. Allergic asthma occurs following sensitization to naturally occurring aeroallergens such as house dust mites (HDM),[3] roaches, pollens, or animal dander. We have previously shown that HDM administered through the mucosal route and without the use of additional adjuvant leads to the generation of Th2-mediated inflammation with all of the cardinal features of asthma (1, 2). Furthermore, we have recently furnished a comprehensive computational view of the impact of dose and length of allergen exposure on allergic sensitization and inflammation (3). Although many facets of asthma have been uncovered, the origins of asthma pathogenesis remain unclear. Importantly, allergen exposure does not ever occur in isolation and, thus, exposure to other entities such as biologics and chemicals may impact the immune status of the lung such as to alter the levels and thresholds of allergen responsiveness.

A growing number of epidemiologic studies have shown that certain respiratory viral infections in infancy are associated with increased atopy to common allergens and an overall increased risk of asthma in school-aged children (4, 5). Such an outcome seems intuitive in the case of infections with respiratory syncytial virus (RSV) which induces Th2-polarized immunity (6, 7) and can, thereby, establish a lung environment that facilitates subsequent sensitization to allergens. In contrast to RSV infections, infections with influenza A, also a significant cause of lower respiratory illness in young children (8, 9), involve the generation of archetypical Th1 immunity (10) that has been historically regarded as able to inhibit or down-regulate Th2-mediated processes.

In this study, we have investigated the immunologic, structural, and functional impact of exposure to a threshold concentration of HDM in mice during the course of an influenza A infection. Our data show that exposure to HDM during the acute phase of a flu infection, but not during the resolution phase, reduces the threshold responsiveness to allergen exposure, resulting in a robust allergic inflammatory response that is associated with enhanced mucus production and a marked alteration in lung mechanics. Importantly, this response emerges in a lung environment that contains a preferential increase in plasmacytoid dendritic cells (pDCs) over myeloid dendritic cells (mDCs), the presence of activated CD8$^+$ T cells, and high levels of IFN-γ. Thus, these findings intimate that allergen exposure in a flu-induced innate proinflammatory lung environment might precipitate overt allergic disease, hence increasing the susceptibility to develop asthma.

*Division of Respiratory Diseases and Allergy, Center for Gene Therapeutics and Department of Pathology and Molecular Medicine and †Department of Medicine, McMaster University, Hamilton, Ontario, Canada; and ‡Department of Inflammation and Autoimmunity, MedImmune, Inc., Gaithersburg, MD 20878

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[2] Address correspondence and reprint requests to Dr. Manel Jordana, Department of Pathology and Molecular Medicine, Division of Respiratory Diseases and Allergy, McMaster University, MDCL Room 4013, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5. E-mail address: jordanam@mcmaster.ca

[3] Abbreviations used in this paper: HDM, house dust mite; BAL, bronchoalveolar lavage; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; RSV, respiratory syncytial virus; p.i., postinfection; PAS, periodic acid-Schiff; MCh, methacholine; Rn, airway resistance; G, tissue resistance; H_{TE}, tissue elastance; MHCII, MHC class II; KLH, keyhole limpet hemocyanin; NES, *Nippostrongylus brasiliensis*.

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Materials and Methods

Animals

Female BALB/c mice (6–8 wk old) were purchased from Charles River Laboratories. The mice were housed under specific pathogen-free conditions and maintained on a 12-h light-dark cycle with food and water ad libitum. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University (Hamilton, Ontario, Canada).

Influenza A virus and infection protocol

Influenza type A virus strain A/PR/8/34 (H1N1) was prepared as described previously (11) and provided by MedImmune, Inc.. The viral stock suspension (10^9 PFU/ml) was diluted and 10 PFU were administered intranasally to isoflurane-anesthetized BALB/c mice in 35 μl of sterile PBS solution. Animals were monitored for signs of illness twice daily for a period of 10 days following infection.

Sensitization protocols

Allergen administration. HDM extract (Greer Laboratories) was resuspended in sterile PBS at a concentration of 0.5 mg (protein)/ml and 10 μl (5-μg dose) was administered to isoflurane-anesthetized mice intranasally.

Allergen exposure during acute phase infection. Animals that were infected with 10 PFU of PR8 flu virus were exposed intranasally daily to 5 μg of HDM for 10 consecutive days, starting at day 7 postinfection (p.i.) (F plus H group). Age-matched flu-infected animals, received 10 μl of sterile saline (F group). Uninfected animals received either 5 μg of HDM (H group) or 10 μl of sterile saline (SAL group). Three days after the last exposure, animals were sacrificed and the inflammatory response was assessed.

Long-term rechallenge protocol. Animals were infected with A/PR8 virus on day 0 and then groups of animals were exposed to 10 days of allergen (or saline) as described above. After the last exposure, mice were rested for a period of 30 days, at which point they were rechallenged with 5 μg of HDM daily for 3 days. Seventy-two hours after the last challenge, animals were sacrificed and the inflammatory response was assessed.

Allergen exposure during resolution phase. Groups of animals that were infected with 10 PFU of A/PR8 were exposed daily to 5 μg of HDM for 10 consecutive days, starting at day 40 p.i. (F plus H group). Age-matched flu-infected animals received 10 μl of PBS for 10 consecutive days (F group). Uninfected animals received either 5 μg of HDM (H group) or 10 μl of PBS (SAL group) for 10 consecutive days. Animals were sacrificed 3 days after the last exposure and the inflammatory response was assessed.

Collection and measurement of specimens

Bronchoalveolar (BAL) fluid, lungs, and blood were collected at the time of sacrifice. BAL was performed as previously described (12). Briefly, lungs were dissected and the tracheae were cannulated with a polyethylene tube (outer/inner diameter = 0.965/0.58 mm; BD Biosciences). Lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml) and ~0.25–0.3 ml of the instilled fluid was consistently retrieved. Total cell counts were then determined using a hemocytometer. Each BAL sample was then centrifuged and the supernatants were collected and stored at −20°C. Cell pellets were subsequently resuspended in PBS and smears were prepared by centrifugation (Shandon) at 300 rpm for 2 min. A protocol Hema 3 stain set (Fisher Scientific) was used to stain all smears. Differential cell counts of BAL were determined from at least 300 leukocytes using standard hemocytologic criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells. Peripheral blood was collected by retro-orbital bleeding and serum was obtained and stored at −20ºC. Where applicable, after BAL collection, lungs were inflated with 10% formalin at a constant pressure of 20 cm of H_2O and then fixed in 10% formalin for 48–72 h until further processing.

Histology and morphometric analysis

Lung tissues were collected, the left lung was dissected and embedded in paraffin, and 3-μm-thick sections were cut and stained with H&E and periodic acid-Schiff (PAS). Multiple images (9–12 photographs) of the primary airway were taken. Images for morphometric analysis were captured with OpenLab software (version 3.0.3; Improvision) via Leica camera and microscope (Leica Microsystems). Image analysis was performed using a custom-computerized analysis system (Northern Eclipse software version 5; Empix Imaging). Analysis of PAS-stained sections was performed as previously described (13). Briefly, a line is drawn immediately below the airway epithelium. The software creates a parallel line 30 μm away, creating a "band" which encompasses the airway epithelium/goblet cells/mucous. Within this region of interest, a color range is selected that allows for the selection of mucous and excludes nonmucous elements. A weighted average of the region of interest positive for PAS staining is calculated for each mouse.

Lung cell isolation and flow cytometric analysis of lung cells

Total lung cells were isolated as previously described (14). Briefly, total lung cells were isolated by collagenase digestion (collagenase type I; Life Technologies) and washed twice in PBS and stained with a panel of Abs. To minimize nonspecific binding, cells were first preincubated with FcBlock (anti-CD16/CD32; BD Pharmingen). For each Ab combination, 2×10^6 cells were incubated with mAbs at 4°C for 30 min. Cells were then washed in FACS buffer (PBS/0.5% BSA) and data were collected using an LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star and Stanford University, Palo Alto, CA). The following Abs were used for the identification of mDCs, pDCs, macrophages, and B cells (15–17): CD45-allophycocyanin-Cy7, MHC class II (MHCII)-allophycocyanin, CD11b-PE, CD11c-FITC, B220-allophycocyanin, GR1-PE-Cy7, and CD19-PE-Cy5.5. T cells were identified using CD3-PerCp-Cy5.5 or PE-Cy7, CD4-FITC or allophycocyanin, CD8-allophycocyanin-Alexa Fluor 750 (eBioscience) or PerCP, CD69-Pe-Cy7 or PE, CD25-allophycocyanin (all BD Biosciences), and T1/ST2-FITC (MD Bioscience). Where applicable, total lung eosinophils were quantified on the basis of the following analysis: briefly, total lung cells were stained with anti-CD45-allophycocyanin- Cy7 (to select leukocytes) and anti-GR1-PE-Cy7 (to exclude neutrophils–Gr-1^{bright} population). Eosinophils were then identified on the basis of forward scatter and side scatter as shown in supplemental Fig. 4S.[4] All appropriate isotype controls were used (BD Biosciences). Abs were titrated to determine optimal concentration. See online supplemental material for additional details on the methods used to make these measurements.

Mononuclear cell isolation and in vitro cytokine production

Total lung cells were isolated by collagenase digestion and mononuclear cells were purified over a Percoll gradient as described previously (14). After washing in RPMI 1640, cells were resuspended in complete RPMI 1640 (RPMI 1640, 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.1% 2-ME) and 5×10^5 cells/well (in 100 μl of complete RPMI 1640) plated on 96-well plates. Cells were stimulated with 3 μg of HDM extract (2.5 μg/μl) and incubated at 37°C/5% CO_2 for 5 days. Thereafter, supernatants were collected and stored at −70°C for cytokine analysis.

Cytokine analysis and Ig measurements

Levels of proinflammatory cytokines in BAL were measured using a Luminex 100 Total System based on xMAPmultiplexing technology. 5-Plex cytokine kits containing microbeads with capture Ab and biotinylated reporter specific for mouse IL-6, TNF-α, IFN-γ, and IL-10 were purchased from Upstate Biotechnology while type I IFN-α and IFN-β were measured by ELISA kits purchased from PBL Biomedical Laboratories. Levels of IL-5, IL-4, IL-13, and IFN-γ in supernatants from mononuclear cell cultures were measured by DuoSet ELISA kits purchased from R&D Systems and used according to the manufacturer's instruction. Levels of HDM-specific IgE, IgG1, and IgG2 were measured by sandwich ELISA as previously described (2, 12).

Preparation of influenza A/PR8 flu lysate and measurement of influenza-specific IgG1 and IgG2a

Madin-Darby canine kidney cells were seeded at 10^7 cells/15-cm dish and grown to confluency. Cells were washed twice with PBS and infected with A/PR8/34 at an multiplicity of infection of 2. Cells were incubated for 24 h in serum-free medium (α-MEM, 1% L-glutamine, and 1% penicillin/streptomycin) at 37°C in 5% CO_2 or until 50% lysis was achieved. Cells were harvested and spun at 1200 rpm/4°C for 10 min. The cell pellet was resuspended in 1 ml of PBS and then lysed by three sequential freeze-thaw cycles in liquid N_2 followed by a 37ºC water bath. Cell lysates were spun down and supernatants were collected and stored at −70ºC. Protein concentration was determined using a Bradford assay (Bio-Rad) as per the manufacturer's instructions.

For the detection of influenza-specific IgG1 and IgG2a, 96-well MaxiSorp plates (Nunc and VWR) were coated overnight at 4ºC with 50 μl of 10 μg/ml solution of flu-PR8 cell lysate in PBS. Coated wells were blocked with 0.05% BSA in TBS with 0.05% Tween 20 for 2 h at room temperature. After washing, serum samples (diluted 1/20, 1/200, 1/2,000, and 1/20,000 for IgG1 and 1/50, 1/500, 1/5,000, and 1/50,000 for IgG2a, 50 μl/well) were added to the wells and incubated overnight at 4°C, washed,

[4] The online version of this article contains supplemental material.

and then incubated with 0.25 μg/ml biotin-labeled IgG1 or IgG2a (Southern Biotechnology Associates) overnight at 4°C. Plates were then incubated with alkaline phosphatase-streptavidin (Zymed Laboratories) in 50 μl/well at a concentration of 1/1000. The color reaction was developed with *p*-nitrophenyl phosphatase tablets (Sigma-Aldrich) in 50 μl/well and stopped with 25 μl/well 2 N NaOH. ODs were read at 405 nm. Blank OD values were based on the average of 20 control wells that were loaded with diluent instead of sample. Flu-specific IgG1 and IgG2a units corresponded to the maximal dilution that resulted in an OD that exceeded the average OD value of 20 zero standard replicates plus 2 SDs. The formula used to calculate is as follows: relative units = (OD reading − OD blank) × dilution of OD reading.

Airway responsiveness measurements

Airway responsiveness was assessed 2 days after the last exposure to HDM in response to increasing doses of nebulized methacholine (MCh; Sigma-Aldrich) using a previously described protocol (3). Briefly, mice were anesthetized with inhaled isoflurane (3% with 1 liter/min of O_2), paralyzed with pancuronium bromide (1 mg i.p.), tracheotomized with a blunted 18-gauge needle, and mechanically ventilated with a small animal computer-controlled piston ventilator (flexiVent; SCIREQ) (18). Mice received 200 breaths/min and a tidal volume of 0.25 ml; the respiratory rate was slowed during nebulization (10 s) to provide five large breaths of aerosol at a tidal volume of 0.8 ml. The response to nebulized saline and increasing doses (3.125, 12.5, and 50 mg/ml) of MCh were measured and the data fit with the constant phase model. Model parameters of airway resistance (Rn), tissue resistance (G), and tissue elastance (H_{TE}) were calculated as described previously (19). Model fits that resulted in a coefficient of determination <0.8 were excluded.

Data analysis

Data were analyzed using SigmaStat version 3.1 (SPSS). Data are expressed as mean ± SEM. Results were interpreted using ANOVA Fisher's least significance difference post hoc test, unless otherwise indicated. Differences were considered statistically significant when p values were <0.05.

Results

Experimental schematics

To comprehensively investigate the impact of aeroallergen exposure during the course of an influenza infection, we designed the four protocols outlined in Fig. 1. First, we determined the kinetics of the inflammatory response to a flu infection (Fig. 1*A*). Then, we chose the time point at which acute inflammation is at its peak and exposed mice to a low dose of 5 μg of HDM daily. This dose of HDM was chosen because it induces a mild eosinophilic inflammation and no airway dysfunction. The inflammatory and functional responses were evaluated 3 days after the last challenge (Fig. 1*B*). To investigate whether changes in the inflammatory response are transient in nature or, in fact, induced long-lasting immune changes, we recapitulated the protocol in Fig. 1*B*, but allowed animals to rest for a period of at least 30 days, after which they were re-exposed to 5 μg of HDM for 3 consecutive days. The inflammatory responses were then evaluated 3 days after the last exposure (Fig. 1*C*). Lastly, we examined whether the response to HDM was affected by the phase of the influenza infection. To that end, we repeated the infection protocol but then allowed the inflammatory response to resolve. At day 40 p.i., animals were then exposed to the same low dose of HDM and the inflammatory response was evaluated 3 days after the last exposure (Fig. 1*D*).

Kinetics of influenza A/PR8/34 infection in BALB/c mice

As described in Fig. 1*A*, mice were inoculated intranasally with 10 PFU of PR8/34 virus at day 0. This concentration of virus was chosen after an initial dose-response study revealed that this was a sublethal dose that induced a robust inflammatory response from which all animals fully recovered. Groups of mice were sacrificed at days 1, 3, 5, 7, 10, and 28, up to and including day 39 p.i. After infection, we observed that the acute inflammatory response in the BAL peaks at days 3–7 p.i. and was considerably, albeit not completely, resolved at day 10 p.i. (Fig. 2*A* and supplemental Fig. 1S). At day 3, the inflammatory response consisted mainly of infiltrating neutrophils and macrophages while at day 7 this response was replaced by infiltrating mononuclear cells. Inflammatory cells remained elevated over PBS-treated groups even at days 28–39 p.i.

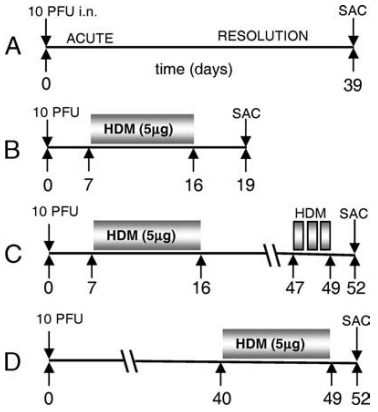

FIGURE 1. Experimental schematics. *A*, Mice were infected with flu strain A/PR8/34 intranasally at a dose of 10 PFU in 35 μl of sterile PBS. Separate groups of mice were sacrificed at various time points and the inflammatory response was delineated through the acute and resolution phase. *B*, Mice were infected as described in *A* and then separate groups of mice were exposed to 5 μg of HDM or SAL daily for a period of 10 days, starting at day 7 p. i. Seventy-two hours after the last exposure, animals were sacrificed and the inflammatory response was examined. *C*, Separate groups of animals were treated as described in *B*, but then allowed to rest for a period of 30 days after the last exposure to HDM. Animals were then re-exposed to 5 μg of HDM on 3 consecutive days and the inflammatory response was assessed for long-term immune changes. *D*, Separate mice were infected with flu-PR8 as described in *A* and allowed to rest for a period of 40 days. Groups of mice were then exposed to 5 μg of HDM, SAL, or no treatment daily for 10 consecutive days. Animals were sacrificed 3 days after the last exposure to HDM and the inflammatory response was assessed.

Flu A infection on cytokine production

In addition to cellular infiltrate, we evaluated the cytokine response. An initial antiviral response, involving the generation of type I IFNs, can be detected as indicated by increased levels of IFN-α and IFN-β in the BAL at day 3 (Fig. 2*B*). This increase was transient and significantly diminished by day 5 p.i. At day 10 p.i., type I IFNs were no longer detectable. The levels of the proinflammatory cytokines IL-6, IFN-γ, and TNF-α were significantly elevated in the BAL at days 3 through 7. TNF-α and IL-6 peaked early in the response, at days 3–7 p.i., while peak levels of IFN-γ and IL-10 were detected at day 7 p.i. and coincided with the influx of mononuclear cells. Again, these increases were transient and all proinflammatory cytokines were undetectable at day 10 p.i.

Lung immune status at days 7 and 10 following flu A infection

Next, we sought to evaluate the immune status of the lung by identifying the different types of APCs and T cells during the acute phase of the inflammatory response, i.e., at days 7–10. As can be seen in Fig. 3*A* (and supplemental Fig. 2S), we identified different populations of APCs by flow cytometry. The total number of $MHCII^+$ cells was dramatically increased in flu-infected animals vs PBS control mice (supplemental Fig. 2S). In addition, in flu-infected animals, there was a remarkable 26-fold increase in the absolute number of pDCs present in the lung as compared with

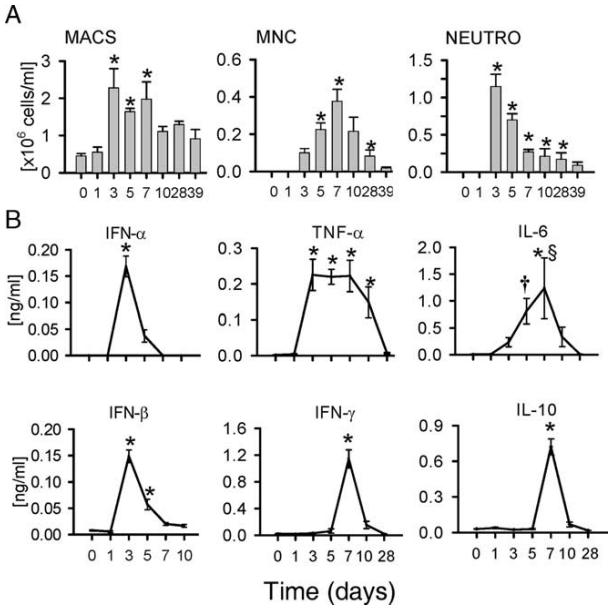

FIGURE 2. Kinetics of influenza A/PR8-induced BAL inflammation and cytokine responses. *A*, Animals were infected intranasally with 10 PFU of PR8 virus and the inflammatory response in BAL was evaluated at days 0, 1, 3, 5, 7, 10, 28, and 39 p.i. Cellular differentials showing absolute number of macrophages, neutrophils, and mononuclear cells. $n = 5$ mice/group. One of two representative experiments is shown. Data represent mean ± SEM. ∗, $p < 0.05$ compared with day 0 (Dunnet's post hoc test). *B*, Kinetics of type I IFNs and proinflammatory cytokine levels in BAL fluid as evaluated by ELISA at various time points. $n = 5$ mice/group. One of two representative experiments is shown. Data represented as mean ± SEM. ∗, $p < 0.001$ compared with day 0 and †, $p < 0.05$ and §, $p < 0.05$ compared with days 3 and 6, respectively. MACS, Macrophages; MNC, mononuclear cells; NEUTRO, neutrophils.

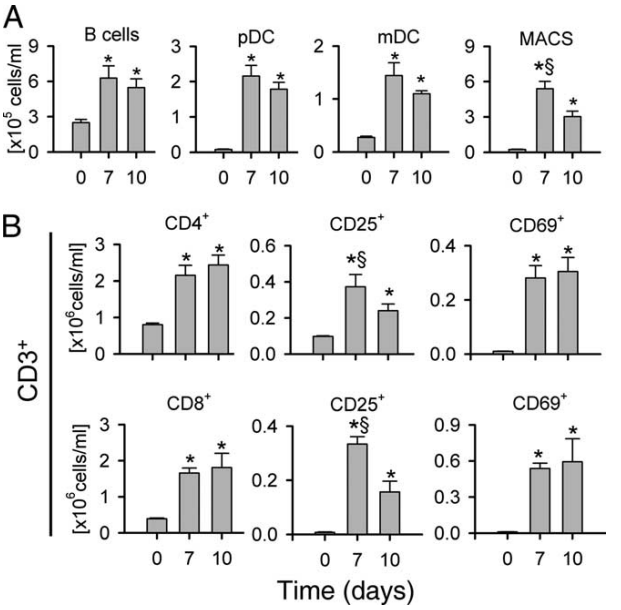

FIGURE 3. Immune status of lung at days 7 and 10 after PR8 infection. Total lung cells were isolated at days 7 and 10 p.i. and stained with MHCII, CD11b, CD11c, B220, and GR1 cell surface markers. More than 300,000 events were collected for each group. *A*, Number of B cells, pDCs, mDCs, and macrophages at days 7 and 10 p.i. as compared with control mice at day 0. $n = 5$ mice/group. One of two representative experiments is shown. Data represent mean ± SEM and $p < 0.05$ compared with ∗, day 0 and §, day 10, respectively (Tukey's post hoc test). *B*, Absolute number of CD4 and CD8 T cells ($CD3^+$) and their activation states ($CD25^+$ and $CD69^+$) at days 7 and 10 after PR8 infection. $n = 5$ mice/group. One of two experiments with similar results is shown. Data represent mean ± SEM. $p < 0.05$ compared with ∗, day 0 and §, day 10, respectively. MACS, Macrophages.

PBS controls, whereas mDCs experienced an~5-fold increase. Similarly, there was an 24-fold increase in $B220^{low}CD11c^{int}CD11b^{high}$ cells, a population representing alveolar macrophages and an ~2.5-fold increase in B cells. Although the absolute number of mDCs increased with infection, it was notable that the relative percent contribution of mDCs did not change after flu infection (3% before and after flu A). In contrast, the pDC percent contribution increased from 1 to 5% at day 7 after flu infection (data not shown).

In addition to APCs, we also assessed T cell populations (Fig. 3*B*). At days 7 and 10 after flu infection, both $CD4^+$ and $CD8^+$ T cells increased in absolute numbers, with $CD8^+$ T cells showing a 4-fold increase while $CD4^+$ T cells increased by ~2.7-fold. We observed an increased number of activated cells as indicated by CD25 and CD69 expression at days 7 and 10 in both CD4 and CD8 T cells. The number of $CD25^+CD8^+$ T cells was 10-fold higher than in $CD4^+$ T cells, with a notable 44-fold increase as compared with a more modest increase of ~4.8-fold in $CD25^+$ activated $CD4^+$ T cells. Furthermore, the relative contribution of $CD25^+$ activated T cells increased to 20% after flu A, corresponding to a 1.7- and 10.5-fold increase for $CD4^+$ and $CD8^+$ T cells, respectively (data not shown). Similarly, $CD69^+CD8^+$ T cells experienced a 55-fold expansion, whereas $CD69^+CD4^+$ T cells increased 31-fold over PBS-treated animals; this corresponded to a 15-fold increase in the activation state for $CD8^+CD69^+$ T cells (2.5% before vs 32% after flu A), whereas the relative contribution of $CD4^+CD69^+$ changed from 1.2% before vs 12.8% after infection (data not shown). Overall, flu infection changed the CD4:CD8 ratio from 2:1 in PBS-treated animals to 1:1 after flu infection, thus indicating a preferential increase in activated $CD8^+$ over activated $CD4^+$ T cells.

Taken together, these results indicate that a proinflammatory environment is rapidly established during the acute phase of flu infection and is associated with an expansion in the APC as well as the T cell compartment, with a preferential increase in both pDCs and activated $CD8^+$ T cells.

Responses to HDM exposure in the context of flu-induced acute inflammation

Next, we investigated the impact of a viral-induced proinflammatory environment on the response to allergen exposure. To this end, we exposed groups of animals to the protocol outlined in Fig. 1*B*. We found enhanced BAL inflammation in mice previously infected with flu virus (F plus H) as compared with HDM (H) alone, those infected only with flu (F), or saline (SAL)-treated animals (Fig. 4*A*). The increase in total inflammation in HDM-exposed animals previously infected with flu could be mainly attributed to an increase in both mononuclear cells and eosinophils. Indeed, prior flu infection resulted in a doubling of the proportion of eosinophils in the BAL from 7% in HDM-treated animals to 15% in the flu plus HDM group (data not shown).

To further evaluate the nature of T cell subsets in the lung, total lung cells were analyzed by flow cytometry. The total number of $CD4^+$ or $CD8^+$ T cells in mice exposed to HDM alone, flu alone, and HDM after flu infection was not significantly different compared with saline at this time point (supplemental Fig. 3S and data not shown). However, we observed

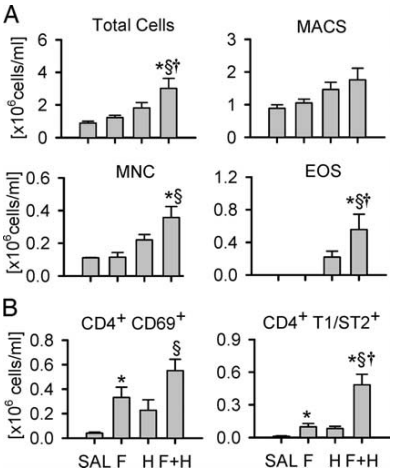

FIGURE 4. Impact of influenza A infection on subsequent HDM exposure. Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. Mice were sacrificed 3 days after the last exposure and the inflammatory response was evaluated in BAL fluid and lung. *A*, Cellular profile in BAL fluid showing the number of total cells, macrophages, mononuclear cells, and eosinophils. $n = 5$ mice/group. One of three representative experiments is shown. Data represent mean ± SEM. $p < 0.05$ compared with ∗, SAL; †, F; and §, HDM, respectively. *B*, Flow cytometric analysis of lung immune cells showing the absolute number of $CD4^+$ T cells expressing the activation marker CD69 and Th2 marker T1/ST2. $n = 5$ mice/group. Data represent mean ± SEM. $p < 0.05$ compared with ∗, SAL; †, F; and §, HDM, respectively. MACS, Macrophages; MNC, mononuclear cells; EOS, eosinophils.

a significant increase in the number of activated cells, as evaluated by $CD69^+$ expression (Fig. 4*B*). In animals exposed to flu only, we found an 8.2-fold increase in CD69-activated $CD4^+$ cells and a 2.1-fold increase in $CD8^+$ T cells (supplemental Fig. 3S). HDM exposure further increased this by 1.6- and 1.5-fold for $CD4^+$ and $CD8^+$ T cells, respectively. Indeed, the level of $CD4^+$ T cell activation in flu-infected animals increased from 26 to 39% after HDM exposure (data not shown). In contrast, exposure to 5 μg of HDM alone led to only a modest increase in CD69 expression as compared with control animals.

Finally, to examine whether the pronounced eosinophilic response was associated with an expansion in Th2 cells, we evaluated the expression level of $T1/ST2^+$, a cell surface marker expressed on effector Th2 cells (20, 21). We observed a modest increase in $CD4^+T1/ST2^+$ cells in animals exposed to 5 μg of HDM over saline-treated animals; this level was significantly increased in the flu plus HDM-treated group (5.9-fold) as compared with HDM and corresponds to a doubling in the percent contribution of this cell type in the flu-infected and HDM-exposed group vs HDM alone, ~%8 vs ~4%, respectively (data not shown).

Cytokine recall responses of lung mononuclear cells

Next, we explored whether the enhanced inflammatory and eosinophilic response was associated with changes in immune responsiveness. We examined the production of Th1/Th2 cytokines by lung mononuclear cells cultured with 25 μg of HDM. We found that in animals exposed to HDM alone, the average level of IL-5 production was 621 pg/ml, whereas in animals infected with flu virus and subsequently exposed to HDM, this level increased to an average of 1613 pg/ml (Fig. 5). Similarly to IL-5, IFN-γ production was also increased significantly in

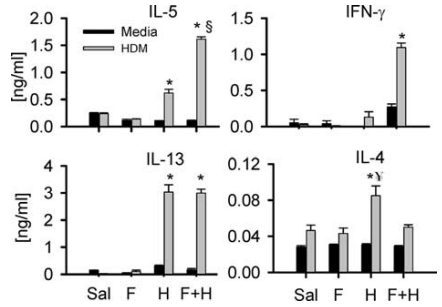

FIGURE 5. Cytokine production by lung mononuclear cells. Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. Mice were sacrificed 3 days after the last exposure and the inflammatory response was evaluated. Evaluation of IL-4, IL-5, IL-13, and IFN-γ expression by lung mononuclear cells cultured for 5 days in the presence of HDM (▨) or medium alone (■). Samples were pooled within groups and plated in triplicate ($n = 5$). Data represent mean ± SEM. $p < 0.001$ compared with §, HDM; ¥, F + H; and ∗, F or SAL, respectively.

animals previously infected with flu and subsequently exposed to HDM, from 130 pg/ml in the HDM group to 1094 pg/ml in the flu plus HDM-treated animals.

In contrast to increases in IL-5 and IFN-γ, we observed a significant decrease in IL-4 production in animals previously infected with flu and exposed to HDM, 85 pg/ml vs 50 pg/ml, in HDM vs flu plus HDM, respectively. Finally, we observed no difference in IL-13 production between HDM and flu plus HDM-treated animals, but found it to be significantly increased over flu alone or SAL control.

To determine the impact on Ig production, we evaluated flu and HDM-specific IgGs. Whereas, flu-specific IgG responses were not affected by subsequent HDM exposure, there was a significant increase in both HDM-specific IgG1 and IgG2a responses in F plus H compared with HDM alone (Fig. 6). HDM-specific IgE responses at this time point could not be evaluated due to the short duration of the experimental protocol used.

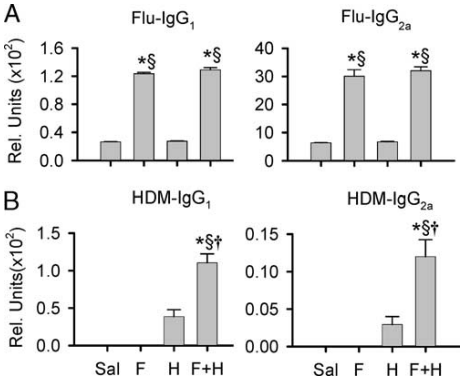

FIGURE 6. Impact of influenza A infection on HDM-specific Igs. Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. Mice were sacrificed 3 days after the last exposure. Serum Ig levels measured by ELISA showing flu-specific IgG1 and IgG2a (*A*) and HDM-specific IgG1 and IgG2a (*B*). $n = 5$ mice/group. One of two representative experiments is shown. Data represent mean ± SEM. $p < 0.05$ compared with ∗, SAL; §, HDM; and †, flu, respectively.

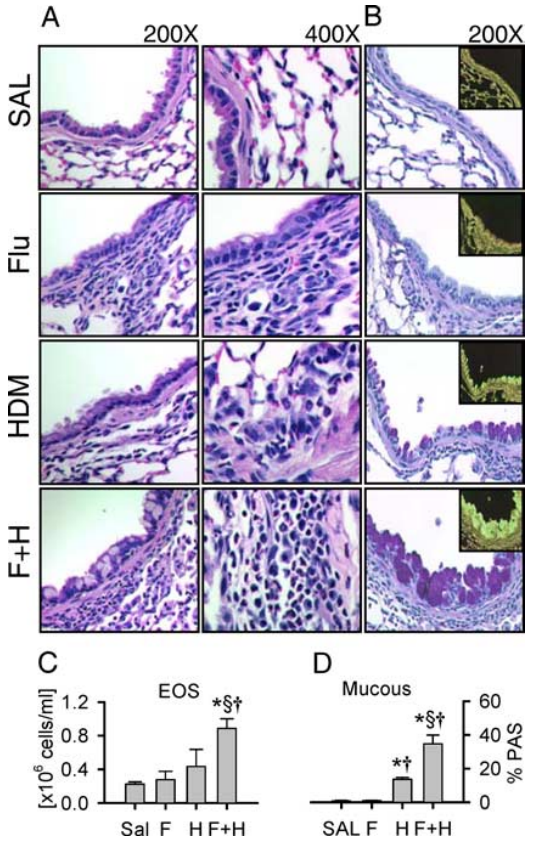

FIGURE 7. Lung histopathology after HDM exposure in the context of a prior flu infection. *A* and *B*, Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. Mice were sacrificed 3 days after the last exposure. Lung histopathology was evaluated by H&E to show degree of lung eosinophilia (*A*) and PAS staining (*B*) indicating mucous production by epithelial goblet cells (magenta; *inset*s show color-inverted image used for morphometric analysis). *C*, Quantitative analysis of tissue eosinophils by flow cytometry. *D*, Morphometric measurement of PAS-stained tissues. $n = 5$ mice/group. One of two representative experiments is shown. Micrographs were taken at ×200 and ×400 original magnification as noted. Data represent mean ± SEM. $p < 0.05$ compared with ∗, SAL; †, F; and §, HDM.

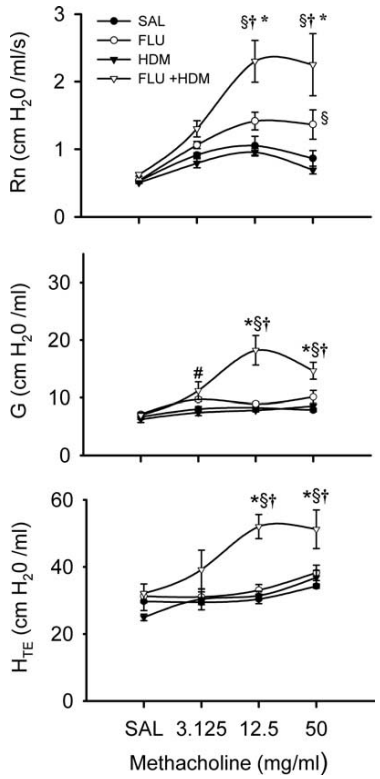

FIGURE 8. Impact of HDM exposure on airway responsiveness in the context of acute flu infection. Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. Mice were sacrificed 3 days after the last exposure. Airway responsiveness to increasing doses of MCh was assessed and shown as maximum Rn, G, and H_{TE}. $n = 6$ mice/group. One of two representative experiments is shown. Data represent mean ± SEM. $p < 0.01$ compared with ∗, SAL; §, HDM; and †, flu, respectively. #, $p < 0.05$ compared with HDM or SAL. Two-way ANOVA (Tukey's post hoc test).

The changes in inflammation observed in the BAL were also associated with changes in tissue inflammation, goblet cell metaplasia, and enhanced mucous production. Animals exposed to 10 days of HDM exhibited a very modest degree of tissue inflammation characterized by peribronchiolar mononuclear cell and eosinophil accumulation, which was enhanced in animals previously exposed to flu virus (Fig. 7*A*). In contrast, tissue inflammation in animals exposed to flu alone was limited to mononuclear cell infiltration with no accumulation of eosinophils. The degree of tissue eosinophilia was evaluated both qualitatively by H&E staining and quantitatively by flow cytometric analysis (Fig. 7*C* and supplemental Fig. 4S), which confirmed the elevated eosinophilic inflammation observed in the BAL of flu plus HDM-treated animals.

Based on the increased inflammatory responses, we examined whether flu infection also impacted structural remodeling, in particular goblet cell metaplasia and mucous production (Fig. 7*B*). After 10 days of HDM exposure, goblet cell metaplasia and mucous production was evident in HDM-treated animals. This effect was almost doubled in the HDM-treated group previously infected with flu virus (Fig. 7*D*). In contrast, control animals treated with flu or saline alone did not exhibit any mucous production.

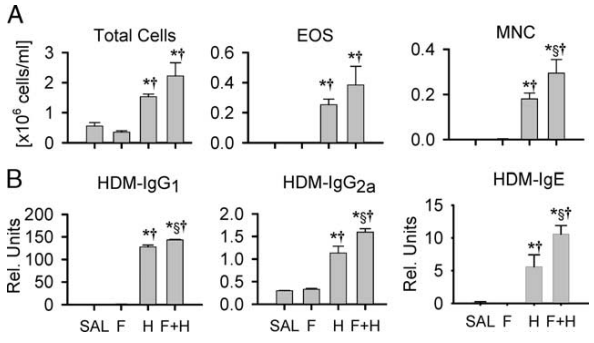

FIGURE 9. Impact of acute flu infection on long-term allergen-specific immunity. Groups of mice were infected with 10 PFU of PR8 virus or PBS alone. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 7 p.i. After a 30-day rest period, mice were re-exposed to three doses of 5 μg of HDM or SAL. The inflammatory response was evaluated 3 days after the last exposure. *A*, Cellular profile in BAL fluid showing the number of total cells, eosinophils, and mononuclear cells. *B*, HDM-specific IgG1, IgG2a, and IgE levels in the serum. One of two representative experiments is shown. Data represent mean ± SEM. $p < 0.05$ as compared with ∗, SAL; †, flu; and §, HDM, respectively. EOS, Eosinophils; MNC, mononuclear cells.

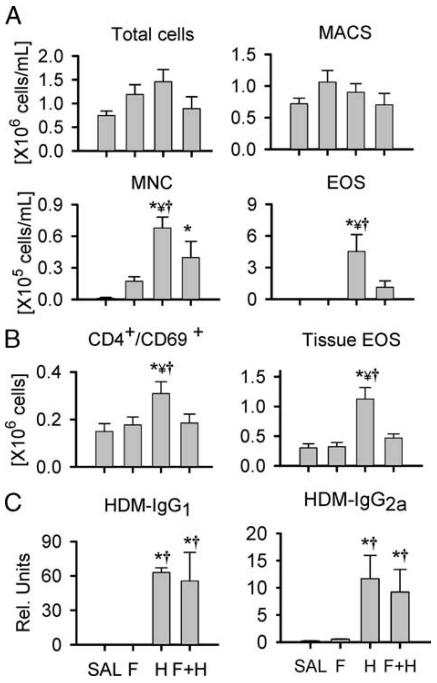

FIGURE 10. Allergen exposure during the resolution phase of flu infection. Mice were infected with 10 PFU of PR8 virus or mock infected (PBS) and then allowed to rest for 40 days. Flu-infected (F) and PBS groups were subsequently exposed to HDM (H) or saline (SAL) for 10 days starting at day 40 p.i. Mice were sacrificed 3 days after the last exposure and the inflammatory response was evaluated. *A*, BAL inflammation showing the number of total cells, macrophages, mononuclear cells, and eosinophils. $n = 5$ mice/group. One of three representative experiments is shown. Data represent mean ± SEM. $p < 0.05$ compared with ∗, SAL; †, F; and ¥, F + H, respectively. *B*, Flow cytometric analysis of lung cells showing the absolute number of activated $CD4^+$ T cells expressing CD69 and tissue eosinophils. *C*, Serum Ig levels of HDM-specific IgG1 and IgG2a. $n = 6$ animals/group. One of two representative experiments is shown. Data represent mean ± SEM. $p < 0.05$ compared with ∗, SAL; †, F; and ¥, F + H, respectively. MACS, Macrophages; MNC, mononuclear cells; EOS, eosinophils.

Lung function after HDM exposure in the context of flu-induced acute inflammation

We investigated whether the enhanced allergic response to HDM in flu-infected animals resulted in altered lung function by evaluating respiratory mechanics; this was accomplished by evaluating Rn, G, and H_{TE}. A low dose of HDM exposure (5 μg for 10 days), which caused a mild inflammatory response, did not elicit any measurable changes in lung function (Fig. 8). In contrast, animals previously infected with flu and subsequently exposed to HDM exhibited significantly enhanced dysfunction as measured by Rn, G, and H, and this was statistically significant at both 12.5 and 50 mg/ml of MCh exposure. Interestingly, animals infected with flu only also experienced a small but significant increase in Rn over HDM and saline-treated animals at the highest dose of MCh used (Fig. 8).

Long-term immune-inflammatory changes

To investigate whether the flu-mediated changes in allergen responsiveness were transient in nature, we examined long-term immunologic events. Thus, flu-infected, HDM-exposed animals were rechallenged at day 31 after the last HDM exposure for 3 consecutive days (Fig. 1*C*).

Similar to what we observed at day 19, we found an increase in total BAL inflammation in animals with a prior flu infection as compared with the HDM-only group and the respective control groups (Fig. 9*A*). Furthermore, this increase in inflammation was characterized by a significant increase in mononuclear cells as well as eosinophils; although the latter did not reach statistical significance. In addition, we found that the serum HDM-specific IgG1 and IgG2a responses remained statistically elevated at day 52 p.i. in flu plus HDM-treated animals, as compared with HDM only (Fig. 9*B*). Importantly, HDM-specific IgE levels were significantly increased at this time in animals previously infected with flu virus and exposed to HDM over the HDM-alone group or the respective control animals.

HDM exposure during the resolution phase

The responses to HDM exposure observed during the acute phase of influenza infection raised the question whether exposure to HDM long after resolution of the acute phase would lead to a similar outcome. To that end, animals were exposed to the experimental protocol outlined in Fig. 1*D*. We found that animals infected with flu and subsequently exposed to HDM had lower total inflammation and that this was associated with a 4-fold reduction in eosinophils in BAL and lung tissue (Fig. 10, *A* and *B*). Furthermore, we observed a significant decrease in BAL mononuclear cells, which upon further examination by flow cytometry revealed a significant decrease in the number of activated $CD4^+CD69^+$ cells (Fig. 10*B*). At variance with these findings, we found similar serum levels of HDM-specific IgG1 and IgG2a in animals exposed to HDM and previously infected with flu as compared with the HDM-only group (Fig. 10*C*) suggesting that allergic sensitization was not affected.

Discussion

There has been a great deal of interest to uncover the impact of respiratory viral infections on asthma. Understandably, much of this research has focused on the potential of such infections to exacerbate asthma; much less evidence is available investigating their ability to facilitate the emergence of the asthmatic phenotype. This is due, at least in part, to the fact that it is nearly impossible to directly investigate the development of asthma in humans. With respect to experimental research, the vast majority of studies investigating the impact of flu infection on allergic asthma have been conducted using the innocuous Ag OVA as a surrogate allergen (22–27). Indeed, this model system has become a prolific tool to elucidate specific molecular mechanisms of Th2-mediated inflammation. It should be noted that, in the absence of exogenous adjuvants, mucosal exposure to OVA leads to the induction of tolerance and, in this regard, Tsitoura et al. (26) clearly demonstrated that a concurrent flu infection is able to prevent the induction of tolerance. In addition, others have shown that a flu infection can either enhance or suppress allergic responses, interestingly both effects being mediated by IFN-γ (28, 29). The controversial and, in some instances, even contradictory nature of these and other data in OVA-based systems intimate that models of allergic asthma that require the introduction of Ag into the peritoneal cavity along with chemical adjuvants to elicit productive immunity are limited. Indeed, not only do they introduce confounding variables, but, importantly, preclude the investigation of allergic sensitization via the mucosal route. Thus, the precise impact mucosal allergen exposure has following influenza A infection has not been examined.

In this study, we have investigated the impact of a preexisting lung viral infection on cardinal immune, structural, and functional features of the asthmatic phenotype. The experimental system that we used has three central characteristics. First, we used the most

pervasive and common aeroallergen worldwide, HDM, a biochemically complex material with a wide array of protein and nonprotein components with numerous immunogenic properties (30–32). These constituents enable these extracts to initiate Th2-polarized immune-inflammatory responses when delivered intranasally without additional adjuvants (1, 2), thus permitting the study of incipient mucosal responses. Second, we used a low concentration of HDM, 5 μg per day for 10 days, that elicits only very mild airway inflammation and, most importantly, no lung dysfunction. Third, a preexisting immune-inflammatory environment was established with influenza A, a virus that elicits an archetypic Th1-type response. This is in contrast to many studies that have investigated the impact of RSV infection which by itself tends to promote a Th2 environment.

To precisely identify the time points at which aeroallergen exposure was to be conducted, we first delineated the inflammatory response to a sublethal dose of influenza A/PR8 virus. Our data show that the pulmonary environment after flu infection exhibits an initial antiviral as well as a proinflammatory state characterized by a robust, albeit transient, infiltration of inflammatory cells largely consisting of neutrophils and mononuclear cells. In addition to cellular immunity, this acute phase is characterized by increased amounts of an array of proinflammatory cytokines in the BAL including TNF-α, IFN-γ, IL-10, and IL-6.

To better define the immune status of the lung, we evaluated the profile of immune cells at the peak of the PR8-induced mononuclear cell infiltration, i.e., at days 7 and 10 p.i. We first evaluated the APCs compartment because it is clear that APCs play an important role in enhancing allergen responses after flu infection (24, 25, 27). Our findings demonstrate an overall increase in all APC populations, namely, macrophages, B cells, mDCs, and pDCs. Notably, our data also show a particularly dramatic increase in pDCs and macrophages as compared with mDCs and B cells. These findings are consistent with the idea that pDCs are the major cell type responding after respiratory viral infections and the major producers of type I IFNs (15). With respect to T cell subsets, our data show that although the number of both $CD4^+$ and $CD8^+$ T cells increased, there was a preferential increase in the activation of $CD8^+$ T cells over $CD4^+$ T cells. This preferential increase is expected after a viral infection and particularly interesting in terms of its potential impact on the outcome of subsequent allergen exposure.

This detailed kinetic study allowed us to clearly separate the response to PR8 into acute and resolution phases. Exposure to a low dose of HDM during the acute phase of a flu infection led to a significantly increased number of mononuclear cells as well as eosinophils in the lung. A closer examination by flow cytometry revealed that the increase in mononuclear cells was associated with a significant increase in the number of activated $CD4^+$ T cells and, particularly, activated $CD4^+$ T cells expressing T1/ST2, a cell surface marker expressed by effector Th2 cells (20, 21) over and above that induced by this concentration of HDM alone. In terms of humoral immunity, our data show that HDM exposure during the acute phase of a flu infection results in significantly increased levels of HDM-specific IgG1 and IgG2a, although this time point is too premature to detect a HDM-specific IgE response. In contrast, the levels of flu-specific Igs remained unaltered. Collectively, these data indicate that the environment established during the acute phase of an infection with flu-PR8 virus leads to enhanced sensitization and attendant allergic inflammation to a low dose of aeroallergen administered through the mucosal route. Interestingly, this occurs at a time when there is an accumulation of activated $CD8^+$ T cells in the lung and increased IFN-γ production, both of which have been proposed to inhibit allergic airway inflammation (33). Thus, our findings demonstrate that a vigorous flu-induced Th1 response does not deviate HDM-induced Th2 immunity.

We next asked whether the enhancement in allergic sensitization and inflammation was transient in nature. Thus, animals that had been exposed to allergen during the acute response of a flu infection were allowed to rest for a period of 30 days so that the HDM-mediated inflammatory response would fully resolve. Allergen rechallenge, at this time point, led to a substantial increase in BAL inflammation, eosinophilia, and mononuclear cells. Similar to the observations during the acute phase, HDM exposure at this time resulted in enhanced humoral immunity and was now associated with increases in HDM-specific IgE. These data show that HDM exposure during the acute phase of a flu infection resulted in long-lasting immune changes.

Our findings may appear to contrast with those of Wohlleben et al. (29) showing that, in a conventional OVA model, a prior flu infection inhibited airway eosinophilia and Th2 cell recruitment and that these effects were dependent on IFN-γ. However, the timing of viral and allergen exposures were fundamentally different from those used in our study. Indeed, Wohlleben et al. (29) examined already sensitized mice that were subsequently infected with influenza A 1–9 wk before OVA challenge. Hence, this study examined the impact of a flu infection on OVA-specific recall responses rather than on mucosal allergic sensitization.

Our data demonstrate that the presence of a large number of pDCs and macrophages in the lung at the time of allergen exposure did not result in inhibition of allergic sensitization and inflammation. Rather, our study shows that these responses were enhanced. Interestingly, in a model of experimental asthma to the inert Ag OVA, it has been shown that pDCs inhibit allergen sensitization and, in fact, promote tolerance (17), while mDCs are thought to mediate allergic sensitization (16). These observations led to the suggestion that immature pDCs possess tolerogenic properties and have poor Ag-presenting capacity, whereas activated pDCs can convert to an immunogenic phenotype able to prime T cells and drive potent Th1 polarization. However, there is a considerable amount of conflicting data regarding this simple dichotomy. Indeed, pDCs have been shown capable of mediating both Th1 and Th2 polarization (15, 34). Furthermore, studies in humans show that both mDCs as well as pDCs are present in the allergic immune response to allergen (35, 36) and, in patients with allergic asthma, allergen challenge increased both mDC and pDC numbers with an overall greater percent increase in pDCs (37). Clearly, the precise role of different dendritic cells subsets in allergic diseases is far from resolved. With respect to experimental studies, it seems increasingly apparent that the Ag used, an inert protein such as OVA or a complex allergen with inherent immunogenic properties, may have a decisive impact on the outcome.

The immune interactions that occur between respiratory viral infection and aeroallergen exposure defy a simple classification into Th1- or Th2-type responses. For example, our data show that lung mononuclear cells from HDM-treated animals previously infected with flu virus express, upon in vitro stimulation with HDM, an atypical profile characterized by increased amounts of IL-5 as well as IFN-γ while IL-4 is significantly down-regulated and IL-13 production is unchanged. Whether enhanced allergic responses to HDM in the context of an ongoing flu infection involve an IL-4-independent mechanism is not known. However, a recent study by Kurowska-Stolarska et al. (38) has shown, in an OVA system, that IL-33, the ligand for the T1/ST2 receptor expressed on $CD4^+$ T cells, can induce IL-5 and IL-13 production and promote allergic airway inflammation in the absence of IL-4. It is, thus, conceivable

to postulate that the increased IFN-γ level that we detected down-regulates IL-4 production, whereas the enhanced IL-5 production is driven by IL-33. However, whether a flu infection induces the expression of IL-33 is not known. We should also note that other innate molecules may contribute to the enhanced allergic responses that we have documented in flu- infected, HDM-exposed animals. These may include, for example, GM-CSF and IL-6, both of which are elevated in flu- infected animals. Studies to uncover the mechanisms underlying the enhanced allergic responses that we have documented are receiving intense attention in our laboratory.

Within a larger perspective, studies have demonstrated that several hundred genes are up-regulated shortly after a flu infection (39, 40). There are no similar data available to date for HDM-elicited immune-inflammatory responses, although a similarly rich array of gene up-regulation would be expected given the immunogenic potential of HDM extracts (30–32). Furthermore, the interplay that surely occurs between flu-induced and HDM-induced gene products would elevate the degree of complexity to a higher level. In regard to the mechanisms underlying the enhancement in allergic sensitization and inflammation that we have documented when HDM exposure occurs during the acute phase of a flu infection, we can say that such an enhancement is connected to the presence of an innate, proinflammatory environment and that it seems unlikely that a single molecule is responsible for this effect.

To elucidate the health impact of those immunologic events, we investigated their structural and functional consequences. Airway remodeling is, indeed, a typical hallmark of asthma and a growing number of studies have found that remodeling, or at least certain aspects of it does contribute to airway dysfunction and, ultimately, clinical symptoms (41, 42). Typical features of remodeling occurring below the epithelial lining, such as collagen deposition, take several weeks to develop in mice. Thus, within the interval of the short protocol of HDM exposure that we used (10 days), only goblet cell metaplasia and mucous production can be evaluated. We observed that exposure to even a low dose of HDM induced mild but significant mucous production and that influenza A infection alone did not lead to any mucous production. However, animals that were exposed to HDM in the context of an acute flu infection had significantly greater goblet cell metaplasia and mucous production as compared with those treated with a low dose of HDM only. Thus, this provides evidence that a prior flu infection can enhance aspects of airway remodeling that could have an impact on function.

Previous studies, using conventional OVA models, have found either no effect (28) or shown increased airway hyperreactivity (24) after flu infection. Not only are the outcomes controversial but the actual methodology used to assess lung function performed in these studies (enhanced pause (Penh)) has serious drawbacks such that its physiologic significance has been questioned (43, 44). We evaluated lung physiology using a protocol that furnishes data on lung mechanics including Rn, G, and H_{TE}, an indicator of the degree of airway closure in response to MCh exposure. This approach has been validated in other models and is considered a superior predictor of changes in lung function (45). Exposure of animals to a low dose of HDM did not result in any changes in lung mechanics as compared with saline-treated animals. Intriguingly, flu infection alone induced significant changes in airway resistance. Moreover, a low dose of HDM exposure during an acute flu infection increased the response to MCh challenge, leading to enhanced values for Rn, G, and H_{TE}. Thus, the immunologic and structural changes that occurred as a result of flu infection lowered the threshold of allergen needed to generate significant lung dysfunction. In other words, a low dose of HDM that in an otherwise healthy lung environment would not lead to significant lung dysfunction results in severe functional abnormalities if exposure had occurred during the acute phase of a flu infection.

Lastly, to address whether timing of allergen exposure played a role in the response to HDM, we exposed animals to HDM starting at 40 days after flu infection. In contrast to the enhancement observed during the acute phase of infection, the inflammatory response, including eosinophils, elicited by HDM exposure was significantly reduced compared with uninfected mice. Importantly, these changes in the BAL were substantiated by a reduction in $CD4^+CD69^+$ T cells and eosinophils in the tissue. The decrease in lung inflammation cannot be attributed to decreased allergic sensitization because the levels of HDM-specific IgG1 and IgG2a were similar between animals that had been infected with the flu virus 40 days earlier and uninfected animals. Moreover, we did not detect any alteration in lung function (data not shown). There is no direct evidence of reference for these findings. However, two studies have examined the consequences of exposure to a heterologous Ag during the resolution phase of a flu infection with divergent results (28, 33). Our findings are in sharp contrast to those of Dahl et al. (28) in that these authors reported an increase in lung eosinophilia as well as primary, but not secondary, Ag-specific IgG1 and IgE responses upon exposure to the surrogate allergen keyhole limpet hemocyanin (KLH) 30 days after flu infection, an effect that was shown to be mediated by activated MHCII/$CD11c^+$ cells (28). Not only the Ag but also the experimental protocol was different from that which we used in that, at day 30, mice had to be exposed first to KLH i.p. twice along with aluminum hydroxide to achieve sensitization and later exposed to KLH intranasally. At variance with these findings, Marsland et al. (33) reported a reduction of allergic airway inflammation in mice exposed to the parasite *Nippostrongylus brasiliensis* (NES) 14 days after a flu infection, an effect mediated by $CD8^+$ T cells and IFN-γ (33). Although it may be argued whether 14 days represents true resolution, this time point is indeed past the acute proinflammatory phase of the flu infection. In this study as well, mice were exposed first to a NES extract i.p. along with aluminum hydroxide and exposed to NES intranasally 7 days later. Thus, neither of these studies was able to investigate the effect of a resolving, or resolved, flu infection on mucosal sensitization to allergen. With this background, our study shows that mucosal exposure to the common aeroallergen HDM during the resolution phase of a flu infection does not alter allergic sensitization and is associated with a decrease of allergic airway inflammation.

In summary, our findings demonstrate that mucosal exposure to the common aeroallergen HDM during the acute proinflammatory phase of a flu infection results in increased allergic sensitization and enhanced allergic inflammation, goblet cell metaplasia, and mucous production. In addition, allergen exposure under these conditions lowers the threshold for the generation of a definitely abnormal functional response (lung mechanics). The direct, and particularly important, implication of these findings is that the environment established during the acute phase of a flu infection increases the susceptibility to elicit allergic disease. However, this increased susceptibility is transient since allergic inflammation is, in fact, reduced when allergen exposure occurs during the resolution phase. It should be noted that the enhancement of allergic responses observed when allergen is introduced during the acute phase of the flu infection occurs at a time when there is a preferential increase in the lung of pDCs over mDCs as well as the presence of increased numbers of activated $CD8^+$ T cells and high levels of IFN-γ, all of which have been proposed to inhibit allergic responses. Hence, perhaps a broader implication of our findings is that of caution against attachment to oversimplified paradigms.

The biologic responses induced by HDM and influenza A are each complex; it seems that decoding the regulation of this complexity may require ontologies more sophisticated than traditional reductionist dichotomies.

Acknowledgments

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Disclosures

A.J.C. is an employee of MedImmune and J.R. is a former employee of MedImmune, Inc.

References

1. Cates, E. C., R. Fattouh, J. Wattie, M. D. Inman, S. Goncharova, A. J. Coyle, J. C. Gutierrez-Ramos, and M. Jordana. 2004. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J. Immunol.* 173: 6384–6392.
2. Johnson, J. R., R. E. Wiley, R. Fattouh, F. K. Swirski, B. U. Gajewska, A. J. Coyle, J. C. Gutierrez-Ramos, R. Ellis, M. D. Inman, and M. Jordana. 2004. Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am. J. Respir. Crit. Care Med.* 169: 378–385.
3. Llop-Guevara, A., M. Colangelo, D. K. Chu, C. L. Moore, N. A. Stieber, T. D. Walker, S. Goncharova, A. J. Coyle, L. K. Lundblad, P. M. O'Byrne, et al. 2008. In vivo-to-in silico iterations to investigate aeroallergen-host interactions. *PLoS ONE* 3: e2426.
4. Nafstad, P., B. Brunekreef, A. Skrondal, and W. Nystad. 2005. Early respiratory infections, asthma, and allergy: 10-year follow-up of the Oslo Birth Cohort. *Pediatrics* 116: e255–e262.
5. Sigurs, N., P. M. Gustafsson, R. Bjarnason, F. Lundberg, S. Schmidt, F. Sigurbergsson, and B. Kjellman. 2005. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. *Am. J. Respir. Crit. Care Med.* 171: 137–141.
6. Roman, M., W. J. Calhoun, K. L. Hinton, L. F. Avendano, V. Simon, A. M. Escobar, A. Gaggero, and P. V. Diaz. 1997. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. *Am. J. Respir. Crit. Care Med.* 156: 190–195.
7. Bendelja, K., A. Gagro, A. Bace, R. Lokar-Kolbas, V. Krsulovic-Hresic, V. Drazenovic, G. Mlinaric-Galinovic, and S. Rabatic. 2000. Predominant type-2 response in infants with respiratory syncytial virus (RSV) infection demonstrated by cytokine flow cytometry. *Clin. Exp. Immunol.* 121: 332–338.
8. Jansen, A. G., E. A. Sanders, A. W. Hoes, A. M. van Loon, and E. Hak. 2007. Influenza- and respiratory syncytial virus-associated mortality and hospitalisations. *Eur. Respir. J.* 30: 1158–1166.
9. Poehling, K. A., K. M. Edwards, G. A. Weinberg, P. Szilagyi, M. A. Staat, M. K. Iwane, C. B. Bridges, C. G. Grijalva, Y. Zhu, D. I. Bernstein, et al. 2006. The underrecognized burden of influenza in young children. *N. Engl. J. Med.* 355: 31–40.
10. Cella, M., F. Facchetti, A. Lanzavecchia, and M. Colonna. 2000. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. *Nat. Immunol.* 1: 305–310.
11. Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, et al. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. USA* 96: 9345–9350.
12. Fattouh, R., N. G. Midence, K. Arias, J. R. Johnson, T. D. Walker, S. Goncharova, K. P. Souza, R. C. Gregory, Jr., S. Lonning, J. Gauldie, and M. Jordana. 2008. Transforming growth factor-β regulates house dust mite-induced allergic airway inflammation but not airway remodeling. *Am. J. Respir. Crit. Care Med.* 177: 593–603.
13. Ellis, R., R. Leigh, D. Southam, P. M. O'Byrne, and M. D. Inman. 2003. Morphometric analysis of mouse airways after chronic allergen challenge. *Lab. Invest.* 83: 1285–1291.
14. Fattouh, R., M. A. Pouladi, D. Alvarez, J. R. Johnson, T. D. Walker, S. Goncharova, M. D. Inman, and M. Jordana. 2005. House dust mite facilitates ovalbumin-specific allergic sensitization and airway inflammation. *Am. J. Respir. Crit. Care Med.* 172: 314–321.
15. Colonna, M., G. Trinchieri, and Y. J. Liu. 2004. Plasmacytoid dendritic cells in immunity. *Nat. Immunol.* 5: 1219–1226.
16. Lambrecht, B. N. 2005. Dendritic cells and the regulation of the allergic immune response. *Allergy* 60: 271–282.
17. de Heer, H. J., H. Hammad, T. Soullie, D. Hijdra, N. Vos, M. A. Willart, H. C. Hoogsteden, and B. N. Lambrecht. 2004. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J. Exp. Med.* 200: 89–98.
18. Schuessler, T. F., and J. H. Bates. 1995. A computer-controlled research ventilator for small animals: design and evaluation. *IEEE Trans. Biomed. Eng.* 42: 860–866.
19. Hantos, Z., B. Daroczy, B. Suki, S. Nagy, and J. J. Fredberg. 1992. Input impedance and peripheral inhomogeneity of dog lungs. *J. Appl. Physiol.* 72: 168–178.
20. Coyle, A. J., C. Lloyd, J. Tian, T. Nguyen, C. Erikkson, L. Wang, P. Ottoson, P. Persson, T. Delaney, S. Lehar, et al. 1999. Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. *J. Exp. Med.* 190: 895–902.
21. Lohning, M., A. Stroehmann, A. J. Coyle, J. L. Grogan, S. Lin, J. C. Gutierrez-Ramos, D. Levinson, A. Radbruch, and T. Kamradt. 1998. T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc. Natl. Acad. Sci. USA* 95: 6930–6935.
22. Sakamoto, M., S. Ida, and T. Takishima. 1984. Effect of influenza virus infection on allergic sensitization to aerosolized ovalbumin in mice. *J. Immunol.* 132: 2614–2617.
23. Suzuki, S., Y. Suzuki, N. Yamamoto, Y. Matsumoto, A. Shirai, and T. Okubo. 1998. Influenza A virus infection increases IgE production and airway responsiveness in aerosolized antigen-exposed mice. *J. Allergy Clin. Immunol.* 102: 732–740.
24. Yamamoto, N., S. Suzuki, A. Shirai, M. Suzuki, M. Nakazawa, Y. Nagashima, and T. Okubo. 2000. Dendritic cells are associated with augmentation of antigen sensitization by influenza A virus infection in mice. *Eur. J. Immunol.* 30: 316–326.
25. Yamamoto, N., S. Suzuki, Y. Suzuki, A. Shirai, M. Nakazawa, M. Suzuki, T. Takamasu, Y. Nagashima, M. Minami, and Y. Ishigatsubo. 2001. Immune response induced by airway sensitization after influenza A virus infection depends on timing of antigen exposure in mice. *J. Virol.* 75: 499–505.
26. Tsitoura, D. C., S. Kim, K. Dabbagh, G. Berry, D. B. Lewis, and D. T. Umetsu. 2000. Respiratory infection with influenza A virus interferes with the induction of tolerance to aeroallergens. *J. Immunol.* 165: 3484–3491.
27. Brimnes, M. K., L. Bonifaz, R. M. Steinman, and T. M. Moran. 2003. Influenza virus-induced dendritic cell maturation is associated with the induction of strong T cell immunity to a coadministered, normally nonimmunogenic protein. *J. Exp. Med.* 198: 133–144.
28. Dahl, M. E., K. Dabbagh, D. Liggitt, S. Kim, and D. B. Lewis. 2004. Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nat. Immunol.* 5: 337–343.
29. Wohlleben, G., J. Muller, U. Tatsch, C. Hambrecht, U. Herz, H. Renz, E. Schmitt, H. Moll, and K. J. Erb. 2003. Influenza A virus infection inhibits the efficient recruitment of Th2 cells into the airways and the development of airway eosinophilia. *J. Immunol.* 170: 4601–4611.
30. Cates, E. C., R. Fattouh, J. R. Johnson, A. Llop-Guevara, and M. Jordana. 2007. Modeling responses to respiratory house dust mite exposure. *Contrib. Microbiol.* 14: 42–67.
31. Thomas, W. R., B. J. Hales, and W. A. Smith. 2005. Structural biology of allergens. *Curr. Allergy Asthma Rep.* 5: 388–393.
32. Thomas, W. R., W. A. Smith, B. J. Hales, K. L. Mills, and R. M. O'Brien. 2002. Characterization and immunobiology of house dust mite allergens. *Int. Arch. Allergy Immunol.* 129: 1–18.
33. Marsland, B. J., N. L. Harris, M. Camberis, M. Kopf, S. M. Hook, and G. Le Gros. 2004. Bystander suppression of allergic airway inflammation by lung resident memory $CD8^+$ T cells. *Proc. Natl. Acad. Sci. USA* 101: 6116–6121.
34. Rissoan, M. C., V. Soumelis, N. Kadowaki, G. Grouard, F. Briere, R. de Waal Malefyt, and Y. J. Liu. 1999. Reciprocal control of T helper cell and dendritic cell differentiation. *Science* 283: 1183–1186.
35. Matsuda, H., T. Suda, H. Hashizume, K. Yokomura, K. Asada, K. Suzuki, K. Chida, and H. Nakamura. 2002. Alteration of balance between myeloid dendritic cells and plasmacytoid dendritic cells in peripheral blood of patients with asthma. *Am J. Respir. Crit. Care Med.* 166: 1050–1054.
36. Jahnsen, F. L., F. Lund-Johansen, J. F. Dunne, L. Farkas, R. Haye, and P. Brandtzaeg. 2000. Experimentally induced recruitment of plasmacytoid ($CD123^{high}$) dendritic cells in human nasal allergy. *J. Immunol.* 165: 4062–4068.
37. Bratke, K., M. Lommatzsch, P. Julius, M. Kuepper, H. D. Kleine, W. Luttmann, and J. Christian Virchow. 2007. Dendritic cell subsets in human bronchoalveolar lavage fluid after segmental allergen challenge. *Thorax* 62: 168–175.
38. Kurowska-Stolarska, M., P. Kewin, G. Murphy, R. C. Russo, B. Stolarski, C. C. Garcia, M. Komai-Koma, N. Pitman, Y. Li, A. N. McKenzie, et al. 2008. IL-33 induces antigen-specific $IL\text{-}5^+$ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J. Immunol.* 181: 4780–4790.
39. Kash, J. C., C. F. Basler, A. Garcia-Sastre, V. Carter, R. Billharz, D. E. Swayne, R. M. Przygodzki, J. K. Taubenberger, M. G. Katze, and T. M. Tumpey. 2004. Global host immune response: pathogenesis and transcriptional profiling of type A influenza viruses expressing the hemagglutinin and neuraminidase genes from the 1918 pandemic virus. *J. Virol.* 78: 9499–9511.
40. Kash, J. C., T. M. Tumpey, S. C. Proll, V. Carter, O. Perwitasari, M. J. Thomas, C. F. Basler, P. Palese, J. K. Taubenberger, A. Garcia-Sastre, et al. 2006. Genomic analysis of increased host immune and cell death responses induced by 1918 influenza virus. *Nature* 443: 578–581.
41. James, A. L., and S. Wenzel. 2007. Clinical relevance of airway remodelling in airway diseases. *Eur. Respir. J.* 30: 134–155.
42. McParland, B. E., P. T. Macklem, and P. D. Pare. 2003. Airway wall remodeling: friend or foe? *J. Appl. Physiol.* 95: 426–434.
43. Bates, J., C. Irvin, V. Brusasco, J. Drazen, J. Fredberg, S. Loring, D. Eidelman, M. Ludwig, P. Macklem, J. Martin, et al. 2004. The use and misuse of Penh in animal models of lung disease. *Am. J. Respir. Cell Mol. Biol.* 31: 373–374.
44. Adler, A., G. Cieslewicz, and C. G. Irvin. 2004. Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice. *J. Appl. Physiol.* 97: 286–292.
45. Bates, J. H. 2007. Point:counterpoint: lung impedance measurements are/are not more useful than simpler measurements of lung function in animal models of pulmonary disease. *J. Appl. Physiol.* 103: 1900–1901; discussion 1904.

Online Data Supplement

ACUTE, BUT NOT RESOLVED, INFLUENZA A INFECTION ENHANCES SUSCEPTIBILITY TO HOUSE DUST MITE INDUCED ALLERGIC DISEASE

Amal A. Al-Garawi, Ramzi Fattouh, Tina D. Walker, Erin B. Jamula, Fernando Botelho, Susanna Goncharova, Jennifer Reed, Martin R. Stampfli, Paul M. O'Byrne, Anthony J. Coyle and Manel Jordana.

Figure Legends

Figure 1S: *Kinetics of influenza A/PR8 induced BAL inflammation:*

Mice were infected intranasally with 10 PFU PR8 virus and the inflammatory response evaluated at days 0, 1, 3, 5, 7, 10, 28, and 39 post infection. Figure showing total cells in BALF. One of two representative experiments shown. Data represent mean ± SEM. **P*<0.05 compared with day 0. Dunnets post hoc test.

Figure 2S: *APC gating strategy.*

Groups of mice were infected with 10 PFU PR8 virus or PBS. Total lung cells were isolated at day 7 and 10 p.i. and stained with MHCII, CD11b, CD11c, B220 and GR1 cell surface markers. In all plots single cells were identified on the basis of FSC-W and FSC-A. (A) Shows the number of $MHCII^{+}$ cells in the lung at various time points post flu infection. (B) Shows the gating strategy used to identify four different APC populations, B cells, plasmacytoid DCs (pDC), myeloid DCs (DC) and macrophages (MACS). To identify these populations, single cells were first gated on MHCII+ and then differentiated on the basis of B220, CD11b, CD11c, expression as shown. More than 300,000 events were collected for each group Shaded histogram shows isotype control for CD11b. Data represent mean ± SEM and **P*< 0.05 compared to day 0.

Figure 3S: *CD69 expression on CD8 T cells in flu infected animals exposed to HDM.* Groups of mice were infected with 10 PFU PR8 virus or PBS. Animals were exposed to 5ug of HDM for 10days starting at day 7 p.i. and sacrificed three days after last exposure

at day 19. Total lung cells were isolated and stained with CD45, CD3, CD4, CD8 and the activation marker CD69 and analyzed by flow cytometry. Cells were gated on CD45/CD3 expression and the absolute number of cells expressing CD8 and CD69 quantified. n=5 mice/group. Data represent mean ± SEM. $P< 0.005$ compared to †HDM and *SAL.

Figure 4S: *Eosinophil quantification using flow cytometric analysis.*

Whole leukocytes were first selected by gating on CD45+ cells in order to exclude non-leukocytes, then single cells were selected on the basis of FSC-W and FSC-A. Three distinct populations were subsequently selected, on the basis of FSC and SSC, and sorted. Cytospins were prepared for each sorted population and differential cell counts were performed using standard hemocytological criteria to classify the cells as neutrophils (NEUT), eosinophils (EOS), monocytes/macrophages (MONO/MACS) or lymphocytes (Lymph).

Figure 1S

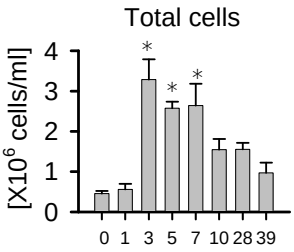


Figure 2S:

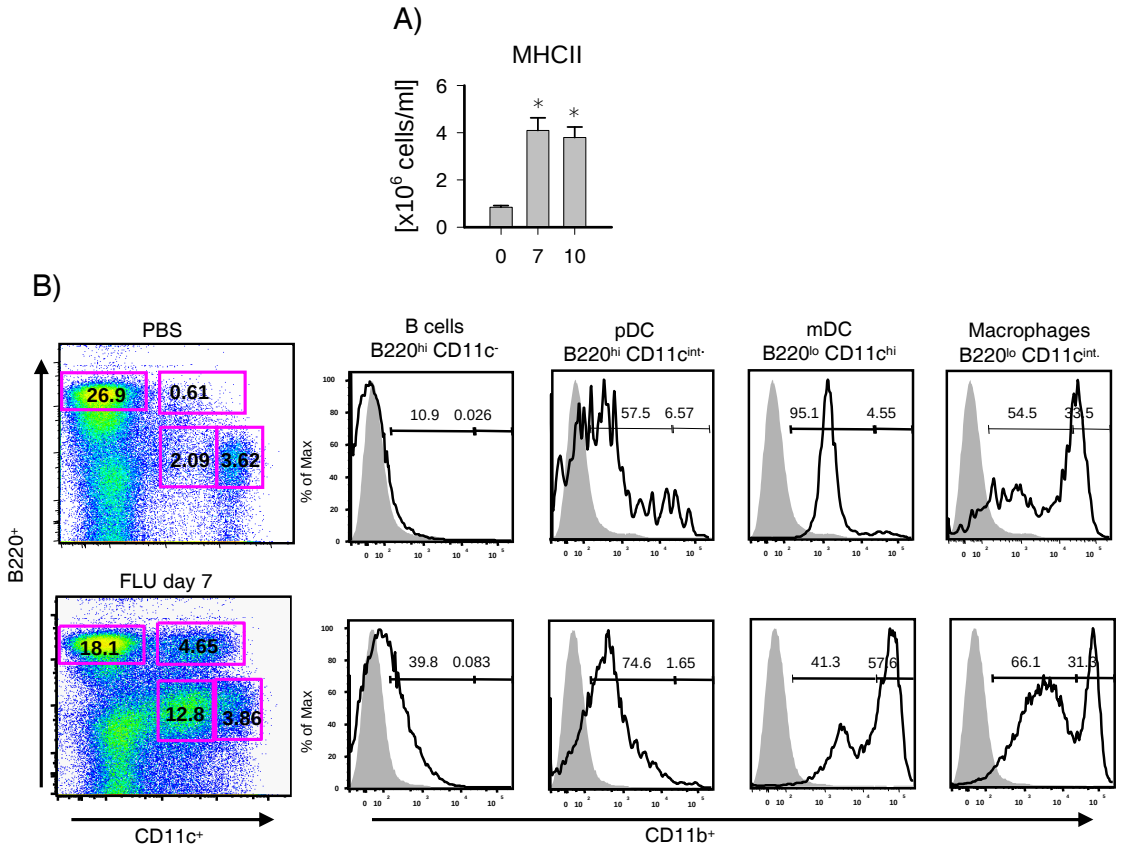

A)
MHCII
[x10^6 cells/ml]
6
4
2
0
0 7 10
B)
PBS
26.9
0.61
2.09
3.62
FLU day 7
18.1
4.65
12.8
3.86
B220+
CD11c+
% of Max
B cells
B220hi CD11c-
10.9 0.026
pDC
B220hi CD11cint.
57.5 6.57
mDC
B220lo CD11chi
95.1 4.55
Macrophages
B220lo CD11cint.
54.5 33.5
39.8 0.083
74.6 1.65
41.3 57.6
66.1 31.8
CD11b+

Figure 3S

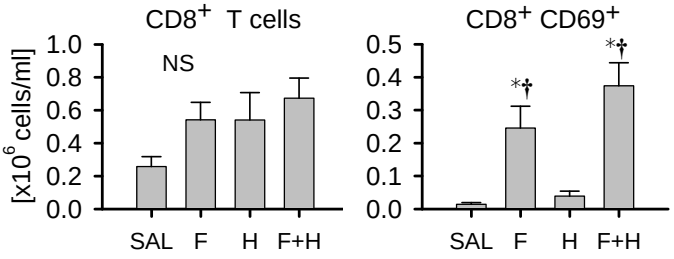
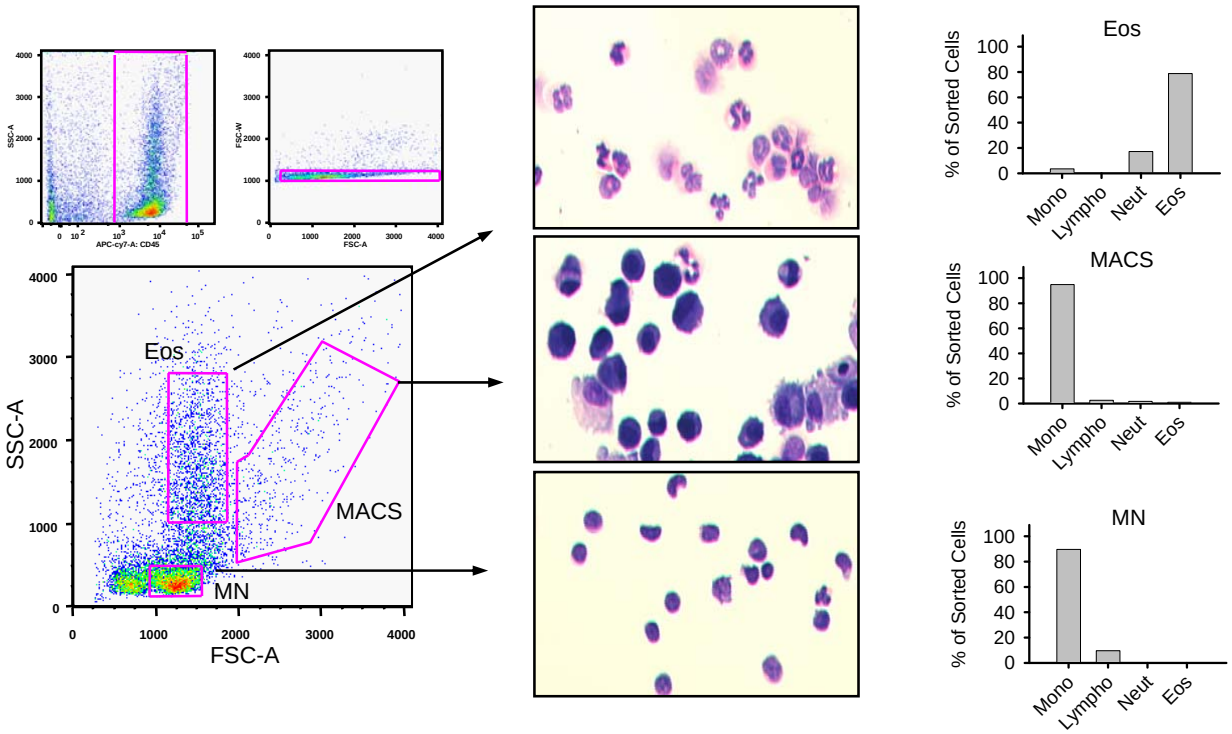

CD8+ T cells
NS
[x10^6 cells/ml]
1.0
0.8
0.6
0.4
0.2
0.0
SAL F H F+H
CD8+ CD69+
*†
*†
0.5
0.4
0.3
0.2
0.1
0.0
SAL F H F+H

Figure 4S


SSC-A
FSC-W
APC-cy7-A: CD45
FSC-A
Eos
MACS
MN
SSC-A
FSC-A
Eos
% of Sorted Cells
Mono
Lympho
Neut
Eos
MACS
% of Sorted Cells
Mono
Lympho
Neut
Eos
MN
% of Sorted Cells
Mono
Lympho
Neut
Eos

6

Chapter 3

SHIFTING OF IMMUNE RESPONSIVENESS TO HOUSE DUST MITE BY INFLUENZA A INFECTION: GENOMIC INSIGHTS

Amal Al-Garawi[*§], Mainul Husain[*§], Dora Ilieva[*], Alison A. Humbles[†], Roland Kolbeck[†], Martin R. Stampfli[*], Paul M. O'Byrne[‡], Anthony J. Coyle[*§§] and Manel Jordana[*, 1].

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Understanding the underlying mechanisms associated with complex viral-allergen interactions are key to developing effective intervention strategies that can protect against the development of allergic disease. To better appreciate the complexity of the interactions between influenza A and HDM established in the first study, we used genome-wide transcriptional profiling to uncover immune pathways by which influenza A enhances the response to subsequent HDM exposure. There are currently no studies that have examined global changes in gene expression during influenza A and HDM interaction. Our data reveal that influenza infection led to a pervasive upregulation of genes associated with the general response to stimuli and stress and markedly increased the expression of multiple gene classes capable of sensing allergens and amplifying the ensuing immune-inflammatory response. Importantly, the context of prior influenza A infection exposure to a low dose of HDM resulted in the expression of hundreds of unique genes, absent in mice exposed to HDM only. Thus, these data demonstrate that influenza A infection primes the lung environment in ways that are conducive to lowering the threshold of allergen responsiveness, thus facilitating the emergence of a clinically significant allergic phenotype.

The Journal of Immunology

Shifting of Immune Responsiveness to House Dust Mite by Influenza A Infection: Genomic Insights

Amal Al-Garawi,[*,†,1] Mainul Husain,[*,†,1] Dora Ilieva,[*,†] Alison A. Humbles,[‡] Roland Kolbeck,[‡] Martin R. Stampfli,[*,†] Paul M. O'Byrne,[§] Anthony J. Coyle,[*,†,¶] and Manel Jordana[*,†]

Respiratory viral infections have been associated with an increased incidence of allergic asthma. However, the mechanisms by which respiratory infections facilitate allergic airway disease are incompletely understood. We previously showed that exposure to a low dose of house dust mite (HDM) resulted in enhanced HDM-mediated allergic airway inflammation, and, importantly, marked airway hyperreactivity only when allergen exposure occurred during an acute influenza A infection. In this study, we evaluated the impact of concurrent influenza infection and allergen exposure at the genomic level, using whole-genome microarray. Our data showed that, in contrast to exposure to a low dose of HDM, influenza A infection led to a dramatic increase in gene expression, particularly of TLRs, C-type lectin receptors, several complement components, as well as FcεR1. Additionally, we observed increased expression of a number of genes encoding chemokines and cytokines associated with the recruitment of proinflammatory cells. Moreover, HDM exposure in the context of an influenza A infection resulted in the induction of unique genes, including calgranulin A (*S100a8*), an endogenous damage-associated molecular pattern and TLR4 agonist. In addition, we observed significantly increased expression of serum amyloid A (*Saa3*) and serine protease inhibitor 3n (*Serpina3n*). This study showed that influenza infection markedly increased the expression of multiple gene classes capable of sensing allergens and amplifying the ensuing immune-inflammatory response. We propose that influenza A infection primes the lung environment in such a way as to lower the threshold of allergen responsiveness, thus facilitating the emergence of a clinically significant allergic phenotype. ***The Journal of Immunology*****, 2012, 188: 832–843.**

Allergic asthma is a chronic immune-inflammatory disease of the airways that occurs following sensitization to common aeroallergens, such as house dust mite (HDM), the most ubiquitous indoor aeroallergen worldwide. However, despite universal exposure, only ~20% of the population develops the disease (1). This suggests that the natural response to allergens is immunologic homeostasis and that additional factors contribute to triggering aberrant immune responses to these allergens (2). In addition to a genetic predisposition, environmental factors, such as respiratory viral infections, have been implicated with the clinical expression of allergic airway inflammation (3, 4). In this regard, there is abundant evidence in humans of an association between viral infections and the expression of asthma (5, 6). In mice, we previously reported that exposure to a concentration of HDM, which by itself elicits negligible airway inflammation and no changes in lung function, results in a phenotype characterized by robust allergic airway inflammation, enhanced mucus production, and marked lung dysfunction in the context of an acute influenza A virus (Flu) infection (7).

In this study, we used genome-wide transcriptional profiling to investigate the nature of the Flu-induced environment in the lung, by examining global gene expression during the early phase (EP; 4 d) and late phase (LP; 7 d) of HDM exposure. We found that exposure to a low concentration of HDM alone elicited minimal alterations in the gene profile, whereas Flu infection led to a pervasive upregulation of genes associated with the general response to stimuli and stress. In particular, Flu infection dramatically increased the expression of a number of cell surface receptors, notably TLRs, C-type lectin receptors (CLRs), and FcRs, and a prolific number of chemokines and chemokine receptors. Interestingly, exposure to HDM in the context of this Flu-induced environment led to the increased expression of several hundred genes, which were not expressed in mice exposed to allergen alone. These data suggested that Flu lowered the threshold of HDM responsiveness by establishing a global, heightened state of immune sensing in the lung and launching multiple pathways involved in inflammatory responses to exogenous Ags.

*Division of Respiratory Diseases and Allergy, Center for Gene Therapeutics, McMaster University, Hamilton, Ontario L8S 4K1, Canada; [†]Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario L8S 4K1, Canada; [‡]Department of Respiratory, Inflammation and Autoimmunity, MedImmune LLC, Gaithersburg, MD 20878; [§]Department of Medicine, McMaster University, Hamilton, Ontario L8S 4K1, Canada; and [¶]Centers for Therapeutic Innovation, Pfizer Inc., Cambridge, MA 02138

[1]A.A.-G. and M.H. contributed equally to this work.

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The sequences presented in this article have been submitted to the European Molecular Biology Laboratory-European Bioinformatics Institute ArrayExpress database (http://www.ebi.ac.uk/microarray-as/ae) under accession number E-MEXP-3325.

Address correspondence and reprint requests to Dr. Manel Jordana, Division of Respiratory Diseases and Allergy and Department of Pathology and Molecular Medicine, McMaster University, 1280 Main Street West, Hamilton, Ontario L8S 4K1, Canada. E-mail address: jordanam@mcmaster.ca

The online version of this article contains supplemental material.

Abbreviations used in this article: CLR, C-type lectin receptor; DC, dendritic cell; EP, early phase; Flu, influenza A virus; Flu+HDM, house dust mite in the context of influenza A virus infection; GO, gene ontology; HDM, house dust mite; LP, late phase; qPCR, quantitative real-time PCR.

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Materials and Methods

Animals

Female BALB/c mice (6–8 wk old) were purchased from Charles River Laboratories (Saint-Constant, QC, Canada). The mice were housed under specific pathogen-free conditions and maintained on a 12-h light-dark cycle, with food and water ad libitum. All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

Flu and infection protocol

Flu strain A/PR/8/34 (H1N1) was prepared, as described previously (8), and kindly provided by MedImmune. The viral stock suspension (10^9 PFU/ml) was diluted, and 10 PFU was administered intranasally to isoflurane-anesthetized BALB/c mice in 35 μl sterile PBS solution. Animals were monitored for signs of illness twice daily for 10 d following infection.

Sensitization protocols

Allergen administration. HDM extract (Greer Laboratories, Lenoir, NC) was resuspended in sterile PBS at a concentration of 0.5 mg (protein)/ml, and 10 μl (5-μg dose) was administered to isoflurane-anesthetized mice intranasally. Groups of mice were infected with Flu or exposed to PBS. Seven days later, separate groups of mice were exposed to saline or HDM for 3 or 6 d, and lungs were harvested 24 h after the last exposure, at day 4 (EP) or day 7 (LP) (Fig. 1).

RNA isolation and hybridization of Affymetrix GeneChip array

Lungs were harvested 24 h after the last allergen challenge, snap-frozen in liquid nitrogen, and stored at −80°C until further processing. Total RNA was extracted using RNA-STAT60 reagent (Tel-Test, Friendwood, TX), as per the manufacturer's protocol. The extracted total RNA was further purified using an RNeasy Mini Kit (QIAGEN, Valencia, CA), and quality was assessed with the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). Preparation of wild-type sense cDNA targets, hybridization to Affymetrix Mouse Gene 1.0 ST arrays (Affymetrix, Santa Clara, CA), and scanning were performed according to standard Affymetrix protocols. In brief, 100 ng total RNA from each sample was used for the synthesis of dsDNA with random hexamers tagged with a T7 promoter sequence. The double-stranded cDNA was subsequently used as template and amplified by T7 RNA polymerase, producing many copies of antisense cRNA. In the second cycle of cDNA synthesis, random hexamers were used to prime reverse transcription of the cRNA from the first cycle to produce ssDNA in the sense orientation with incorporated deoxyuridine triphosphate. Fragmentation of the ssDNA was performed using a combination of uracil DNA glycosylase and apurinic/apyramidine endonuclease 1 that breaks DNA at deoxyuridine triphosphate residues. Labeling of fragmented DNA by terminal deoxynucleotidyl transferase with the Affymetrix DNA labeling reagent covalently linked to biotin was used in the final step of target preparation. Fragmented and biotin-labeled cDNA was hybridized at 45°C for 17 h to Affymetrix Mouse Gene 1.0 ST arrays. The arrays were washed and stained with streptavidin-PE, followed by signal amplification with a biotinylated anti-streptavidin Ab. The arrays were scanned according to the manufacturer's instructions.

Processing of image files and gene-expression analysis

Gene-expression measurements were generated from quantified Affymetrix image files ("*CEL" files) using the robust multiarray analysis algorithm (9) and GeneSpring 10.0 software (Agilent Technologies). All 21 CEL files were analyzed simultaneously with quantile normalization and median polish probe summarization using the PBS samples as a baseline (control). Transcripts with expression levels in the first quantile were filtered out to remove noise from downstream statistical analyses. One-way ANOVA was applied to the filtered and log2-transformed probe sets. A Tukey post hoc test was applied successively to identify transcripts with a statistically significant expression among the treatment groups. Genes were defined as differentially expressed if they had fold changes of at least ±1.5, with p values ≤ 0.05. This selection criterion includes the maximum number of differentially expressed genes used to identify biologically relevant gene families and pathways. Functional annotations of the differentially expressed genes were identified using NetAffx (Affymetrix) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (10, 11). Gene ontology (GO) analysis of the differentially expressed genes was performed using GoMiner software (12), and GO biological processes that had a Fisher exact p value < 0.05 (false discovery rate corrected; $p < 0.05$) were considered significantly enriched. Microarray data files from this study are available at European Molecular Biology Laboratory-European Bioinformatics Institute ArrayExpress database (http://www.ebi.ac.uk/microarray-as/ae). ArrayExpress accession number for these files is E-MEXP-3325.

Quantitative real-time PCR for gene validation

RNA was quantified and normalized, and RNA integrity was assessed by Agilent Bioanalyzer. cDNA was generated using the Super Script III Reverse Transcriptase kit (Life Technologies, Carlsbad, CA), according to the manufacturer's instructions. Relative transcript expression assay was conducted, as described previously (13), using the Fluidigm Biomark system (Fluidigm, San Francisco, CA). BestKeeper (version 1) (14) was used to identify the stably expressed housekeeping gene to be used as an internal reference. Among the four housekeeping genes, β-actin (*Actb*), β-2-microglobulin (*B2m*), *Gapdh*, and hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), *Hprt1* emerged as the most stably expressed and, thus, was selected for normalizing genes of interest. Data analysis of cycle threshold values was conducted using the Relative Expression Software Tool-384 (REST-384) version 1, and the pair-wise fixed reallocation randomization test was performed to determine the fold changes and statistical significance (15).

Results

Impact of Flu infection and HDM exposure on global gene-expression profiles

To investigate the global impact of exposure to a low dose of HDM, Flu, and HDM in the context of Flu infection (Flu+HDM) on gene-expression profiles (Fig. 1), we conducted a principal component analysis, which allows visualization of the effects of multiple treatments on gene-expression profiles. To this end, the analysis included all genes from all arrays without any prior filtering. Our analysis revealed the differential effects of HDM exposure, Flu infection, or Flu+HDM coexposure, during the EP and LP of the response, on gene expression by placing them along the planes of the x-, y-, and z-axes (Fig. 2). Our data showed that EP and LP HDM treatment falls in close proximity to the PBS control group along the x- and z-axes, thus suggesting that exposure to such a low dose of HDM (5 μg) for 3 or 6 d had only minimal effects

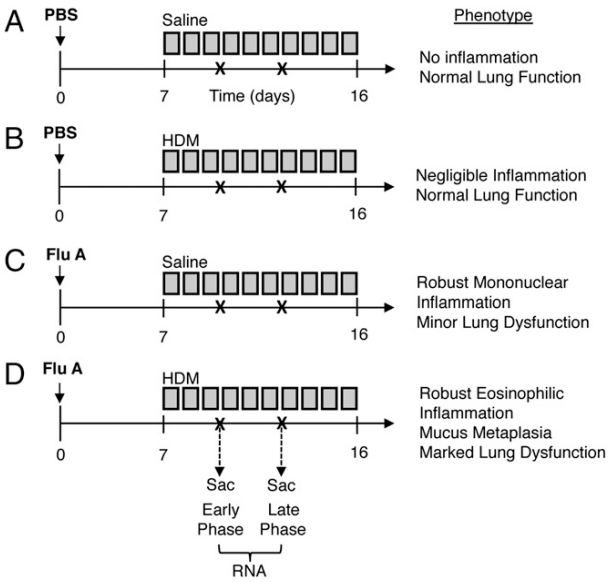

FIGURE 1. Experimental plan. *A–D*, Separate groups of mice were infected with Flu or exposed to PBS. Seven days later, mice were either exposed to saline or HDM for 10 d, resulting in four treatment groups: PBS (*A*), HDM (*B*), Flu (*C*), and Flu+HDM (*D*) (7). Lungs were harvested, and RNA was isolated 24 h after the third or sixth dose of HDM at day 4 or 7, representing the EP or LP response, respectively.

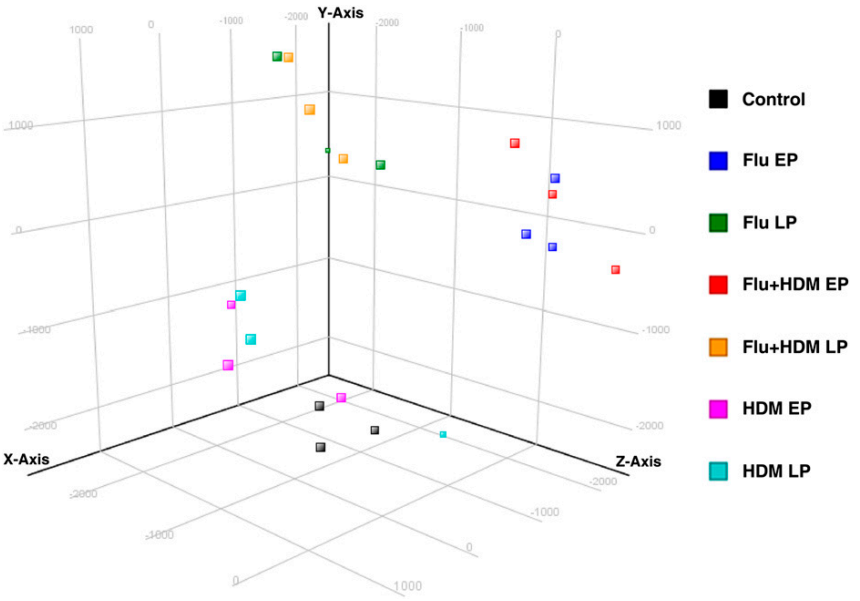

FIGURE 2. Principal component analysis evaluating the impact of treatment and time on gene expression. Separate groups of mice were either infected with Flu or exposed to PBS; 7 d later, they were exposed to 5 μg HDM or treated with saline for 3 or 6 d. Gene-expression profiles were evaluated 24 h after the last exposure, at day 4 (EP) or 7 (LP) (n = 3 mice/group).

on gene expression. In contrast, Flu LP and Flu+HDM LP groups clustered together along the y-axis, farthest from the x–z plane; similarly, the expression profile during the EP of Flu and Flu+HDM clustered together along the y–z plane, indicating that Flu infection and Flu+HDM coexposure during EP or LP had similar effects on lung gene-expression profiles, which is distinct from that observed in the lungs of mice exposed to PBS or HDM. Interestingly, the genes expressed during the EP in Flu and Flu+HDM groups were clustered apart from those expressed during the LP, highlighting the impact of time on gene expression in these two treatment groups.

To quantify the number of genes differentially regulated in response to HDM, Flu, or Flu+HDM, each individual treatment group was compared with PBS controls. Genes with a differential regulation of at least ±1.5-fold changes ($p \leq 0.05$) were considered statistically significant. Using this selection criterion, we identified a total of 51 differentially expressed genes in mice exposed to HDM only during the EP (Fig. 3*A*). In contrast, we identified a total of 1443 genes in mice infected with Flu only at the same time point. Exposure to Flu+HDM led to the regulation of a total of 1592 genes compared with PBS controls. However, a majority of the genes regulated in the Flu+HDM group (1235 genes) were also expressed in mice infected with Flu only (common genes), whereas 330 genes were only expressed in the Flu+HDM group. Heretofore, we refer to these 330 genes as "unique genes" (Fig. 3*A*). During the LP of the response, representing the time point at which Flu infection enters into the resolving phase (i.e., 14 d after the initial infection), the number of differentially expressed genes was reduced to 1378 in the Flu-only group and 1362 in the Flu+HDM-treated group. In addition, during the LP, Flu and Flu+HDM groups shared 1038 genes, whereas a total of 240 genes was uniquely expressed in the Flu+HDM group (Fig. 3*B*).

Evaluation of differentially expressed genes

To gain an appreciation of the various functions associated with the dramatic increase in differentially expressed genes following Flu and Flu+HDM exposure, we next performed a GO analysis and compared the biological profiles between these two groups (Fig. 4). Our data showed that, during both the EP and LP of the response, Flu- and Flu+HDM-treated mice have similar profiles with respect to various GO biological processes (Fig. 4). Interestingly, at least five of these GO processes, including "response to stimulus" (GO:0050896), "response to stress" (GO:0006950), "response to wounding" (GO:0009611), "inflammatory response" (GO:0006954), and "chemotaxis" (GO:0006935) were enriched with a greater number of genes in the Flu+HDM-treated group compared with the Flu-alone–treated group in both EP and LP; specifically, the "response to stimulus" process was enriched with 265 and 218 genes during EP and LP, respectively, in mice infected with Flu only, whereas in the Flu+HDM group this same process was enriched with 283 (EP) and 243 (LP) genes. To gain greater insight into the biological significance of these diverse processes, we identified specific genes associated with these functional groups. Our analysis revealed that these biological processes are associated with the expression of genes encoding complement components and their receptors (Table I) or various C-type lectins (including CLRs), FcRs, and TLRs (Table II). In addition, we identified genes encoding chemokines and chemokine receptors (Table III), as well as various cytokines and cytokine receptors (Table IV). The majority of these genes was found to be upregulated in both Flu and Flu+HDM groups, during both the EP and LP responses.

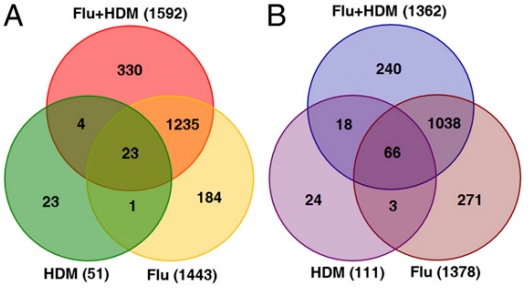

FIGURE 3. Venn analysis of differentially expressed genes. Separate groups of mice were either infected with Flu or exposed to PBS; 7 d post-infection, they were either exposed to 5 μg HDM or saline treated for 3 or 6 d. Gene-expression profiles were evaluated 24 h after the last exposure, at day 4 (EP) or 7 (LP). Venn diagram showing number of common and unique genes expressed following Flu, HDM, and Flu+HDM treatment during EP (*A*) and LP (*B*) (n = 3 mice/group). Genes were filtered based on fold change (≥1.5). $p < 0.05$, one-way ANOVA, Tukey post hoc test.

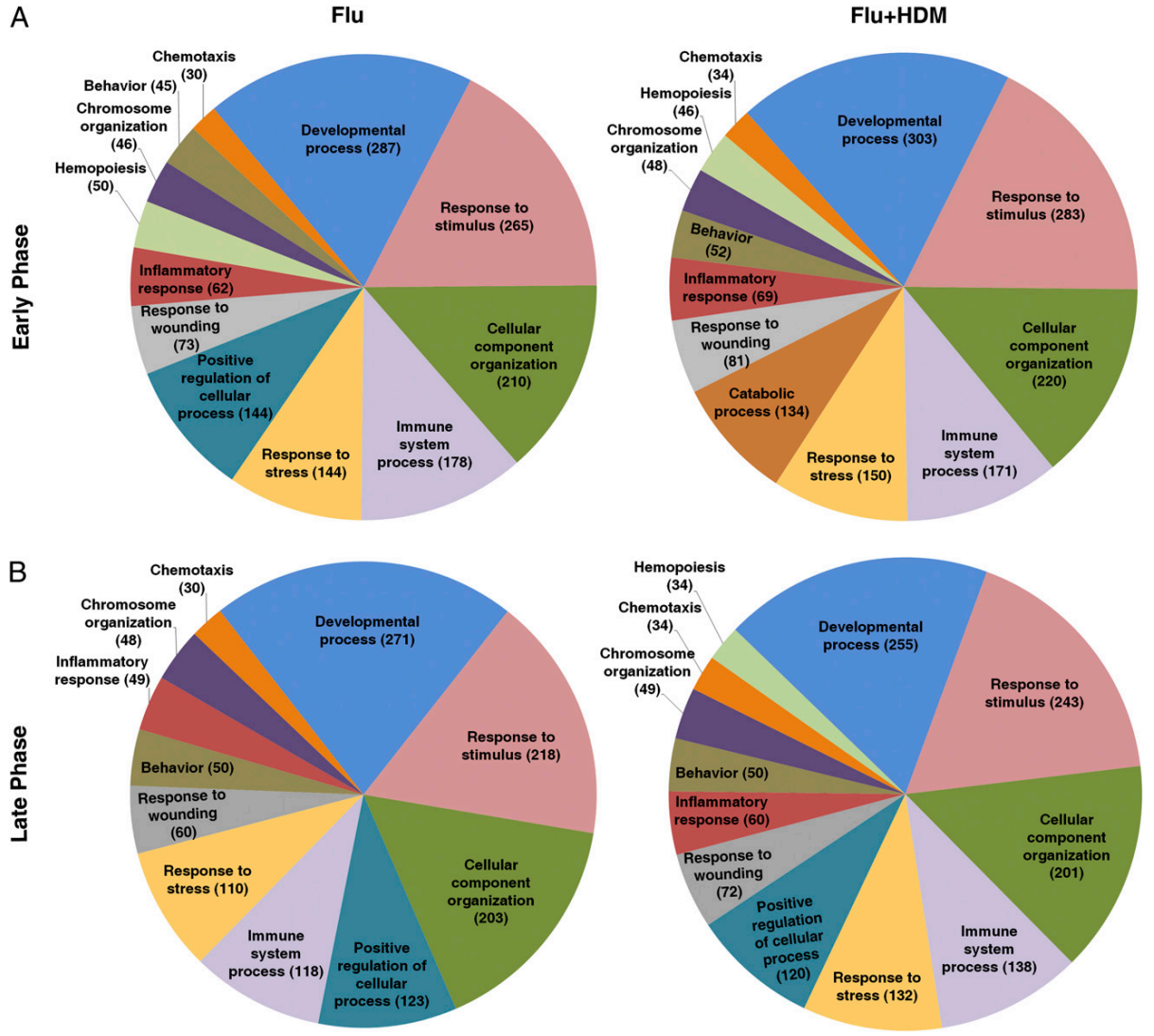

FIGURE 4. GO biological processes in mice infected with Flu and exposed to HDM. GO biological processes enriched with genes from Flu and Flu+HDM during EP (*A*) and LP (*B*) response. Numbers in parentheses represent the number of genes enriching each process (n = 3 mice/group). $p <$ 0.05, Fisher exact test (false discovery rate corrected).

Impact of HDM exposure on gene expression during ongoing Flu infection

In addition to the 330 and 240 uniquely expressed genes in the Flu+HDM-treated mice during the EP and LP, respectively (Fig. 3), we selected genes whose expression was shared among the three treatment groups but that exhibited additional regulation in response to HDM when allergen exposure occurred during ongoing Flu infection. A selection criterion of a 1.5-fold difference in expression was applied to the 1262 commonly expressed genes in the EP that resulted in the inclusion of 34 genes shared between Flu and Flu+HDM groups and 2 genes shared among all three treatments (HDM, Flu, and Flu+HDM). Collectively, a total of 366 genes were additionally regulated in response to HDM exposure in the context of Flu infection (Supplemental Table I). The same selection criterion was applied to the genes commonly expressed in the LP, which resulted in a total of 263 genes (240 were unique to Flu+HDM; 11 were common among all three treatments; 8 were common among Flu and Flu+HDM; 4 were common among HDM and Flu+HDM) (Supplemental Table I). According to available functional annotations, these genes could be classified into 13 major functional categories. Functional annotations for 146 genes from EP and 86 genes from LP are not available and, thus, were classified in the "other" category (Table V).

To further understand the contribution of immune genes in regulating the response to HDM during acute viral infection, we examined in more detail those genes that were part of the "immune/inflammatory response" category. Our data identified 32 genes in the EP, of which 8 genes were shared between Flu and Flu+HDM (Table VI), and 46 genes expressed during the LP, of which 7 genes were shared between these two treatment groups (Table VII). These shared genes showed additional regulation to HDM exposure, resulting in additional fold increases in expression levels following HDM exposure in mice infected with influenza (Flu+HDM) compared with those infected with influenza only. Importantly, for the majority of these immune/inflammatory-responsive genes, the expression levels were up-

Table I. Differentially expressed genes associated with complement pathway during EP and LP responses after exposure to HDM, Flu, or Flu+HDM

		EP			LP		
Gene Symbol	Gene Name	HDM	Flu	F+H	HDM	Flu	F+H
C3ar1	Complement component 3a receptor 1	–	8.66	8.71	1.56	4.47	3.71
C1qc	Complement component 1, q C chain	–	5.73	6.59	–	5.46	4.70
C1qb	Complement component 1, q, β polypeptide	–	6.11	5.67	1.53	4.30	4.52
C1qa	Complement component 1, q, α polypeptide	–	5.17	4.91	–	4.26	3.76
Cfb	Complement factor B	−1.52	3.55	3.68	–	–	1.69
C5ar1	Complement component 5a receptor 1	–	–	1.89	–	–	–
C1rb	Complement component 1, r	–	1.74	1.88	–	–	–
C4b|C4a	Complement component 4B /4A	–	1.70	1.80	–	–	–
C1s	Complement component 1, s	–	2.36	1.80	–	–	–
C2	Complement component 2 (within H-2S)	–	–	1.78	–	–	–
C1qbp	Complement component 1, q binding protein	–	–	1.59	–	1.84	1.79
C1r	Complement component 1, r	–	–	1.56	–	–	–
Cr2	Complement receptor 2	–	–	−1.68	–	–	–
C1qtnf7	C1q and TNF related protein 7	–	−1.71	−2.16	–	−1.72	−1.67
Cfd	Complement factor D (adipsin)	–	−2.12	−2.38	−2.78	−1.60	−2.12
C7	Complement component 7	–	–	–	–	−2.24	−1.75
C3	Complement component 3	–	1.51	–	1.74	–	1.55
Cfh	Complement component factor h	–	–	–	–	−1.83	–

Values represent mean fold changes compared with PBS (n = 3 mice/group).
$p < 0.05$, one-way ANOVA, Tukey post hoc test.
–, Genes that showed no significant regulation in that treatment group; F+H, Flu+HDM.

regulated and, for only a few of these genes (11 genes in EP; 4 genes in LP), the levels were downregulated. We identified serum amyloid A (*Saa3*), tissue inhibitor of metalloproteinase 1 (*Timp1*), and serine protease inhibitor A3 (*Serpina3n*) among those genes that were additionally upregulated by HDM exposure during EP and LP, whereas S100 calcium-binding protein A8 (*S100a8*) was one of the uniquely expressed genes that emerged during the EP.

Gene validation by quantitative real-time PCR

To validate the gene-expression data obtained by microarray analysis, we examined gene-expression levels for a number of genes using quantitative real-time PCR (qPCR). To this end, we chose to examine genes with various fold changes across different gene classes as representative samples; these included a selection of genes expressed following Flu infection, as well as representative genes from the "uniquely expressed" list of genes following HDM

Table II. Differential expression of genes encoding cell surface receptors during EP and LP responses after exposure to HDM, Flu, or Flu+HDM

		EP			LP		
Gene Symbol	Gene Name	HDM	Flu	F+H	HDM	Flu	F+H
C-Type Lectins							
Clec12a	C-type lectin domain family 12, a	–	5.56	4.48	–	2.74	2.81
Clec5a	C-type lectin domain family 5, a	–	4.34	4.11	–	2.93	3.75
Clec4a2	C-type lectin domain family 4, a2	–	2.74	3.03	–	1.66	1.81
Clec4a1	C-type lectin domain family 4, a1	–	4.23	2.95	–	1.75	1.97
Clec4a3	C-type lectin domain family 4, a3	–	3.81	2.54	1.63	1.79	1.75
Clec4d	C-type lectin domain family 4, d	–	2.07	2.39	–	1.84	2.38
Clec4n	C-type lectin domain family 4, n	–	2.39	2.10	–	2.81	3.45
Clec7a	C-type lectin domain family 7, a	–	2.63	1.77	–	2.49	2.99
Clec14a	C-type lectin domain family 14, a	–	−2.92	−3.07	–	−4.26	−3.74
Clec1a	C-type lectin domain family 1, a	−1.86	−2.09	–	−2.59	−2.23	–
FcRs							
Fcgr4	FcR, IgG, low affinity IV	–	8.10	8.18	–	2.68	2.56
Fcgr1	FcR, IgG, high affinity I	–	5.42	6.24	–	2.37	2.30
Fcer1g	FcR, IgE, high affinity I, γ	–	3.17	3.80	–	2.44	2.25
Fcgr2b	FcR, IgG, low affinity IIb	–	3.14	3.17	2.12	2.43	3.88
Fcgr3	FcR, IgG, low affinity III	–	2.60	2.26	–	2.04	2.09
Fcer2a	FcR, IgE, low affinity II, α	–	−1.51	−2.20	–	–	–
TLRs							
Tlr13	TLR13	–	3.40	3.70	–	2.05	2.41
Tlr2	TLR2	–	2.02	2.10	–	–	–
Tlr7	TLR7	–	1.81	1.91	–	–	–
Tlr1	TLR1	–	2.15	1.80	–	–	–
Tlr8	TLR8	–	1.88	1.77	–	–	–

Values represent mean fold changes compared with PBS (n = 3 mice/group).
$p < 0.05$, one-way ANOVA, Tukey post hoc test.
–, Genes that showed no regulation in that treatment group; F+H, Flu+HDM.

Table III. Differential expression of genes encoding chemokines and chemokine receptors during EP and LP responses after exposure to HDM, Flu, or Flu+HDM

Gene Symbol			EP			LP		
Chemokines	Common Name	Receptor	HDM	Flu	F+H	HDM	Flu	F+H
Cxcl10	IP-10	CXCR3	–	22.20	23.30	2.26	5.91	7.41
Cxcl9	MIG	CXCR3	–	14.50	12.93	–	7.03	6.64
Ccl8	MCP-2	CCR1, CCR2b, CCR5	–	11.81	9.78	4.37	7.54	6.62
Ccl3	MIP1a	CCR1	–	7.36	7.69	–	2.62	3.78
Ccl12	MCP-5	–	–	5.34	6.12	1.74	2.82	2.82
Cxcl13	BCA-1/BLC	CXCR5	–	5.34	5.57	1.64	3.37	5.62
Ccl2	MCP-1	CCR2	–	5.21	5.46	–	1.64	1.68
Ccl7	MCP-3/MARC	CCR2	–	4.00	5.13	1.61	1.56	1.94
Ccl5	RANTES	CCR5	–	3.32	2.69	–	2.11	1.54
Cxcl16	SRPSOX	CXCR6	–	2.44	2.26	–	1.79	1.83
Cxcl5	ENA-78	CXCR2	–	2.46	2.26	2.02	2.33	4.18
Ccl9	RP-2, CCF18, MIP-1?	CCR1	–	1.67	2.04	1.70	1.72	2.25
Cxcl17	DMC, VCC-1	–	–	–	1.51	–	2.48	2.49
Cx3cl1	Fractalkine	CX3CR1	–	−1.75	−1.55	–	–	–
Ccl21a	6Ckine, Exodus-2	CCR7	1.51	–	–	–	1.69	1.50
Ccl22	MDC	CCR4	–	–	–	–	–	1.58
Ccl11	Eotaxin	CCR2, CCR3, CCR5	–	–	–	2.88	–	1.59
Ccl20	LARC, Exodus-1	CCR6	–	–	–	–	–	1.71
Ccl17	TARC	CCR4	–	–	–	–	1.64	1.81
Ccl6	C10, MRP-2	CCR1	–	–	–	–	1.89	1.90
Ccl19	ELC, Exodus-3	CCR7	2.20	–	–	–	–	–
Receptors	Cellular Expression	Ligands						
Ccr5	DC and memory Th1 cells	CCL2, CCL3, CCL4, CCL5, CCL11, CCL13, CCL14, CCL16	–	9.28	8.78	1.71	3.21	3.56
Cxcr6	–	CXCL16	–	6.54	4.58	–	2.84	3.56
Cxcr3	T, NK, and B cell	CXCL9, CXCL10, CXCL11	–	4.37	4.17	–	2.58	2.14
Ccr2	Monocytes, memory T cells, B cells, basophils, macrophages	CCL2, CCL8, CCL16	–	3.06	2.28	–	–	1.72
Cxcr7	T and B cells	CXCL12	–	–	1.69	–	–	–
Cxcr2	Neutrophils	CXCL1 to CXCL7	–	–	1.63	1.63	–	1.62
Ccrl1	–	CCL19/CCL21/SLC and CCL25/TECK	–	−1.71	−2.07	–	–	−1.75
Cxcr4	Hematopoietic cells	CXCL12	–	−1.68	−2.11	–	−1.73	−1.71
Ccrl2	Neutrophils and monocytes		–		–	–	−1.69	−1.87
Xcr1	–	–	–	2.11	–	–	1.50	1.59
Cx3cr1	–	CX3CL1	–	1.68	–	–	1.83	–

Values represent mean fold changes compared with PBS (n = 3 mice/group).
$p < 0.05$, one-way ANOVA, Tukey post hoc test.
–, Genes that showed no significant regulation in that treatment group; F+H, Flu+HDM.

exposure in the context of an ongoing Flu infection. These included complement component 1, q subcomponent, C chain (*C1qc*); chemokine (CC motif) ligand 7 (*Ccl7*); chemokine (CC motif) ligand 8 (*Ccl8*); C-type lectin domain family 14, member a (*Clec14a*); chemokine (CXC motif) ligand 10 (*Cxcl10*); chemokine (CXC motif) ligand 5 (*Cxcl5*); chemokine (CXC motif) ligand 9 (*Cxcl9*); chemokine (CXC motif) receptor 3 (*Cxcr3*); FcR, IgG, low affinity IV (*Fcgr4*); Ig μ chain V region AC38 205.12 (*IghmAC38.205.12*); *Saa3*; and *Timp1* (Fig. 5). All of these genes demonstrated the same directional fold changes (11 genes upregulated and 1 gene downregulated) analyzed by qPCR as that observed by microarray analysis. *Ccl7*, *Ccl8*, *Cxcl10*, *Cxcl5*, *Saa3*, and *Timp1* from the microarray experiment were found to be upregulated in the HDM-treatment group in the LP only, which was confirmed by qPCR analysis. Furthermore, none of these genes was significantly expressed in the HDM treatment group in the EP, as analyzed either by microarray or qPCR.

Discussion

The conditions under which allergen sensitization and ultimately, allergic inflammation, occur remain to be fully elucidated. HDM, the most ubiquitous aeroallergen worldwide, comprises a biochemically complex mixture of hundreds of protein and nonprotein components that confer immunogenic activities (16). Studies examining mechanisms associated with HDM-induced inflammation have identified various molecular constituents capable of activating innate defense mechanisms at mucosal surfaces (17, 18). However, these studies relied on the administration of concentrations of allergen designed to elicit maximal immune responses. In this regard, we recently reported detailed dose responses to HDM in a mouse system (19). Our data showed that although exposure to a concentration of 25 μg daily elicited near-maximal responses, exposure to 1 μg represented the threshold of visible responsiveness. Although it is exceedingly difficult to determine, on clinical and epidemiological grounds, the precise amount of HDM that humans inhale, it is reasonable to argue that most individuals are exposed to amounts that are insufficient to elicit allergic sensitization and, particularly, allergic asthma. Furthermore, we must be mindful that, in humans, allergen exposure rarely occurs in isolation but, rather, coupled to concurrent exposures to a plethora of chemicals (e.g., pollution) and biological entities, such as respiratory viruses, which can elicit substantial immune changes in the lung. Thus, priming of the immune environment of the lung through environmental coexposures may shift HDM responsiveness and, thus, increase the susceptibility to develop allergic sensitization and, eventually, allergic asthma.

The research we report in this article is based on a previous study in which we described a distinctive asthmatic phenotype in mice

Table IV. Differential expression of genes encoding cytokines and cytokine receptors during EP and LP responses after exposure to HDM, Flu, or Flu+HDM

Gene Symbol	Gene Name	EP			LP		
		HDM	Flu	F+H	HDM	Flu	F+H
Receptors							
Tnfrsf12a	TNFR superfamily, member 12a	–	1.72	2.75	–	1.60	1.50
Il1r2	IL-1R, type II	–	1.61	2.48	–	–	2.14
Il2rb	IL-2R, β-chain	–	2.22	2.43	–	–	–
Il1rn	IL-1R antagonist	–	2.00	1.97	–	1.58	2.03
Tnfrsf9	TNFR superfamily, member 9	–	2.00	1.87	–	–	1.97
Il21r	IL-21R	–	1.90	1.82	–	1.67	1.72
Il2rg	IL-2R, γ-chain	–	2.18	1.55	–	–	1.63
Tnfsf10	TNF (ligand) superfamily, member 10	–	–	−1.72	–	−1.94	−1.63
Il17rd	IL-17R D	–	−1.68	−1.88	–	−1.62	−1.68
Ifngr2	IFN-γR 2	–	–	–	–	–	1.52
Il18rap	IL-18R accessory protein	–	1.57	–	–	–	–
Il10ra	IL-10Rα	–	1.63	–	–	–	–
Cytokines							
Irf7	IFN regulatory factor 7	–	3.03	4.44	–	1.72	1.86
Irf1	IFN regulatory factor 1	–	2.22	2.40	–	–	1.51
Il18bp	IL-18 binding protein	–	2.07	2.38	–	–	1.51
Tnf	TNF	–	2.11	2.24	–	–	1.56
Il1b	IL-1β	–	1.95	2.00	1.57	2.23	2.93
Tnfrsf1b	TNFR superfamily, member 1b	–	1.82	1.99	–	–	1.67
Irf8	IFN regulatory factor 8	–	1.99	1.83	–	–	1.61
Tnfaip6	TNF-α–induced protein 6	–	–	–	1.85	–	1.56
Il1f9	IL-1 family, member 9	–	–	–	–	–	1.59
Tnfaip2	TNF-α–induced protein 2	–	–	–	–	1.63	1.88
Il33	IL-33	–	1.64	–	1.58	–	1.93
Il6st	IL 6 signal transducer	–	–	–	–	−1.63	–
Tnfsf13b	TNF (ligand) superfamily, member 13b	–	1.63	–	–	–	–
Il1a	IL-1α	–	–	–	–	–	–

Values represent mean fold changes compared with PBS (n = 3 mice/group).
$p < 0.05$, one-way ANOVA, Tukey post hoc test.
–, Genes that showed no significant regulation in that treatment group; F+H, Flu+HDM.

exposed to a low concentration of HDM (5 μg daily) in the context of an acute influenza infection (7). In this model, exposure to HDM alone elicits minimal allergic sensitization and airway eosinophilia and, importantly, does not alter lung function. Hence, we define this dose as a subclinical dose of allergen exposure. However, if HDM exposure takes place during the course of an ongoing acute influenza infection, strong Th2-mediated immunity, robust eosinophilia and, importantly, the generation of marked lung dysfunction ensue. We sought to investigate the genomic basis of this phenotype. As shown in Fig. 2, our data indicated that the global gene-expression profile in the lungs of mice exposed to HDM alone approximated the expression profile of mice exposed to PBS. In contrast, infection with Flu led to a gene-expression profile that was remarkably different from that observed in mice exposed to either HDM alone or PBS. Interestingly, the expression pattern in the lungs of Flu+HDM-treated mice was similar, but not identical, to that induced by Flu alone, despite the overt differences in phenotype observed between these two groups (7). Venn analysis revealed that 80% of genes expressed in Flu+HDM-treated mice were also expressed in the lungs of mice infected with Flu only (Fig. 3). A similar examination revealed that mice exposed to HDM alone or Flu+HDM shared only 1.5% and 6% of expressed genes in the EP and LP, respectively. In contrast to shared genes, we identified a set of 330 and 240 unique genes expressed only in mice exposed to Flu+HDM during the EP and LP of the response, respectively; these genes represent 23% and 19% of the total regulated genes in this treatment group. These data revealed that, compared with the minimal impact elicited by a subclinical dose of allergen, Flu infection has a major impact on global gene regulation, involving hundreds of genes involved in the regulation of a number of distinct biological pathways.

Table V. Functional categories of uniquely expressed genes in mice concurrently exposed to Flu+HDM during the EP and LP response

Biological Function	F+H Treatment Group	
	EP	LP
	No. of Genes	No. of Genes
Translation regulation	3	8
Other metabolic genes	4	9
Nucleotide/DNA/chromosome binding	4	5
Lipid/fatty acid metabolism	10	6
Ion transport/binding	11	12
Carbohydrate metabolism	11	6
Protein transport	16	6
Tissue/muscle development or reorganization	19	16
Transcription regulation	22	19
Cell division/cell cycle regulation	22	16
Protein metabolism/regulation	30	16
Immune/inflammatory response	32	46
Intracellular signaling	36	12
Others	146	86
Total genes	366	263

Genes were selected based on a mean fold change of ±1.5 compared with PBS control-, HDM only-, or Flu-only–treated groups (n = 3 mice/group).
$p < 0.05$, one-way ANOVA, Tukey post hoc test.
F+H, Flu+HDM.

Table VI. Immune inflammatory genes with additional responsiveness to HDM in the lungs of mice infected with Flu and exposed to HDM for 3 d (EP)

Gene Symbol	Gene Name	Flu	F+H
Saa3	Serum amyloid A 3	15.74	17.35
Cxcl9	Chemokine (CXC motif) ligand 9	14.50	12.93
Timp1	Tissue inhibitor of metalloproteinase 1	8.01	10.07
Ccl8	Chemokine (CC motif) ligand 8	11.81	9.78
Serpina3n	Serine (or cysteine) peptidase inhibitor, clade A, member 3N	4.09	9.25
IghmAC38.205.12	Ig μ chain V region AC38 205.12	3.65	5.65
Igh-Ia	IgH Ia	2.96	4.94
Cd163	CD163 Ag	–	2.54
C5ar1	Complement component 5a receptor 1	–	1.89
C2	Complement component 2 (within H2S)	–	1.78
S100a8	S100 calcium binding protein A8 (calgranulin A)	–	1.70
Ndrg1	Nmyc downstream regulated gene 1	–	1.68
Il8rb	IL-8R, β	–	1.63
Nupr1	Nuclear protein 1	–	1.62
G6pdx	Glucose-6 phosphate dehydrogenase X-linked	–	1.62
Enpp1	Ectonucleotide pyrophosphatase/phosphodiesterase 1	–	1.58
C1r	Complement component 1, r subcomponent	–	1.56
H2Q2|H2t9|H2D1	MHC class Ib T9	–	1.54
Fn1	Fibronectin 1	–	1.53
Cxcl17	Chemokine (CXC motif) ligand 17	–	1.51
H2T24	Histocompatibility 2, T region locus 24	–	1.50
Bpgm	2,3 bisphosphoglycerate mutase	–	−1.62
Cr2	Complement receptor 2	–	−1.68
Tnfsf10	TNF (ligand) superfamily, member 10	–	−1.72
Cbfa2t3	Core-binding factor, runt domain, α subunit 2, translocated to 3 (human)	–	−1.72
Enpp2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	–	−1.74
Ms4a1	Membrane spanning 4 domains, subfamily A, member 1	–	−1.79
Bank1	B cell scaffold protein with ankyrin repeats 1	–	−1.80
Itga1	Integrin α 1	–	−1.82
Lyz1	Lysozyme 1	–	−1.94
Cd209a	CD209a Ag	–	−2.14
Hc	Hemolytic complement	−2.00	−5.56

Values represent mean fold changes compared with PBS (n = 3 mice/group).
$p < 0.05$, one-way ANOVA, Tukey post hoc test.
–, Genes that showed no significant regulation in that treatment group; F+H, Flu+HDM.

Based on the initial global gene-expression analysis, we sought to uncover potential pathways by which influenza may enhance allergen responsiveness. To this end, we analyzed in greater detail the biological roles of genes induced following influenza infection and, then, focused our analysis on specific gene classes that may facilitate innate responsiveness to allergen exposure. From a functional perspective, the gene-expression profile observed following Flu and Flu+HDM treatment showed that these genes pertained to a wide range of biological processes. Major categories included response to stimuli, stress, and wounding and genes mainly associated with inflammatory responses and chemotaxis (Fig. 4). Many of these processes were highly enriched with genes associated with the initiation of immune inflammatory responses encoding proteins for pattern recognition receptors and damage-associated molecular patterns, such as TLRs, CLRs, and several members of the complement pathway (Tables I, II). Among the TLRs, our study showed that Flu infection particularly led to increased and sustained expression of *TLR13*, a recently described member of the TLR family, shown to be expressed largely by myeloid cells, particularly dendritic cells (DCs) (20, 21). Similarly, we observed increased expression for a number of CLR family members, most notably Dectin 1 (*Clec7a*) and Dectin 2 (*Clec4n*) expressed on DCs and macrophages (22, 23). These receptors were shown to respond to complex carbohydrate structures, including glycans present on HDM aeroallergens, such as *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* (24). Interestingly, we detected increased expression of a number of FcR molecules, most notably *FcεR1*. Grayson et al. (25) reported, in a mouse model of sendai virus infection, increased type-1 IFNR-dependent upregulation of FcεR1 on lung DCs, which, upon receptor cross-linking, led to the production of CCL28, a Th2-associated chemokine. Furthermore, evidence from a microarray analysis of PBMCs isolated during acute virus-associated asthma exacerbations from HDM-sensitized children, revealed increased *FcεR1* gene expression on monocytes and DCs (26); interestingly, this was associated with increased expression of *CCR2*, a chemokine receptor necessary for the recruitment of monocytes and DCs to inflamed tissues. In accordance with these findings, our study showed that influenza infection triggers the expression of *FcεRI* in the lung, along with concomitant increases in *CCR2* and type I IFN-stimulated genes (Table III). The nature of our study prevented identification of the specific cell types expressing these molecules. However, these findings collectively suggested that viral infections may amplify the allergic immune-inflammatory response through a mechanism that involves type-1 IFN and FcεR1 and the recruitment of monocytes and DCs into the inflamed lung environment.

Lastly, our data revealed increased expression of several components of the complement pathway, most notably several *C1q* subunits (*C1qa*, *C1qb*, *C1qc*), *C3*, and *C3ar*, which are key components necessary for the generation and signaling of anaphylatoxins C3a and C5a. Anaphylatoxins are involved in the recruitment and activation of a number of leukocytes, including mast cells, eosinophils, and basophils, and were also implicated in the regulation of DC and T cell signaling (27). Thus, Flu infection enhanced the expression of a substantial number of innate molecules involved in the sensing and response to HDM.

Activation of innate-immune pathways through signaling of cell surface receptors leads to the recruitment of immune inflammatory

Table VII. Immune inflammatory genes with additional responsiveness to HDM in the lungs of mice infected with Flu and exposed to HDM for 6 d (LP)

Gene Symbol	Gene Name	Flu	F+H
Igj	Ig joining chain	9.61	11.17
IghmAC38.205.12	Ig μ chain V region AC38 205.12	8.72	10.80
Saa3	Serum amyloid A 3	5.88	10.61
Cxcl10	Chemokine (CXC motif) ligand 10	5.91	7.41
Timp1	Tissue inhibitor of metalloproteinase 1	5.27	6.82
Cxcl13	Chemokine (CXC motif) ligand 13	3.37	5.62
Cxcl5	Chemokine (CXC motif) ligand 5	2.33	4.18
Irg1	Immunoresponsive gene 1	–	2.22
Orm1	Orosomucoid 1	–	1.91
Itgax	Integrin α X	–	1.79
Mpa2l	Guanylate binding protein 10	–	1.77
Mpa2l	Macrophage activation 2 like	–	1.74
Oasl2	2′5′ oligoadenylate synthetase-like 2	–	1.73
H2Eb1	Histocompatibility 2, class II Ag E β	–	1.72
Ccr2	Chemokine (CC motif) receptor 2	–	1.72
Cd274	CD274 Ag	–	1.72
Igbp1	Ig (CD79A) binding protein 1	–	1.71
Ccl20	Chemokine (CC motif) ligand 20	–	1.71
Gbp2	Guanylate binding protein 2	–	1.71
Cfb	Complement factor B	–	1.69
Tnfrsf1b	TNFR superfamily, member 1b	–	1.67
Oas1a	2′5′ oligoadenylate synthetase 1A	–	1.65
H2Q8	Histocompatibility 2, Q region locus 1	–	1.63
Il2rg	IL-2R, γ-chain	–	1.63
Irf8	IFN regulatory factor 8	–	1.61
Vav1	Vav 1 oncogene	–	1.61
Gbp3	Guanylate binding protein 3	–	1.61
H2Q2|H2gs10|H2Q1	Histocompatibility 2, Q region locus 5	–	1.61
Il1f9	IL-1 family, member 9	–	1.59
H2Aa	Histocompatibility 2, class II Ag A, α	–	1.59
Ccl22	Chemokine (CC motif) ligand 22	–	1.58
Plek	Pleckstrin	–	1.57
Psme1	Proteasome (prosome, macropain) 28 subunit, α	–	1.57
Ptafr	Platelet-activating factor receptor	–	1.57
Fn1	Fibronectin 1	–	1.56
Lsp1	Lymphocyte specific 1	–	1.54
Sirpa	Signal regulatory protein α	–	1.54
Lax1	Lymphocyte transmembrane adaptor 1	–	1.53
Mx1	Myxovirus (influenza virus) resistance 1	–	1.52
Il18bp	IL-18 binding protein	–	1.51
Irf1	IFN regulatory factor 1	–	1.51
Pomp	Proteasome maturation protein	–	1.51
H2T24	Histocompatibility 2, T region locus 24	–	−1.50
Mfge8	Milk fat globule EGF factor 8 protein	–	−1.53
Ear2	Eosinophil-associated, RNase A family, member 3	–	−1.56
Alas2	Aminolevulinic acid synthase 2, erythroid	–	−1.87

Values represent mean fold changes compared with PBS (n = 3 mice/group).
$p < 0.05$, one-way ANOVA, Tukey post hoc test.
–, Genes that showed no significant regulation in that treatment group; F+H, Flu+HDM.

cells capable of producing a plethora of cytokines. Consistent with our previous report of increased expression of proinflammatory cytokines and the accumulation of neutrophils, DCs, and monocytes into the lung of mice during the acute phase of the response (7), influenza infection at day 10 postinfection regulated the expression of a substantial number of chemokines and their receptors involved in the recruitment of various leukocytes, such as monocytes, macrophages, DCs' Th cells, eosinophils, and mast cells (Table III). The cytokine gene profile elicited by influenza reflects its potent Th1 cell-promoting effects, with strong induction of members of the type I IFN family, as well as proinflammatory cytokines, such as *TNF-α* and several members of the IL-1 family of proteins, including *IL-1β*, *IL-18bp* and, notably, *IL-33* (7, 28) (Table IV). Interestingly, IL-33 was shown to amplify both Th1- and Th2-type responses by targeting mast cells, basophils, and Th2 cells, as well as NK and NK T cells, hence suggesting an important role in asthma pathogenesis (29). Importantly, our study showed that this cytokine is also increased 7 d following HDM exposure, and its expression is further enhanced in mice exposed to HDM in the context of an ongoing Flu infection. Thus, through the activation of innate and adaptive immune pathways, influenza infection induces a number of gene families involved in the initiation and propagation of a variety of Th-mediated inflammatory responses.

In addition to the pervasive upregulation of genes involved in innate immune responsiveness following influenza infection, we identified a subset of genes uniquely expressed as a result of Flu+HDM coexposure; these consisted of 366 and 263 genes during the EP and LP of the response, respectively (Table V, Supplemental Table I). Of these, 330 and 240 genes were identified as unique genes in mice exposed to Flu+HDM and were not expressed in any other treatment groups (Fig. 3). An additional 36 and 23 genes were shared among HDM-, Flu-, and Flu+HDM–treated groups; however, these shared genes were found to be significantly up- or downregulated in Flu-infected mice concurrently exposed to HDM. Although 30–40% of these genes do not have ascribed

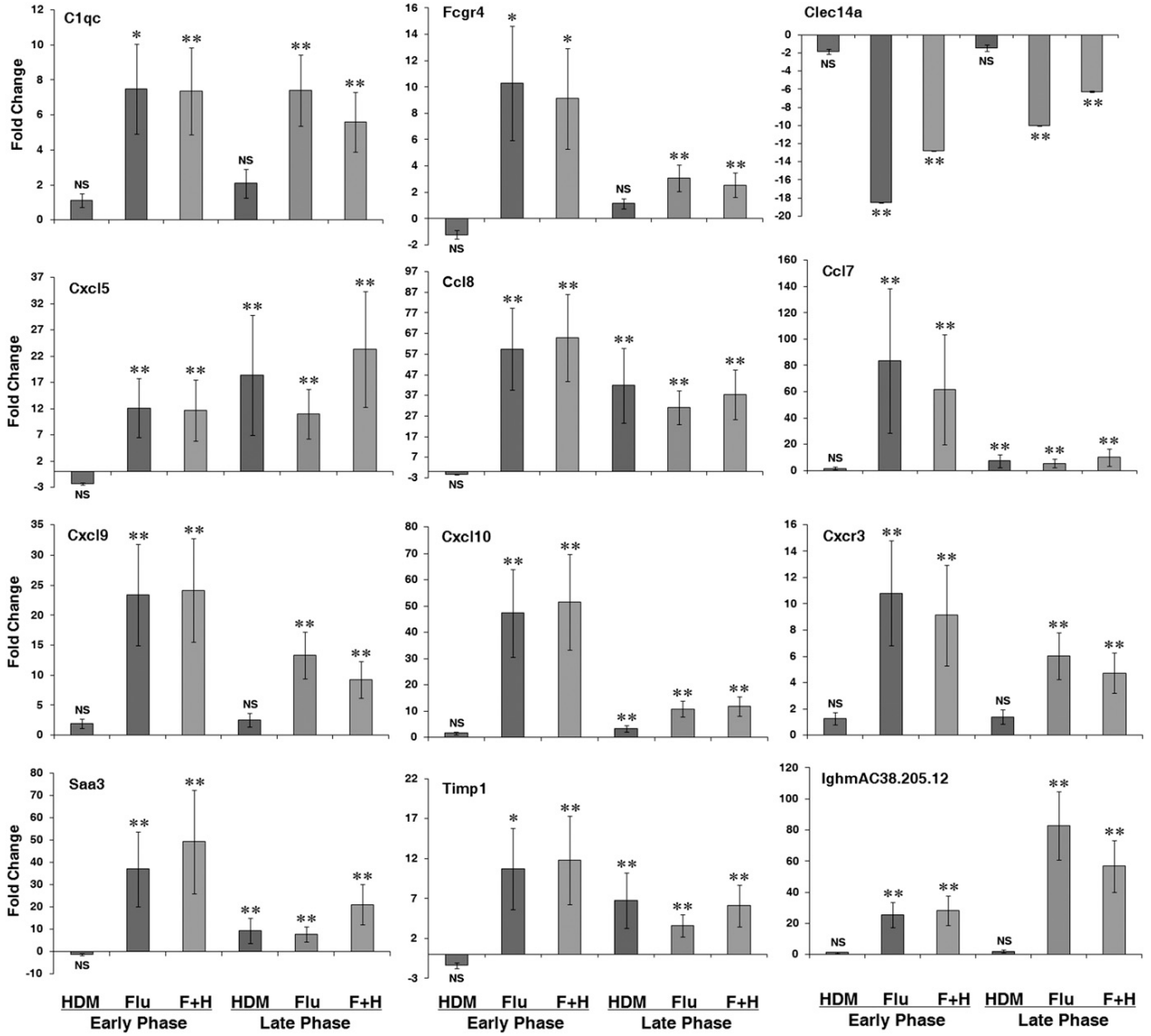

FIGURE 5. Representative genes from microarray experiment validated by qPCR. Data are expressed as mean ± SEM ($n = 4$). *$p < 0.05$, **$p < 0.01$, pair-wise fixed reallocation randomization test.

functions, the remaining 60–70% are known to be involved in a wide range of biological processes; these could be divided into 13 functional categories, including immune inflammatory responses, intracellular signaling, metabolism, and transcriptional and translational regulation, as well as tissue/muscle development and reorganization. These data showed that a subclinical dose of HDM, which by itself did not lead to significantly altered gene expression, elicited distinct, unique changes when administered in the context of an ongoing Flu infection.

To further understand how the interaction between HDM and Flu affected gene expression from an immunological perspective, we evaluated the contribution of genes expressed within the functional group "immune inflammatory-responsive genes." In addition to the increased expression of several members of the chemokine and cytokine family and genes encoding additional complement components, we particularly observed increased expression of genes encoding proteins, such as *Saa3*, *Timp1*, and serine peptidase inhibitor, clade A, member 3N (*Serpina3n*). Although *Timp1* was reported to play a role in extracellular matrix remodeling, and its role in the development of allergic asthma is well described (30, 31), the functional role of *Serpina3n* has not been established. In humans, SERPIN3A, commonly known as antichymotrypsin, is produced by a variety of cell types, including hepatocytes and bronchial epithelial cells during acute inflammatory responses, and studies showed that serum levels of two other serpin family members, SERPINB3 and SERPINB4, are elevated in patients with asthma (32, 33). Interestingly, in a recent animal model of allergic asthma, the murine ortholog, *Serpin3ba*, was shown to mediate HDM-induced mucus production (34). Thus, our study suggested that, similar to other serpin family members, *Serpina3n* may likely play an important role in HDM-mediated allergic disease, particularly in the context of an acute viral infection.

Saa3, is a major acute-phase protein that can act as a chemoattractant for phagocytes (35) and recently was shown to promote Th17-mediated allergic asthma through the activation of the NLRP3 inflammasome complex (36). Our data showed that this gene was significantly upregulated following Flu infection, with an additional 2-fold increase following HDM exposure during the

EP that was sustained through the LP, where we observed a 5-fold difference in expression level between Flu- and Flu+HDM-treated mice (Tables VI, VII). Interestingly, the expression of *Saa3* in the lung is regulated by *S100a8* (37), which was also found to be exclusively expressed in Flu+HDM-treated mice, albeit at lower levels. Indeed, S100A8 was recently identified as an important damage-associated molecular pattern released by activated phagocytes and was shown to be an endogenous activator of TLR4 on monocytes (38, 39). Although the functional role of *S100a8* remains to be elucidated, our findings intimate the potential importance of the *Saa3–S100a8* axis in allergic disease. Considering that S100A8 can activate TLR4 signaling, the increased expression of these genes may amplify inflammatory responses (40). Thus, HDM exposure in the context of a prior Flu infection leads to the expression of a number of unique immune genes that collectively function to either facilitate or amplify allergic inflammatory responses.

We describe the concentration of HDM used in this study as "subclinical" because, per se, it induced minimal immune-inflammatory responses and no airway physiological changes. In this study, we investigated the impact of an ongoing Flu infection on the response to such low concentration of HDM at the genomic level. Under these conditions, the phenotype elicited is characterized by robust allergic airway inflammation and airway hyperreactivity. Our data showed that the potential mechanisms by which Flu infection may facilitate this phenotype are manifold and impact different processes in the generation of allergic-inflammatory responses. A first repercussion may be conceptualized as a heightened state of immune alertness, illustrated by the enhanced expression of a number of innate molecules involved in Ag sensing. A second repercussion may be visualized as an amplification event exhibited by the enhanced expression of chemokines and chemokines receptors that facilitate the recruitment of a variety of immune-inflammatory cell types. We suggest that the overall consequence of these effects is a significant lowering of the threshold of allergen responsiveness required to manifest a clinically meaningful phenotype. Indeed, this outcome is depicted by the increased expression of molecules distinctly associated with features of the allergic phenotype. Clearly, the immune priming induced by Flu, and likely other respiratory viruses, is archetypically complex because it involves the expression of hundreds of genes and multiple interacting pathways and, moreover, additional posttranscriptional and translational regulation. Thus, it seems unlikely that the generation of an allergic phenotype under these conditions could be conceptualized linearly or attributed to a single critical signal. This notion has implications for the design and expectations of therapeutic strategies to prevent viral-induced allergic asthma.

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References

1. Sporik, R., and T. A. Platts-Mills. 2001. Allergen exposure and the development of asthma. *Thorax* 56(Suppl. 2): ii58–ii63.
2. Lloyd, C. M., and J. R. Murdoch. 2010. Tolerizing allergic responses in the lung. *Mucosal Immunol.* 3: 334–344.
3. Bartlett, N. W., G. R. McLean, Y. S. Chang, and S. L. Johnston. 2009. Genetics and epidemiology: asthma and infection. *Curr. Opin. Allergy Clin. Immunol.* 9: 395–400.
4. Sevin, C. M., and R. S. Peebles, Jr. 2010. Infections and asthma: new insights into old ideas. *Clin. Exp. Allergy* 40: 1142–1154.
5. Kusel, M. M., N. H. de Klerk, T. Kebadze, V. Vohma, P. G. Holt, S. L. Johnston, and P. D. Sly. 2007. Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. *J. Allergy Clin. Immunol.* 119: 1105–1110.
6. Jackson, D. J., R. E. Gangnon, M. D. Evans, K. A. Roberg, E. L. Anderson, T. E. Pappas, M. C. Printz, W. M. Lee, P. A. Shult, E. Reisdorf, et al. 2008. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. *Am. J. Respir. Crit. Care Med.* 178: 667–672.
7. Al-Garawi, A. A., R. Fattouh, T. D. Walker, E. B. Jamula, F. Botelho, S. Goncharova, J. Reed, M. R. Stampfli, P. M. O'Byrne, A. J. Coyle, and M. Jordana. 2009. Acute, but not resolved, influenza A infection enhances susceptibility to house dust mite-induced allergic disease. *J. Immunol.* 182: 3095–3104.
8. Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, et al. 1999. Generation of influenza A viruses entirely from cloned cDNAs. *Proc. Natl. Acad. Sci. USA* 96: 9345–9350.
9. Irizarry, R. A., B. M. Bolstad, F. Collin, L. M. Cope, B. Hobbs, and T. P. Speed. 2003. Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res.* 31: e15.
10. Dennis, G., Jr., B. T. Sherman, D. A. Hosack, J. Yang, W. Gao, H. C. Lane, and R. A. Lempicki. 2003. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* 4: P3.
11. Huang, D. W., B. T. Sherman, and R. A. Lempicki. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* 4: 44–57.
12. Zeeberg, B. R., W. Feng, G. Wang, M. D. Wang, A. T. Fojo, M. Sunshine, S. Narasimhan, D. W. Kane, W. C. Reinhold, S. Lababidi, et al. 2003. GoMiner: a resource for biological interpretation of genomic and proteomic data. *Genome Biol.* 4: R28.
13. Yao, Y., B. W. Higgs, C. Morehouse, M. de Los Reyes, W. Trigona, P. Brohawn, W. White, J. Zhang, B. White, A. J. Coyle, et al. 2009. Development of Potential Pharmacodynamic and Diagnostic Markers for Anti-IFN-α Monoclonal Antibody Trials in Systemic Lupus Erythematosus. *Hum. Genomics Proteomics* 2009: pii 374312.
14. Pfaffl, M. W., A. Tichopad, C. Prgomet, and T. P. Neuvians. 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* 26: 509–515.
15. Pfaffl, M. W., G. W. Horgan, and L. Dempfle. 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* 30: e36.
16. Cates, E. C., R. Fattouh, J. R. Johnson, A. Llop-Guevara, and M. Jordana. 2007. Modeling responses to respiratory house dust mite exposure. *Contrib. Microbiol.* 14: 42–67.
17. Karp, C. L. 2010. Guilt by intimate association: what makes an allergen an allergen? *J. Allergy Clin. Immunol.* 125: 955–960; quiz 961–962.
18. Shakib, F., A. M. Ghaemmaghami, and H. F. Sewell. 2008. The molecular basis of allergenicity. *Trends Immunol.* 29: 633–642.
19. Llop-Guevara, A., M. Colangelo, D. K. Chu, C. L. Moore, N. A. Stieber, T. D. Walker, S. Goncharova, A. J. Coyle, L. K. Lundblad, P. M. O'Byrne, et al. 2008. In vivo-to-in silico iterations to investigate aeroallergen-host interactions. *PLoS ONE* 3: e2426.
20. Luber, C. A., J. Cox, H. Lauterbach, B. Fancke, M. Selbach, J. Tschopp, S. Akira, M. Wiegand, H. Hochrein, M. O'Keeffe, and M. Mann. 2010. Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. *Immunity* 32: 279–289.
21. Shi, Z., Z. Cai, A. Sanchez, T. Zhang, S. Wen, J. Wang, J. Yang, S. Fu, and D. Zhang. 2011. A novel Toll-like receptor that recognizes vesicular stomatitis virus. *J. Biol. Chem.* 286: 4517–4524.
22. van Vliet, S. J., J. J. García-Vallejo, and Y. van Kooyk. 2008. Dendritic cells and C-type lectin receptors: coupling innate to adaptive immune responses. *Immunol. Cell Biol.* 86: 580–587.
23. Willart, M. A., and H. Hammad. 2010. Alarming dendritic cells for allergic sensitization. *Allergol. Int.* 59: 95–103.
24. Barrett, N. A., A. Maekawa, O. M. Rahman, K. F. Austen, and Y. Kanaoka. 2009. Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. *J. Immunol.* 182: 1119–1128.
25. Grayson, M. H., D. Cheung, M. M. Rohlfing, R. Kitchens, D. E. Spiegel, J. Tucker, J. T. Battaile, Y. Alevy, L. Yan, E. Agapov, et al. 2007. Induction of high-affinity IgE receptor on lung dendritic cells during viral infection leads to mucous cell metaplasia. *J. Exp. Med.* 204: 2759–2769.
26. Subrata, L. S., J. Bizzintino, E. Mamessier, A. Bosco, K. L. McKenna, M. E. Wikström, J. Goldblatt, P. D. Sly, B. J. Hales, W. R. Thomas, et al. 2009. Interactions between innate antiviral and atopic immunoinflammatory pathways precipitate and sustain asthma exacerbations in children. *J. Immunol.* 183: 2793–2800.
27. Wills-Karp, M. 2007. Complement activation pathways: a bridge between innate and adaptive immune responses in asthma. *Proc. Am. Thorac. Soc.* 4: 247–251.
28. Ichinohe, T., I. K. Pang, and A. Iwasaki. 2010. Influenza virus activates inflammasomes via its intracellular M2 ion channel. *Nat. Immunol.* 11: 404–410.
29. Smithgall, M. D., M. R. Comeau, B. R. Yoon, D. Kaufman, R. Armitage, and D. E. Smith. 2008. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int. Immunol.* 20: 1019–1030.

30. Erlewyn-Lajeunesse, M. D., L. P. Hunt, P. Pohunek, S. J. Dobson, P. Kochhar, J. A. Warner, and J. O. Warner. 2008. Bronchoalveolar lavage MMP-9 and TIMP-1 in preschool wheezers and their relationship to persistent wheeze. *Pediatr. Res.* 64: 194–199.
31. Sands, M. F., P. J. Ohtake, S. D. Mahajan, S. S. Takyar, R. Aalinkeel, Y. V. Fang, J. W. Blume, B. A. Mullan, D. E. Sykes, S. Lachina, et al. 2009. Tissue inhibitor of metalloproteinase-1 modulates allergic lung inflammation in murine asthma. *Clin. Immunol.* 130: 186–198.
32. Nishi, N., M. Miyazaki, K. Tsuji, T. Hitomi, E. Muro, M. Zaitsu, S. Yamamoto, S. Inada, I. Kobayashi, T. Ichimaru, et al. 2005. Squamous cell carcinoma-related antigen in children with acute asthma. *Ann. Allergy Asthma Immunol.* 94: 391–397.
33. Horvath, A. J., J. A. Irving, J. Rossjohn, R. H. Law, S. P. Bottomley, N. S. Quinsey, R. N. Pike, P. B. Coughlin, and J. C. Whisstock. 2005. The murine orthologue of human antichymotrypsin: a structural paradigm for clade A3 serpins. *J. Biol. Chem.* 280: 43168–43178.
34. Sivaprasad, U., D. J. Askew, M. B. Ericksen, A. M. Gibson, M. T. Stier, E. B. Brandt, S. A. Bass, M. O. Daines, J. Chakir, K. F. Stringer, et al. 2011. A nonredundant role for mouse Serpinb3a in the induction of mucus production in asthma. *J. Allergy Clin. Immunol.* 127: 254–261, 261.e1–6.
35. He, R. L., J. Zhou, C. Z. Hanson, J. Chen, N. Cheng, and R. D. Ye. 2009. Serum amyloid A induces G-CSF expression and neutrophilia via Toll-like receptor 2. *Blood* 113: 429–437.
36. Ather, J. L., K. Ckless, R. Martin, K. L. Foley, B. T. Suratt, J. E. Boyson, K. A. Fitzgerald, R. A. Flavell, S. C. Eisenbarth, and M. E. Poynter. 2011. Serum amyloid A activates the NLRP3 inflammasome and promotes Th17 allergic asthma in mice. *J. Immunol.* 187: 64–73.
37. Hiratsuka, S., A. Watanabe, Y. Sakurai, S. Akashi-Takamura, S. Ishibashi, K. Miyake, M. Shibuya, S. Akira, H. Aburatani, and Y. Maru. 2008. The S100A8-serum amyloid A3-TLR4 paracrine cascade establishes a pre-metastatic phase. *Nat. Cell Biol.* 10: 1349–1355.
38. Perera, C., H. P. McNeil, and C. L. Geczy. 2010. S100 Calgranulins in inflammatory arthritis. *Immunol. Cell Biol.* 88: 41–49.
39. Vogl, T., K. Tenbrock, S. Ludwig, N. Leukert, C. Ehrhardt, M. A. van Zoelen, W. Nacken, D. Foell, T. van der Poll, C. Sorg, and J. Roth. 2007. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat. Med.* 13: 1042–1049.
40. Ehrchen, J. M., C. Sunderkötter, D. Foell, T. Vogl, and J. Roth. 2009. The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *J. Leukoc. Biol.* 86: 557–566.

Supplementary table 1: Differentially expressed genes that showed additional responsiveness to house dust mite exposure in the context of flu infection (Flu+HDM) during the early phase (EP) and late phase (LP).

Affy Transcript ID	Accession ID	Gene Name	Gene Symbol	Biological Function	Fold Change					
					HDM [EP]	Flu [EP]	Flu + HDM [EP]	HDM [LP]	Flu [LP]	Flu + HDM [LP]
Immune/Inflammatory Response										
10541644	NM_001170395	CD163 antigen	Cd163	Acute inflammatory response	-	-	2.54	-	-	-
10355403	NM_010233	Fibronectin 1	Fn1	Acute inflammatory response	-	-	1.53	-	-	1.56
10567995	NM_019738	Nuclear protein 1	Nupr1	Acute inflammatory response	-	-	1.62	-	-	-
10505438	NM_008768	Orosomucoid 1	Orm1	Acute inflammatory response	-	-	-	-	-	1.91
10563597	NM_011315	Serum amyloid A 3	Saa3	Acute inflammatory response	-	15.74	17.35	4.14	5.88	10.61
10398075	NM_009252	Serine (or cysteine) peptidase inhibitor, clade A, member 3N	Serpina3n	Acute inflammatory response	-	4.09	9.25	-	-	-
10541678	NM_023143	Complement component 1, r subcomponent	C1r	Adaptive immune response	-	-	1.56	-	-	-
10450344	NM_013484	Complement component 2 (within H2S)	C2	Adaptive immune response	-	-	1.78	-	-	-
10450325	NM_008198	Complement factor B	Cfb	Adaptive immune response	-	-	-	-	-	1.69
10361292	NM_007758	Complement receptor 2	Cr2	Adaptive immune response	-	-	-1.68	-	-	-
10450154	NM_010378	Histocompatibility 2, class II antigen A, alpha	H2Aa	Adaptive immune response	-	-	-	-	-	1.59
10481962	NM_010406	Hemolytic complement	Hc/C5	Adaptive immune response	-	-2.00	-5.56	-	-	-
10403018	---	Immunoglobulin heavy chain Ia	Igh-Ia	Adaptive immune response	-	2.96	4.94	-	-	-
10403015	---	Ig mu chain V region AC38 205.12	IghmAC38.205.12	Adaptive immune response	-	3.65	5.65	-	8.72	10.80
10566050	NM_010531	Interleukin 18 binding protein	Il18bp	Adaptive immune response	-	-	-	-	-	1.51
10415282	NM_011189	Proteasome (prosome, macropain) 28 subunit, alpha	Psme1	Antigen processing and presentation	-	-	-	-	-	1.57
10444298	NM_010382	Histocompatibility 2, class II antigen E beta	H2Eb1	Antigen processing and presentation of exogenous peptide antigen	-	-	-	-	-	1.72
10502335	NM_001033350	Bcell scaffold protein with ankyrin repeats 1	Bank1	B cell activation	-	-	-1.80	-	-	-

Affy Transcript ID	Accession ID	Gene Name	Gene Symbol	Biological Function	Fold Change					
					HDM [EP]	Flu [EP]	Flu + HDM [EP]	HDM [LP]	Flu [LP]	Flu + HDM [LP]
10600973	NM_008784	Immunoglobulin (CD79A) binding protein 1	Igbp1	B cell activation	-	-	-	-	-	1.71
10466172	NM_007641	Membranespanning 4domains, subfamily A, member 1	Ms4a1	B cell activation	-	-	-1.79	-	-	-
10560242	NM_001173550	Complement component 5a receptor 1	C5ar1	Chemotaxis	-	-	1.89	-	-	-
10347888	NM_016960	Chemokine (CC motif) ligand 20	Ccl20	Chemotaxis	-	-	-	-	-	1.71
10574213	NM_009137	Chemokine (CC motif) ligand 22	Ccl22	Chemotaxis	-	-	-	-	-	1.58
10379535	NM_021443	Chemokine (CC motif) ligand 8	Ccl8	Chemotaxis	-	11.81	9.78	-	-	-
10531415	NM_021274	Chemokine (CXC motif) ligand 10	Cxcl10	Chemotaxis	-	-	-	2.26	5.91	7.41
10561047	NM_153576	Chemokine (CXC motif) ligand 17	Cxcl17	Chemotaxis	-	-	1.51	-	-	-
10523120	NM_009141	Chemokine (CXC motif) ligand 5	Cxcl5	Chemotaxis	-	-	-	2.20	2.33	4.18
10414262	NM_007895	Eosinophilassociated, ribonuclease A family, member 3	Ear2	Chemotaxis	-	-	-	-	-	-1.56
10428619	NM_015744	Ectonucleotide pyrophosphatase/phosphodiesterase 2	Enpp2	Chemotaxis	-	-	-1.74	-	-	-
10347291	NM_009909	Interleukin 8 receptor, beta	Il8rb	Chemotaxis	-	-	1.63	-	-	-
10412298	NM_001033228	Integrin alpha 1	Itga1	Chemotaxis	-	-	-1.82	-	-	-
10559207	NM_019391	Lymphocyte specific 1	Lsp1	Chemotaxis	-	-	-	-	-	1.54
10493831	NM_013650	S100 calcium binding protein A8 (calgranulin A)	S100a8	Chemotaxis	-	-	1.70	-	-	-
10605338	NM_008062	Glucose6phosphate dehydrogenase Xlinked	G6pdx	Cytokine production	-	-	1.62	-	-	-
10508734	NM_001081211	Plateletactivating factor receptor	Ptafr	Cytokine production	-	-	-	-	-	1.57
10531407	NM_008599	Chemokine (CXC motif) ligand 9	Cxcl9	Defense response	-	14.50	12.93	-	-	-
10372652	NM_013590	Lysozyme 1	Lyz1	Defense response	-	-	-1.94	-	-	-
10441233	NM_010846	Myxovirus (influenza virus) resistance 1	Mx1	Defense response	-	-	-	-	-	1.52
10518300	NM_011610	Tumor necrosis factor receptor superfamily, member 1b	Tnfrsf1b	Defense response	-	-	-	-	-	1.67
10576784	NM_133238	CD209a antigen	Cd209a	Endocytosis	-	-	-2.14	-	-	-
10468898	NM_001159649	Lymphocyte transmembrane adaptor 1	Lax1	Immune effector process	-	-	-	-	-	1.53
10368289	NM_008813	Ectonucleotide pyrophosphatase/phosphodiesterase 1	Enpp1	Immune response	-	-	1.58	-	-	-
10496592	NM_010260	Guanylate binding protein 2	Gbp2	Immune response	-	-	-	-	-	1.71
10496580	NM_018734	Guanylate binding protein 3	Gbp3	Immune response	-	-	-	-	-	1.61
10444814	---	Histocompatibility 2, Q region locus 5	H2Q2|H2gs10|H2Q1	Immune response	-	-	-	-	-	1.61
10450699	AB359227	MHC class Ib T9	H2Q2|H2t9|H2D1	Immune response	-	-	1.54	-	-	-

Affy Transcript ID	Accession ID	Gene Name	Gene Symbol	Biological Function	Fold Change					
					HDM [EP]	Flu [EP]	Flu + HDM [EP]	HDM [LP]	Flu [LP]	Flu + HDM [LP]
10450675	NM_008207	Histocompatibility 2, T region locus 24	H2T24	Immune response	-	-	1.50	-	-	-1.50
10531126	NM_152839	Immunoglobulin joining chain	Igj	Immune response	-	-	-	-	9.61	11.17
10469786	NM_153511	Interleukin 1 family, member 9	Il1f9	Immune response	-	-	-	-	-	1.59
10531994	NM_194336	Guanylate binding protein 10	Mpa2l	Immune response	-	-	-	-	-	1.77
10532019	NM_001039647	Macrophage activation 2 like	Mpa2l	Immune response	-	-	-	-	-	1.74
10533256	NM_145211	2'5' oligoadenylate synthetase 1A	Oas1a	Immune response	-	-	-	-	-	1.65
10524621	NM_011854	2'5' oligoadenylate synthetaselike 2	Oasl2	Immune response	-	-	-	-	-	1.73
10527598	NM_025624	Proteasome maturation protein	Pomp	Immune response	-	-	-	-	-	1.51
10491091	NM_009425	Tumor necrosis factor (ligand) superfamily, member 10	Tnfsf10	Immune response	-	-	-1.72	-	-	-
10602372	NM_009653	Aminolevulinic acid synthase 2, erythroid	Alas2	Immune system development	-	-	-	-	-	-1.87
10537179	NM_007563	2,3bisphosphoglycerate mutase	Bpgm	Immune system development	-	-	-1.62	-	-	-
10582429	NM_009824	Corebinding factor, runt domain, alpha subunit 2, translocated to, 3 (human)	Cbfa2t3	Immune system development	-	-	-1.72	-	-	-
10590631	NM_009915	Chemokine (CC motif) receptor 2	Ccr2	Immune system development	-	-	-	-	-	1.72
10576034	NM_008320	Interferon regulatory factor 8	Irf8	Immune system development	-	-	-	-	-	1.61
10598976	NM_001044384	Tissue inhibitor of metalloproteinase 1	Timp1	Immune system development	-	8.01	10.07	3.55	5.27	6.82
10523359	NM_018866	Chemokine (CXC motif) ligand 13	Cxcl13	inflammatory response	-	-	-	1.64	3.37	5.62
10564713	NM_008594	Milk fat globuleEGF factor 8 protein	Mfge8	Phagocytosis	-	-	-	-	-	-1.53
10476021	NM_007547	Signalregulatory protein alpha	Sirpa	Phagocytosis	-	-	-	-	-	1.54
10446253	NM_011691	Vav 1 oncogene	Vav1	Phagocytosis	-	-	-	-	-	1.61
10444821	NM_023124	Histocompatibility 2, Q region locus 1	H2Q8	Regulation of cytokine production	-	-	-	-	-	1.63
10376060	NM_008390	Interferon regulatory factor 1	Irf1	Regulation of cytokine production	-	-	-	-	-	1.51
10557895	NM_021334	Integrin alpha X	Itgax	T cell proliferation	-	-	-	-	-	1.79
10384458	NM_019549	Pleckstrin	Plek	Platelet degranulation	-	-	-	-	-	1.57
10429140	NM_008681	Nmyc downstream regulated gene 1	Ndrg1	Mast cell activation	-	-	1.68	-	-	-
10416837	NM_008392	Immunoresponsive gene 1	Irg1	Response to molecule of bacterial origin	-	-	-	-	-	2.22
10606016	NM_013563	Interleukin 2 receptor, gamma chain	Il2rg	Positive regulation of immune system process	-	-	-	-	-	1.63

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10462390	NM_021893	CD274 antigen	Cd274	Negative regulation of immune system process	-	-	-	-	-	1.72
Ion Transport/Binding										
10540241	NM_022992	ADPribosylation factorlike 6 interacting protein 5	Arl6ip5	Ion transport	-	-	1.55	-	-	-
10442691	NM_011930	Chloride channel 7	Clcn7	Ion transport	-	-	1.52	-	-	-
10562211	NM_052992	FXYD domaincontaining ion transport regulator 1	Fxyd1	Ion transport	-	-	-	-	-	-1.57
10385083	NM_146017	Gammaaminobutyric acid (GABA) A receptor, pi	Gabrp	Ion transport	-	-	-1.53	-	-	1.53
10607403	NM_175429	Potassium channel tetramerisation domain containing 12b	Kctd12b	Ion transport	-	-	-	-	-	-1.56
10431326	NM_133241	Megalencephalic leukoencephalopathy with subcortical cysts 1 homolog (human)	Mlc1	Ion transport	-	-	-1.65	-	-	-1.54
10483353	NM_009135	Sodium channel, voltagegated, type VII, alpha	Scn7a	Ion transport	-	-1.82	-3.45	-	-	-
10599719	NM_172780	Solute carrier family 9 (sodium/hydrogen exchanger), member 6	Slc9a6	Ion transport	-	-	-	-	-	-1.52
10356886	NM_172658	Solute carrier organic anion transporter family, member 4C1	Slco4c1	Ion transport	-	-	-1.71	-	-	-
10357239	NM_019432	Transmembrane protein 37	Tmem37	Ion transport	-	-	1.78	-	-	-
10439268	NM_001013371	Deltex 3like (Drosophila)	Dtx3l	Zinc ion binding	-	-	-	-	-	1.52
10466659	NM_010266	Guanine deaminase	Gda	Zinc ion binding	-	-	-	-	-	1.56
10509596	NM_025786	Ring finger protein 186	Rnf186	Zinc ion binding	-	-	-1.62	-	-	-
10399588	ENSMUST0000(	Zinc finger protein 125	Zfp125	Zinc ion binding	-	-	-	-	-	1.61
10505270	NM_025286	Solute carrier family 31, member 2	Slc31a2	Transition metal ion transport	-	-	1.52	-	-	-
10470948	NM_013901	Solute carrier family 39 (zinc transporter), member 1	Slc39a1	Transition metal ion transport	-	-	1.58	-	-	-
10421309	NM_001135151	Solute carrier family 39 (zinc transporter), member 14	Slc39a14	Transition metal ion transport	-	-	-	-	-	1.67
10399854	NM_011867	Solute carrier family 26, member 4	Slc26a4	Sulfate transport	-	-	-	8.65	3.19	6.43
10502622	NM_017474	Chloride channel calcium activated 3	Clca3	Calcium ion transport	-	-	-	61.17	-	8.42
10363157	NM_001141927	Phospholamban	Pln	Calcium ion transport	-	-	-1.91	-	-	-
10521830	NM_026404	Solute carrier family 35, member A4	Slc35a4	Carbohydrate transport	-	-	-	-	-	-1.61

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					HDM [EP]	Flu [EP]	Flu + HDM [EP]	HDM [LP]	Flu [LP]	Flu + HDM [LP]
Lipid/Fatty Acid Metabolism										
10439442	NM_134102	Phospholipase A1 member A	Pla1a	Lipid catabolic process	-	2.45	6.00	-	-	-
10394538	NM_133360	Acetyl-Coenzyme A carboxylase alpha	Acaca	Lipid metabolism	-	-	-2.03	-	-	-
10580649	NM_133660	Esterase 22	Es22	Lipid metabolism	-	-	-1.98	-	-	-
10514221	NM_007408	Adipose differentiation related protein	Adfp	Lipid transport	-	-	1.92	-	-	-
10466606	NM_010730	Annexin A1	Anxa1	Lipid transport	-	-	-	-	-	1.65
10424695	NM_026730	GPIanchored HDLbinding protein 1	Gpihbp1	Lipid transport	-	-	-	-	-	-1.64
10475845	NM_028765	AcylCoenzyme A oxidaselike	Acoxl	Fatty acid metabolic process	-	-2.15	-3.88	-	-	-
10387838	NM_145684	Arachidonate lipoxygenase, epidermal	Alox12e	Fatty acid metabolic process	-	-	-	1.75	2.88	4.56
10457071	NM_025797	Cytochrome b5	Cyb5	Fatty acid metabolic process	-	-	-1.58	-	-	-
10481518	NM_022415	Prostaglandin E synthase	Ptges	Fatty acid metabolic process	-	-	1.77	-	-	1.60
10467979	NM_009127	StearoylCoenzyme A desaturase 1	Scd1	Fatty acid metabolic process	-	-	-2.20	-	-	-
10474526	NM_207206	Lysophosphatidylcholine acyltransferase 4	Agpat7	Phospholipid metabolic process	-	-	-	-	-	1.52
10524698	NM_011107	Phospholipase A2, group IB, pancreas	Pla2g1b	Phospholipid metabolic process	-	-	-2.24	-	-	-
10516765	NM_172702	Serine incorporator 2	Serinc2	Phospholipid metabolic process	-	-	1.66	-	-	-
10565712	NM_025408	Alkaline ceramidase 3	Phca	Membrane lipid metabolic process	-	-	-	-	-	1.58
Transcription Regulation										
10391504	NM_010791	Mesenchyme homeobox 1	Meox1	Regulation of transcription	-	-	-1.68	-	-	-
10472514	NM_181547	Nitric oxide synthase trafficker	Nostrin	Regulation of transcription	-	-	-1.77	-	-	-
10520950	NM_016861	PDZ and LIM domain 1 (elfin)	Pdlim1	Regulation of transcription	-	-	-	-	-	1.53
10566926	NM_025999	Ring finger protein 141	Rnf141	Regulation of transcription	-	-	-1.65	-	-	-
10552740	---	Nucleoporin 62	Nup62	RNA localization	-	-	1.57	-	-	-
10574033	NM_172410	Nucleoporin 93	Nup93	RNA localization	-	-	1.60	-	-	-
10394353	NM_025323	RIKEN cdna 0610009D07 gene	0610009D07Rik	RNA processing	-	-	-	-	-	1.65
10397081	NM_027349	RNA binding motif protein 25	Rbm25	RNA processing	-	-	-	-	-	1.89
10358454	NM_001166409	RNA binding motif protein 3	Rbm3	RNA processing	-	-	-	-	-	1.55

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10501649	NM_025517	RNA terminal phosphate cyclase domain 1	Rtcd1	RNA processing	-	-	1.58	-	-	-
10529299	NM_009193	Stemloop binding protein	Slbp	RNA processing	-	-	-	-	-	1.79
10476620	NM_021335	U2 small nuclear ribonucleoprotein B	Snrpb2	RNA processing	-	-	-	-	-	1.54
10442341	NM_175229	Serine/arginine repetitive matrix 2	Srrm2	RNA processing	-	-	-1.50	-	-	-
10472605	NM_009278	Sjogren syndrome antigen B	Ssb	RNA processing	-	-	-	-	-	1.51
10541091	NM_026053	Gem (nuclear organelle) associated protein 6	Gemin6	RNA splicing	-	-	-	-	-	1.74
10392098	NM_025310	Ftsj homolog 3 (E. Coli)	Ftsj3	rRNA modification	-	-	1.63	-	-	-
10449971	NM_028543	Zinc finger protein 763	1700065O13Rik	Transcription	-	-	-1.68	-	-	-
10490302	NM_001162922	RIKEN cdna 2810021G02 gene	2810021G02Rik	Transcription	-	-	-1.59	-	-	-
10586064	NM_009672	Acidic (leucinerich) nuclear phosphoprotein 32 family, member A	Anp32a	Transcription	-	-	-1.63	-	-	-
10421950	NM_007826	Dachshund 1 (Drosophila)	Dach1	Transcription	-	-	-1.99	-	-	-
10375358	ENSMUST00000	Early Bcell factor 1	Ebf1	Transcription	-	-	-2.07	-	-	-
10358027	NM_001163131	E74like factor 3	Elf3	Transcription	-	-	1.50	-	-	-
10584142	NM_011808	E26 avian leukemia oncogene 1, 5' domain	Ets1	Transcription	-	-	-1.53	-	-	-
10404404	NM_010225	Forkhead box F2	Foxf2	Transcription	-	-	-	-	-	-1.61
10402063	BC029185	Forkhead box N3	Foxn3	Transcription	-	-	-1.50	-	-	-
10399897	NM_153198	High mobility group box transcription factor 1	Hbp1	Transcription	-	-	-	-	-	-1.54
10542750	NM_025315	Mediator complex subunit 21	Med21	Transcription	-	-	-	-	-	1.60
10507529	NM_020000	Mediator of RNA polymerase II transcription, subunit 8 homolog (yeast)	Med8	Transcription	-	-	-	-	-	1.54
10539574	NM_008723	Nucleoplasmin 3	Npm3ps1	Transcription	-	-	1.51	-	-	-
10510215	NM_001083918	RIKEN cdna 1700029I01 gene	OTTMUSG00000010657	Transcription	-	-	-	-	-	1.73
10601878	NM_146236	Transcription elongation factor A (SII)like 1	Tceal1	Transcription	-	-	-2.21	-	-	-1.61
10543239	NM_031198	Transcription factor EC	Tcfec	Transcription	-	-	-	-	-	1.56
10566358	NM_009099	Tripartite motifcontaining 30	Trim30	Transcription	-	-	-	-	-	1.58
10442250	---	Zinc finger protein	---	Transcription	-	-	-1.62	-	-	-
10408693	NM_028784	Coagulation factor XIII, A1 subunit	F13a1	tRNA metabolic process	-	-	2.06	-	-	-
10432006	NM_019572	Histone deacetylase 7	Hdac7	Negative regulation of transcription from RNA polymerase II promoter	-	-	-1.50	-	-	-

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10417713	NM_011243	Retinoic acid receptor, beta	Rarb	Negative regulation of transcription from RNA polymerase II promoter	-	-	-1.53	-	-	-
10489127	NM_011249	Retinoblastomalike 1 (p107)	Rbl1	Negative regulation of transcription from RNA polymerase II promoter	-	-	-	-	-	1.55
10450501	NM_013693	Tumor necrosis factor	Tnf	Negative regulation of transcription from RNA polymerase II promoter	-	-	-	-	-	1.56
10491036	NM_026623	Nudix (nucleoside diphosphate linked moiety X)type motif 21	Nudt21	mRNA cleavage	-	-	-	-	-	1.60
Translation Regulation										
10397543	NM_001177565	eukaryotic translation initiation factor 1A	Eif1a	Translation	-	-	1.54	-	-	-
10396795	NM_026114	Eukaryotic translation initiation factor 2, subunit 1 alpha	Eif2s1	Translation	-	-	-	-	-	1.61
10578683	NM_026030	Eukaryotic translation initiation factor 2, subunit 2 (beta)	Eif2s2	Translation	-	-	-	-	-	1.65
10425226	NM_145139	Eukaryotic translation initiation factor 3, subunit L	Eif3eip	Translation	-	-	-	-	-	1.55
10407870	NM_029271	Mitochondrial ribosomal protein L32	Mrpl32	Translation	-	-	-	-	-	1.64
10497703	NM_029017	Mitochondrial ribosomal protein L47	Mrpl47	Translation	-	-	-	-	-	1.76
10450640	NM_025878	Mitochondrial ribosomal protein S18B	Mrps18b	Translation	-	-	-	-	-	1.54
10436694	NM_007475	Ribosomal protein, large, P0	Rplp0	Translation	-	-	1.52	-	-	-
10586604	NM_026467	Ribosomal protein S27like	Rps27l	Translation	-	-	1.64	-	-	-
10385043	NM_008722	Nucleophosmin 1	Npm1	Ribosome export from nucleus	-	-	-	-	-	1.62
10462623	NM_008331	Interferoninduced protein with tetratricopeptide repeats 1	Ifit1	Posttranslational modification, protein turnover, chaperones,	-	-	-	-	-	1.51
Cell Division/Cell Cycle Regulation										
10562709	NM_001111058	CD33 antigen	Cd33	Cell adhesion	-	-	1.56	-	-	-
10402211	NM_011812	Fibulin 5	Fbln5	Cell adhesion	-	-	-1.58	-	-	-
10584827	NM_007962	Myelin protein zerolike 2	Mpzl2	Cell adhesion	-	-	1.66	-	-	-
10449000	NM_018857	Mesothelin	Msln	Cell adhesion	-	-	2.46	-	-	-
10498018	NM_130448	Protocadherin 18	Pcdh18	Cell adhesion	-	-	-1.93	-	-	-
10392221	NM_008816	Platelet/endothelial cell adhesion molecule 1	Pecam1	Cell adhesion	-	-	-1.65	-	-	-
10492021	NM_015784	Periostin, osteoblast specific factor	Postn	Cell adhesion	-	-	-1.51	-	-	-

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10352792	XR_033810	predicted gene 10124	Gm10124	Cell cycle	-	-	1.50	-	-	-
10346810	NM_001081050	Par3 partitioning defective 3 homolog B (C. Elegans)	Pard3b	Cell cycle	-	-	-1.51	-	-	-
10358389	NM_009061	Regulator of Gprotein signaling 2	Rgs2	Cell cycle	-	-	-1.54	-	-	-
10493820	NM_011313	S100 calcium binding protein A6 (calcyclin)	S100a6	Cell cycle	-	-	2.11	-	-	-
10576140	NM_026014	Chromatin licensing and DNA replication factor 1	Cdt1	Cell cycle checkpoint	-	-	-	-	-	1.58
10456490	NM_027556	Centrosomal protein 192	Cep192	Cell cycle checkpoint	-	-	-	-	-	1.62
10586184	NM_025372	Timeless interacting protein	Tipin	Cell cycle checkpoint	-	-	-	-	-	1.89
10443463	NM_007669	Cyclindependent kinase inhibitor 1A (P21)	Cdkn1a	Cell cycle process	-	-	-	-	-	1.67
10497731	NM_026222	Coiledcoil domain containing 39	Ccdc39	Cell division and chromosome partitioning,	-	-	-1.57	-	-	-
10512470	NM_001110320	CD72 antigen	Cd72	Cell division and chromosome partitioning,	-	5.12	2.95	-	-	-
10515808	NM_026789	WD repeat domain 65	LOC674677	Cell division and chromosome partitioning,	-	-	-1.63	-	-	-
10422728	NM_023118	Disabled homolog 2 (Drosophila)	Dab2	Cell morphogenesis	-	-	1.50	-	-	-
10439514	NM_008083	Growth associated protein 43	Gap43	Cell morphogenesis	-	-	-1.93	-	-	-
10396421	NM_010431	Hypoxia inducible factor 1, alpha subunit	Hif1a	Cell morphogenesis	-	-	-	-	-	1.51
10519717	NM_009152	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	Sema3a	Cell morphogenesis	-	-	-1.57	-	-	-
10438049	NM_015773	Sperm associated antigen 6	Spag6	Cell motion	-	-	-1.50	-	-	-
10440794	NM_025642	RIKEN cdna 2610039C10 gene	2610039C10Rik	M phase of mitotic cell cycle	-	-	1.60	-	-	1.53
10605674	NM_008892	Polymerase (DNA directed), alpha 1	Pola1	S phase of mitotic cell cycle	-	-	-	-	-	1.73
10380067	NM_011129	Septin 4	Sep4	Germ cell development	-	-	-	-	-	-1.54
10492558	NM_133786	Structural maintenance of chromosomes 4	Smc4	Mitotic sister chromatid segregation	-	-	-	-	-	1.60
10409240	NM_013660	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D	Sema4d	Positive regulation of cell development	-	-	-	-	-	1.60
10451993	NM_080837	DNA segment, Chr 17, Wayne State University 104, expressed	D17Wsu104e	Positive regulation of cell proliferation	-	-	1.52	-	-	-

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10371607	NM_027878	RIKEN cdna 1200002N14 gene	1200002N14Rik	Apoptosis	-	-	-1.51	-	-	-
10345666	NM_026850	Phosducinlike 3	Pdcl3	Apoptosis	-	-	-	-	-	1.54
10418480	NM_153547	Guanine nucleotide binding proteinlike 3 (nucleolar)	Gnl3	Regulation of cell proliferation	-	-	-	-	-	1.59
10534667	NM_008871	Serine (or cysteine) peptidase inhibitor, clade E, member 1	Serpine1	Regulation of cell proliferation	-	-	-	-	-	1.64
10436304	NM_001014423	ABI gene family, member 3 (NESH) binding protein	Abi3bp	Regulation of cellsubstrate adhesion	-	-2.46	-4.03	-	-	-
10474201	NM_008505	LIM domain only 2	Lmo2	Regulation of myeloid cell differentiation	-	-	-	-	-	-1.53
10379630	NM_011408	Schlafen 2	Slfn2	Negative regulation of cell proliferation	-	-	-	-	-	1.74
10558325	NM_025392	BRCA2 and CDKN1A interacting protein	Bccip	Regulation of cyclin-dependent protein kinase activity	-	-	-	-	-	1.52
Cell Signaling										
10473444	NM_011784	Apelin receptor	Agtrl1	Cell surface receptor linked signal transduction	-	-3.24	-4.92	-	-	-
10604375	NM_013912	Apelin	Apln	Cell surface receptor linked signal transduction	-	-	-1.61	-	-	-
10590623	NM_030712	Chemokine (CXC motif) receptor 6	Cxcr6	Cell surface receptor linked signal transduction	-	6.54	4.58	-	-	-
10348451	NM_007722	Chemokine (CXC motif) receptor 7	Cxcr7	Cell surface receptor linked signal transduction	-	-	1.69	-	-	-
10445953	NM_139138	EGFlike module containing, mucinlike, hormone receptorlike sequence 4	Emr4	Cell surface receptor linked signal transduction	-	-	-1.92	-	-	-1.67
10416340	NM_008115	Glial cell line derived neurotrophic factor family receptor alpha 2	Gfra2	Cell surface receptor linked signal transduction	-	-	-	-	-	-1.56
10502780	NM_001081298	Latrophilin 2	Lphn2	Cell surface receptor linked signal transduction	-	-	-1.65	-	-	-
10414271	NM_008964	Prostaglandin E receptor 2 (subtype EP2)	Ptger2	Cell surface receptor linked signal transduction	-	-	1.66	-	-	-

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10350733	NM_011267	Regulator of Gprotein signaling 16	Rgs16	Cell surface receptor linked signal transduction	-	-	-	-	-	1.54
10537789	NM_207028	Taste receptor, type 2, member 126	Tas2r126	Cell surface receptor linked signal transduction	-	-	-1.61	-	-	-
10418729	NM_009937	Collagenlike tail subunit (single strand of homotrimer) of asymmetric acetylcholinesterase	Colq	Cell-cell signaling	-	-	-	-	-	-1.73
10480459	NM_080462	Histamine Nmethyltransferase	Hnmt	Cell-cell signaling	-	-	-1.64	-	-	-
10433114	NM_010577	Integrin alpha 5 (fibronectin receptor alpha)	Itga5	Cell-substrate junction assembly	-	-	1.82	-	-	-
10574027	NM_013602	Metallothionein 1	Mt1	Cellular ion homeostasis	-	1.73	3.65	-	-	-
10574023	NM_008630	Metallothionein 2	Mt2	Cellular ion homeostasis	-	3.08	9.58	-	-	-
10450038	NM_020581	Angiopoietinlike 4	Angptl4	Cellular response to starvation	-	-	1.70	-	-	-
10413419	NM_027871	Rho guanine nucleotide exchange factor (GEF) 3	Arhgef3	Intracellular signaling cascade	-	-	-1.51	-	-	-1.51
10352348	NM_030131	Cornichon homolog 4 (Drosophila)	Cnih4	Intracellular signaling cascade	-	-	1.55	-	-	-
10585982	NM_173018	myosin IXa	MYO9A	intracellular signaling cascade	-	-	-1.51	-	-	-
10476401	NM_019677	Phospholipase C, beta 1	Plcb1	Intracellular signaling cascade	-	-	-2.58	-	-	-
10578986	NM_030263	Pleckstrin and Sec7 domain containing 3	Psd3	Intracellular signaling cascade	-	-	-1.64	-	-	-
10497358	NM_001173460	Signalregulatory protein beta 1	Sirpb1	Intracellular signaling cascade	-	-	-	-	-	1.67
10472289	NM_011529	TRAF family memberassociated Nfkappa B activator	Tank	Intracellular signaling cascade	-	-	-	-	-	1.83
10496789	NM_022983	Lysophosphatidic acid receptor 3	Lpar3	MAPKKK cascade	-	-	-	-	-	-1.50
10536505	NM_008591	Met protooncogene	Met	MAPKKK cascade	-	-	-1.77	-	-	-
10606532	NM_021330	Acid phosphatase 1, soluble	Acp1	Protein amino acid dephosphorylation	-	-	1.55	-	-	-
10478897	NM_011201	Protein tyrosine phosphatase, nonreceptor type 1	Ptpn1	Protein amino acid dephosphorylation	-	-	1.67	-	-	-
10357191	NM_019933	Protein tyrosine phosphatase, nonreceptor type 4	Ptpn4	Protein amino acid dephosphorylation	-	-	-2.07	-	-	-
10513957	NM_011211	Protein tyrosine phosphatase, receptor type, D	Ptprd	Protein amino acid dephosphorylation	-	-	-1.85	-	-	-

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10412667	NM_008981	Protein tyrosine phosphatase, receptor type, G	Ptprg	Protein amino acid dephosphorylation	-	-	-1.51	-	-	-
10356880	NM_009183	ST8 alphaNacetylneuraminide alpha2,8sialyltransferase 4	St8sia4	Protein amino acid glycosylation	-	-	-1.78	-	-	-
10606235	NM_175358	Zinc finger, DHHC domain containing 15	Zdhhc15	Protein amino acid lipidation	-	-	1.55	-	-	-
10563014	NM_019830	Protein arginine Nmethyltransferase 1	Prmt1	Protein amino acid methylation	-	-	1.57	-	-	-
10532630	NM_177078	Adrenergic receptor kinase, beta 2	Adrbk2	Protein amino acid phosphorylation	-	-	-1.50	-	-	-
10443408	NM_011950	Mitogenactivated protein kinase 13	Mapk13	Protein amino acid phosphorylation	-	-	1.67	-	-	-
10461934	NM_153417	Transient receptor potential cation channel, subfamily M, member 6	Trpm6	Protein amino acid phosphorylation	-	-	-1.94	-	-	-
10498576	NM_016753	Latexin	Lxn	Sensory perception	-	-	-	-	-	1.56
10510197	NM_144880	protein phosphatase 2, regulatory subunit B (B56), alpha isoform	Ppp2r5a	Signal transduction	-	-	-1.51	-	-	-
10492798	NM_009144	Secreted frizzledrelated protein 2	Sfrp2	Response to extracellular stimulus	-	-	-1.55	-	-	-
10519527	NM_011076	ATP binding cassette, subfamily B (MDR/TAP), member 1A	Abcb1a	Response to inorganic substance	-	-	-1.56	-	-	-
10519555	NM_011075	ATP binding cassette, subfamily B (MDR/TAP), member 1B	Abcb1b	Response to inorganic substance	-	-	-	-	-	1.77
10409031	NM_025900	DEK oncogene (DNA binding)	Dek	Response to organic substance	-	-	-	-	-	1.53
10596575	NM_029103	Mesencephalic astrocytederived neurotrophic factor	Armet	Response to unfolded protein	-	-	1.58	-	-	1.70
10441718	NM_016694	Parkinson disease (autosomal recessive, juvenile) 2, parkin	Park2	Regulation of neurotransmitter levels	-	-	-1.53	-	-	-
10402435	NM_008458	Serine (or cysteine) peptidase inhibitor, clade A, member 3C	Serpina3c	Response to endogenous stimulus	-	-	1.59	-	-	-
Tissue/Muscle Development or Re-organization										
10414537	NM_021472	Angiogenin, ribonuclease, rnase A family, 5	Ang	Angiogenesis	-	-	-1.50	-	-	-
10401607	NM_008827	Placental growth factor	Pgf	Angiogenesis	-	-	-	-	-	1.53
10584288	NM_028783	Roundabout homolog 4 (Drosophila)	Robo4	Angiogenesis	-	-	-1.51	-	-	-
10490731	NM_009236	SRYbox containing gene 18	Sox18	Angiogenesis	-	-	-	-	-	-1.53
10404429	NM_009256	Serine (or cysteine) peptidase inhibitor, clade B, member 9	Serpinb9	Antiapoptosis	-	-	-	-	-	1.54
10458894	NM_010728	Lysyl oxidase	Lox	Blood vessel development	-	2.13	4.04	-	-	1.71

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10519886	NM_013657	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	Sema3c	Blood vessel development	-	-	-1.80	-	-	-
10393449	NM_007707	Suppressor of cytokine signaling 3	Socs3	Blood vessel development	-	-	-	-	-	1.71
10489204	NM_009373	Transglutaminase 2, C polypeptide	Tgm2	Blood vessel development	-	-	1.74	-	-	-
10387932	NM_011072	Profilin 1	Pfn1	Embryonic epithelial tube formation	-	-	1.59	-	-	-
10575976	NM_030209	Cysteinerich secretory protein LCCL domain containing 2	Crispld2	Extracellular matrix organization	-	-	1.53	-	-	-
10559043	NM_010844	Mucin 5, subtypes A and C, tracheobronchial/gastric	Muc5ac	Extracellular matrix organization	-	-	-	6.94	-	2.33
10485982	NM_009608	Actin, alpha, cardiac muscle 1	Actc1	Cytoskeleton organization	-	-	-2.73	-	-	-
10534343	NM_007925	Elastin	Eln	Cytoskeleton organization	1.67	1.97	4.20	-	-	-
10412260	NM_008046	Follistatin	Fst	Hair follicle development	-	2.41	4.53	-	-	-
10391036	NM_008471	Keratin 19	Krt19	In utero embryonic development	-	-	1.67	-	-	-
10543273	NM_080285	Cortactin binding protein 2	Cttnbp2	Microtubule cytoskeleton organization	-	-	-	-	-	-1.60
10450605	NM_011655	Tubulin, beta 5	Tubb5	Microtubule cytoskeleton organization	-	-	-	-	-	1.63
10458428	NM_013840	Ubiquitously expressed transcript	Uxt	Microtubule cytoskeleton organization	-	-	1.50	-	-	-
10545515	NM_001164669	Dynein, axonemal, heavy chain 6	Dnahc6	Microtubule based process	-	-	-2.01	-	-	-
10354563	NM_001160386	Dynein, axonemal, heavy chain 7B	Dnahc7b	Microtubule based process	-	-	-1.58	-	-	-
10587107	NM_010864	Myosin VA	Myo5a	Regulation of action potential	-	-	-	-	-	1.75
10447708	NM_021881	Quaking	Qk	Regulation of action potential	-	-	-1.50	-	-	-
10406598	NM_172588	Serine incorporator 5	Serinc5	Regulation of action potential	-	-	-	-	-	-1.60
10354309	NM_007737	Collagen, type V, alpha 2	Col5a2	Skeletal system development	-	-	-	-	-	1.53

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10494548	NM_008121	Gap junction membrane channel protein alpha 5	Gja5	Skeletal system development	-	-	-2.14	-	-	-
10606714	NM_013463	Galactosidase, alpha	Gla	Skeletal system development	-	-	-	-	-	1.52
10445119	NM_013819	Histocompatibility 2, M region locus 3	H2M3	Skeletal system development	-	-	-	-	-	1.54
10520362	NM_153526	Insulin induced gene 1	Insig1	Skeletal system development	-	-	-	-	-	-1.74
10456046	NM_001146268	Platelet derived growth factor receptor, beta polypeptide	Pdgfrb	Skeletal system development	-	-	-	-	-	-1.50
10554693	NM_023377	Starrelated lipid transfer (START) domain containing 5	Stard5	Steroid biosynthetic process	-	-	1.54	-	-	-
10452815	NM_011723	Xanthine dehydrogenase	Xdh	Tissue homeostasis	-	-	1.54	-	-	-
10423388	NM_133365	Dynein, axonemal, heavy chain 5	Dnahc5	Ciliary or flagellar motility	-	-	-1.65	-	-	-
10493850	NM_011468	Small prolinerich protein 2A	Sprr2a	Ectoderm development	-	-	-	2.11	4.37	5.93
Carbohydrate Metabolism										
10471675	NM_025374	Glyoxalase 1	Glo1	Carbohydrate metabolic process	-	-	-	-	-	1.57
10422655	ENSMUST00000	Glyceraldehyde3phosphate dehydrogenase pseudogene	Gapdh	Carbohydrate Metabolism	-	-	1.55	-	-	-
10498405	NR_002890	glyceraldehyde-3-phosphate dehydrogenase	Gapdh|LOC433845	Glucose metabolic process	-	-	1.51	-	-	-
10474239	ENSMUST00000	glyceraldehyde-3-phosphate dehydrogenase	Gm2606	Glucose metabolic process	-	-	-	-	-	1.58
10497321	NM_023418	phosphoglycerate mutase 1	Pgam1	Glucose metabolic process	-	-	1.71	-	-	-
10605571	NM_212444	Glycerol kinase	Gyk	Glycerol metabolic process	-	-	-	-	-	1.65
10595718	NM_018763	Carbohydrate sulfotransferase 2	Chst2	Monosaccharide metabolic process	-	-	-1.62	-	-	-
10473240	NM_023119	Enolase 1, alpha nonneuron	Eno1	Monosaccharide metabolic process	-	-	-	-	-	1.63
10368612	NM_008084	Glyceraldehyde3phosphate dehydrogenase	Gapdh	Monosaccharide metabolic process	-	-	-	-	-	1.59
10458547	NM_011937	Glucosamine6phosphate deaminase 1	Gnpda1	Monosaccharide metabolic process	-	-	1.54	-	-	-
10585932	NM_011099	Pyruvate kinase, muscle	Pkm2	Monosaccharide metabolic process	-	-	1.75	-	-	-
10502522	NM_011828	Heparan sulfate 2 O sulfotransferase 1	Hs2st1	Polysaccharide biosynthetic process	-	-	-1.61	-	-	-
10349968	NM_007695	Chitinase 3 like 1	Chi3l1	Polysaccharide catabolic process	-	-	-2.28	-	-	-

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10501020	NM_009892	Chitinase 3 like 3	Chi3l3	Polysaccharide catabolic process	-	-	2.13	-	-	-
10501026	NM_145126	Chitinase 3 like 4	Chi3l4	Polysaccharide catabolic process	-	-	-	9.65	-	2.69
10413615	NM_018746	Inter alphatrypsin inhibitor, heavy chain 4	Itih4	Polysaccharide metabolic process	-	-	-2.08	-	-	-
10458569	NM_008173	Nuclear receptor subfamily 3, group C, member 1	Nr3c1	Regulation of carbohydrate metabolic process	-	-	-1.74	-	-	-
Protein or Amino Acid Metabolism/Regulation										
10368343	NM_007482	Arginase, liver	Arg1	Arginine metabolic process	-	1.82	3.83	-	-	-
10487011	NM_025961	Glycine amidinotransferase (Larginine:glycine amidinotransferase)	Gatm	Cellular amino acid derivative metabolic process	-	6.96	4.89	-	-	-
10345203	NM_153783	Polyamine oxidase (exoN4amino)	Paox	Cellular amino acid derivative metabolic process	-	-	1.57	-	-	-
10362129	NM_011979	Vanin 3	Vnn3	Cellular amino acid derivative metabolic process	-	-	-	-	-	1.57
10546113	NM_016906	Sec61 alpha 1 subunit (S. Cerevisiae)	Sec61a1	Intracellular protein transport	-	-	1.54	-	-	-
10360225	NM_016897	Translocase of inner mitochondrial membrane 23 homolog (yeast)	Timm23	Intracellular protein transport	-	-	1.52	-	-	1.60
10539119	NM_019802	Gammaglutamyl carboxylase	Ggcx	Peptidylglutamic acid carboxylation	-	-	-1.65	-	-	-
10543686	NM_009459	Ubiquitin-conjugating enzyme E2H	Ube2h	Protein catabolic process	-	-	-1.54	-	-	-
10469712	NM_019501	Prenyl (solanesyl) diphosphate synthase, subunit 1	Pdss1	Protein complex assembly	-	-	1.78	-	-	-
10434675	NM_026400	Dnaj (Hsp40) homolog, subfamily B, member 11	Dnajb11	Protein folding	-	-	-	-	-	1.53
10381122	NM_010221	FK506 binding protein 10	Fkbp10	Protein folding	-	-	1.78	-	-	-
10544885	NM_153573	FK506 binding protein 14	Fkbp14	Protein folding	-	-	1.82	-	-	-
10449452	NM_010220	FK506 binding protein 5	Fkbp5	Protein folding	-	2.06	5.76	-	-	1.62
10359034	NM_001024945	Quiescin Q6 sulfhydryl oxidase 1	Qsox1	Protein folding	-	-	1.91	-	-	-
10415844	NM_007798	Cathepsin B	Ctsb	Proteolysis	-	-	-	-	-	1.52
10410124	NM_009984	Cathepsin L	Ctsl	Proteolysis	-	-	1.89	-	-	-
10490212	NM_022325	Cathepsin Z	Ctsz	Proteolysis	-	-	-	-	-	1.64
10581434	NM_176913	Dipeptidase 2	Dpep2	Proteolysis	-	-	1.80	-	-	-
10406817	NM_007930	Ectodermalneural cortex 1	Enc1	Proteolysis	-	-	1.76	-	-	-

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10427904	BC050864	Fbox and leucinerich repeat protein 7	Fbxl7	Proteolysis	-	-	-1.55	-	-	-
10583056	NM_008605	Matrix metallopeptidase 12	Mmp12	Proteolysis	-	-	-	-	3.30	4.62
10570855	NM_008872	Plasminogen activator, tissue	Plat	Proteolysis	-	-	2.05	-	-	-
10448409	NM_133731	Protease, serine, 22	Prss22	Proteolysis	-	-	2.01	-	-	1.60
10585474	NM_011966	Proteasome (prosome, macropain) subunit, alpha type 4	Psma4	Proteolysis	-	-	-	-	-	1.62
10365426	NM_011967	Proteasome (prosome, macropain) subunit, alpha type 5	Psma5	Proteolysis	-	-	-	-	-	1.52
10508182	NM_011970	Proteasome (prosome, macropain) subunit, beta type 2	Psmb2	Proteolysis	-	-	1.53	-	-	1.66
10380815	NM_011971	Proteasome (prosome, macropain) subunit, beta type 3	Psmb3	Proteolysis	-	-	1.56	-	-	-
10419810	NM_011186	Proteasome (prosome, macropain) subunit, beta type 5	Psmb5	Proteolysis	-	-	1.56	-	-	-
10561679	NM_026545	Proteasome (prosome, macropain) 26S subunit, nonatpase, 8	Psmd8	Proteolysis	-	-	1.56	-	-	-
10354191	NM_001033135	Ring finger protein 149	Rnf149	Proteolysis	-	-	-	-	-	1.55
10389719	NM_029023	Serine carboxypeptidase 1	Scpep1	Proteolysis	-	-	1.58	-	-	-
10520124	NM_133354	SMT3 suppressor of mif two 3 homolog 2 (yeast)	Sumo2	Proteolysis	-	-	-	-	-	1.50
10516735	NM_023476	Tubulointerstitial nephritis antigenlike 1	Tinagl	Proteolysis	-	-	2.00	-	-	-
10394532	NM_026454	Ubiquitinconjugating enzyme E2F (putative)	Ube2f	Proteolysis	-	-	-	-	-	1.70
10350489	NM_019562	Ubiquitin carboxylterminal esterase L5	Uchl5	Proteolysis	-	-	1.53	-	-	1.80
10599369	NM_009688	Xlinked inhibitor of apoptosis	Xiap	Proteolysis	-	-	-1.51	-	-	-
10412251	NM_010887	NADH dehydrogenase (ubiquinone) FeS protein 4	Ndufs4	Regulation of protein amino acid phosphorylation	-	-	-	-	-	1.57
10492689	NM_019971	Platelet derived growth factor, C polypeptide	Pdgfc	Regulation of protein amino acid phosphorylation	-	-	1.60	-	-	-
10583021	NM_027924	Platelet derived growth factor, D polypeptide	Pdgfd	Regulation of protein amino acid phosphorylation	-	-	-2.30	-	-	-
10408557	NM_025429	Serine (or cysteine) peptidase inhibitor, clade B, member 1a	Serpinb1a	Regulation of protein catabolic process	-	-	-1.61	-	-	-
10365769	NM_010401	Histidine ammonia lyase	Hal	Histidine metabolic process	-	3.28	4.78	-	-	-

Affy Transcript ID	Accession ID	Gene Name	Gene Symbol	Biological Function	Fold Change					
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Protein Transport										
10498024	NM_011990	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 11	Slc7a11	Amino acid transport	-	-	-	-	-	2.21
10582275	NM_011404	Similar to solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	Slc7a5	Amino acid transport	-	-	-	-	-	1.54
10574985	NM_178798	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 6	Slc7a6	Amino acid transport	-	-	1.87	-	-	-
10462822	NM_175353	Exocyst complex component 6	Exoc6	Exocytosis	-	-	-1.51	-	-	-
10374106	NM_019661	YKT6 homolog (S. Cerevisiae)	Ykt6	Exocytosis	-	-	1.50	-	-	-
10531383	NM_172713	SDA1 domain containing 1	Sdad1	Protein localization	-	-	-	-	-	1.60
10538290	NM_028035	Sorting nexin 10	Snx10	Protein localization	-	-	-	-	-	1.73
10488237	NM_024225	Sorting nexin 5	Snx5	Protein localization	-	-	-	-	-	1.51
10501762	NM_029655	Sorting nexin 7	Snx7	Protein localization	-	-	-	-	-	1.53
10420637	NM_008466	Karyopherin (importin) alpha 3	Kpna3	Protein targeting	-	-	-	-	-	1.61
10346634	NM_018868	NOP58 ribonucleoprotein homolog (yeast)	Nol5	Protein targeting	-	-	-	-	-	1.79
10606689	NM_013898	Translocase of inner mitochondrial membrane 8 homolog a1 (yeast)	Timm8a1	Protein targeting	-	-	1.58	-	-	-
10477370	NM_024214	Similar to Translocase of outer mitochondrial membrane 20 homolog (yeast)	Tomm20	Protein targeting	-	-	-	-	-	1.87
Other Metabolic Processes										
10433494	NM_011955	Nucleotide binding protein 1	Nubp1	Cofactor metabolic process	-	-	1.62	-	-	-
10414522	NM_009687	Apurinic/apyrimidinic endonuclease 1	Apex1	DNA metabolic process	-	-	1.59	-	-	-
10451225	NM_030715	Polymerase (DNA directed), eta (RAD 30 related)	Polh	DNA metabolic process	-	-	1.61	-	-	-
10461487	NM_201351	Cytochrome b, ascorbate dependent 3	Cybasc3	Generation of precursor metabolites and energy	-	-	-	-	-	-1.61
10353624	---	NADHubiquinone oxidoreductase chain 4L	ND4L	Generation of precursor metabolites and energy	-	-	-1.79	-	-	-
10598067	---	Predicted gene 4076	ND5	Generation of precursor metabolites and energy	-	-	-1.56	-	-	-

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10452525	NM_028388	NADH dehydrogenase (ubiquinone) flavoprotein 2	Ndufv2	Generation of precursor metabolites and energy	-	-	-	-	-	1.55
10536931	NM_021414	Sadenosylhomocysteine hydrolaselike 2	Ahcyl2	One-carbon metabolic process	-	-	-1.51	-	-	-
10490913	NM_007606	Carbonic anhydrase 3	Car3	One-carbon metabolic process	-	-2.38	-5.61	-	-	-
10556302	NM_009667	Adenosine monophosphate deaminase 3	Ampd3	Purine base metabolic process	-	-	1.65	-	-	-
10413086	NM_134079	Adenosine kinase	Adk	Purine nucleotide metabolic process	-	-	-1.55	-	-	-
10587639	NM_011851	5' nucleotidase, ecto	Nt5e	Purine nucleotide metabolic process	-	-	1.53	-	-	-
10515164	NM_025647	Cytidine monophosphate (UMPCMP) kinase 1	Cmpk1	Pyrimidine nucleotide metabolic process	-	-	-	-	-	1.65
10395039	NM_020557	Cytidine monophosphate (UMPCMP) kinase 2, mitochondrial	Cmpk2	Pyrimidine nucleotide metabolic process	-	-	1.57	-	-	-
10515943	NM_016748	Cytidine 5'triphosphate synthase	Ctps	Pyrimidine nucleotide metabolic process	-	-	-	-	-	1.71
10427461	NM_001136079	Prostaglandin E receptor 4 (subtype EP4)	Ptger4	Regulation of nucleotide metabolic process	-	-	-	-	-	1.64
10561008	NM_001039185	Carcinoembryonic antigenrelated cell adhesion molecule 1	Ceacam1	Regulation of phosphate metabolic process	-	-	-	-	-	1.51
10395273	BC052902	Gangliosideinduced differentiationassociatedprotein 10	Gdap10	Response to nutrient	-	-	1.51	-	-	-
10472538	NM_175512	Dehydrogenase/reductase (SDR family) member 9	Dhrs9	Retinoid metabolic process	-	-	1.81	-	-	-
10463836	NM_010362	Glutathione Stransferase omega 1	Gsto1	Vitamin metabolic process	-	-	1.59	-	-	-
10483439	NM_001081088	Low density lipoprotein receptorrelated protein 2	Lrp2	Vitamin metabolic process	-	-	-1.72	-	-	-
10453057	NM_009994	Cytochrome P450, family 1, subfamily b, polypeptide 1	Cyp1b1	Xenobiotic metabolic process	-	-	2.03	-	-	-
Nucleotide/DNA/Chromosome Binding										
10472782	NM_026115	Histone aminotransferase 1	Hat1	Chromatin organization	-	-	-	-	-	1.83
10530759	NM_080560	Ubiquitinconjugating enzyme E2N	Ube2n	DNA doublestrand break processing	-	-	-	-	-	1.66
10494407	NM_175666	Histone cluster 1, h2bg	Hist2h2bb	DNA packaging	-	-	1.55	-	-	-
10500333	NM_033596	Histone cluster 1, h4k	Hist2h4	DNA packaging	-	-	1.72	-	-	-

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10505894	NM_024433	Methylthioadenosine phosphorylase	Mtap	Nucleoside metabolic	-	-	-	-	-	1.54
10607877	NM_026662	Phosphoribosyl pyrophosphate synthetase 2	Prps2	Nucleoside metabolic process	-	-	-1.59	-	-	-
10381458	NR_004414	U2 small nuclear RNA	Rnu2	Nucleotide binding	-	-	-	-	-	2.22
10523012	NM_007832	Deoxycytidine kinase	Dck	Nucleotide transport and metabolism	-	-	-	-	-	1.62
10359851	NM_030724	Uridine-cytidine kinase 2	Uck2	Nucleotide transport and metabolism	-	-	1.70	-	-	-
Others										
10542953	NM_009364	Tissue factor pathway inhibitor 2	Tfpi2	Blood coagulation	-	-	1.62	-	-	1.62
10395365	NM_011783	Anterior gradient 2 (xenopus laevis)	Agr2	Body fluid secretion	-	-	-	5.41	1.92	4.33
10533504	NM_009879	Intraflagellar transport 81 homolog (chlamydomonas)	Ift81	Gamete generation	-	-	-1.54	-	-	-
10432986	NM_153416	Achalasia, adrenocortical insufficiency, alacrimia	Aaas	Nucleocytoplasmic transport	-	-	1.73	-	-	-
10537146	NM_008012	Aldoketo reductase family 1, member b8	Akr1b8	Oxidation reduction	-	-	1.50	-	-	-
10346410	NM_023617	Aldehyde oxidase 3	Aox3	Oxidation reduction	-	-3.45	-5.64	-	-	-
10551226	NM_009997	Cytochrome p450, family 2, subfamily a, polypeptide 21, pseudogene	Cyp2a4|Cyp2a5	Oxidation reduction	-	-	-	-	-	-1.61
10367691	NM_027391	Iodotyrosine deiodinase	Iyd	Oxidation reduction	-	-	-1.67	-	-	-
10518408	NM_011122	Procollagenlysine, 2oxoglutarate 5dioxygenase 1	Plod1	Oxidation reduction	-	-	1.72	-	-	-
10545682	NM_183138	Tet oncogene family member 3	Tet3	Oxidation reduction	-	-	-1.51	-	-	-
10537316	NM_009729	ATPase, h+ transporting, lysosomal v0 subunit c | atpase, h+ transporting, lysosomal v0 subunit c, pseudogene 2	Atp6v0c|Atp6v0c-ps2	Oxidative phosphorylation	-	-	1.66	-	-	-
10479411	NM_031373	Opioid growth factor receptor	Ogfr	Regulation of growth	-	-	1.58	-	-	-
10414202	NM_023134	Surfactant associated protein a1	Sftpa1	Respiratory gaseous exchange	-	-	-1.79	-	-	-
10492682	NM_133187	Riken cDNA 1110032e23 gene	1110032E23Rik	-	-	-	-	-	-	-1.54
10588109	NR_028123	Riken cDNA 1600029i14 gene	1600029I14Rik	-	-	-	-1.58	-	-	-
10481272	BC099566	Riken cDNA 1700007k13 gene	1700007K13Rik	-	-	-	-1.50	-	-	-
10565241	NM_029335	Riken cDNA 1700026d08 gene	1700026D08Rik	-	-	-	-1.72	-	-	-

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10354275	BC119032	Riken cDNA 1700029f09 gene	1700029F09Rik	-	-	-	-	-	-	1.94
10493626	NM_028567	Riken cDNA 1700094d03 gene	1700094D03Rik	-	-	-	-1.58	-	-	-
10565210	NM_177894	Family with sequence similarity 154, member b	1700129I04Rik	-	-	-	-1.52	-	-	-
10569877	NM_026985	Riken cDNA 1810033b17 gene	1810033B17Rik	-	-	-	1.52	-	-	-
10526452	NM_027158	Riken cDNA 2310043j07 gene	2310043J07Rik	-	-	-	1.68	-	-	-
10485378	NM_001083810	Proline rich 5 like	2600010E01Rik	-	-	-	-1.83	-	-	-
10415377	NM_026403	Riken cDNA 2610027l16 gene	2610027L16Rik	-	-	-	1.50	-	-	-
10601335	BC035042	WD repeat domain 43	2610029G23Rik	-	-	-	-	-	-	1.50
10595614	BC032970	Riken cDNA 2810026p18 gene	2810026P18Rik	-	-	-	1.53	-	-	-
10436598	---	Riken cDNA 2810055g20 gene	2810055G20Rik	-	-	-	-1.70	-	-	-
10397230	NM_028377	Riken cDNA 2900006k08 gene	2900006K08Rik	-	-	-	-1.51	-	-	-
10368041	NM_028440	Riken cDNA 3110003a17 gene	3110003A17Rik	-	-	-	-	-	-	1.76
10425903	---	Riken cDNA 3110043j09 gene | similar to glyceraldehyde-3-phosphate dehydrogenase (gapdh)	3110043J09Rik	-	-	-	-	-	-	1.66
10374578	NM_028672	Family with sequence similarity 161, member a	4930430E16Rik	-	-	-	-1.58	-	-	-
10438178	NM_029053	Riken cDNA 4930451c15 gene	4930451C15Rik	-	-	-	-1.55	-	-	-
10371916	ENSMUST00000	Riken cDNA 4930485b16 gene	4930485B16Rik	-	-	-	-1.51	-	-	-
10527285	ENSMUST00000	Radial spoke head 10 homolog b (chlamydomonas)	4930526H21Rik	-	-	-	-1.67	-	-	-
10476795	BC048610	Riken cDNA 4930529m08 gene	4930529M08Rik	-	-	-	-1.54	-	-	-
10562368	BC060233	Riken cDNA 4931406p16 gene	4931406P16Rik	-	-	-	-1.52	-	-	-
10439881	NR_028300	Riken cDNA 5330426p16 gene	5330426P16Rik	-	-	-	-1.53	-	-	-

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10586484	NM_026635	Family with sequence similarity 96, member a	5730536A07Rik	-	-	-	-	-	-	1.59
10584561	NM_133733	Riken cDNA 9030425e11 gene	9030425E11Rik	-	-	-	1.68	-	-	-
10488409	NM_029960	Riken cDNA 9230104l09 gene	9230104L09Rik	-	-	-2.23	-3.80	-	-	-
10577388	NM_001039553	RIKEN cDNA 4930467E23 gene | pre	4930467E23Rik	-	-	-	-1.56	-	-	-
10466963	NM_172836	Riken cDNA 9930021j03 gene	9930021J03Rik	-	-	-	-1.51	-	-	-
10356271	NM_213615	RIKEN cDNA A530032D15Rik gene	A530032D15Rik	-	-	-	-	-	-	1.71
10519951	NM_175437	Pigeon homolog (drosophila)	A530088I07Rik	-	-	-	-1.51	-	-	-
10355998	ENSMUST00000	Family with sequence similarity 124, member b	A830043J08Rik	-	-	-	-	-	-	-1.51
10443027	NM_029870	Riken cDNA a930001n09 gene	A930001N09Rik	-	-	-	-1.63	-	-	-
10356995	---	Ensmusg00000073605	AC157923.3	-	-	-	-1.65	-	-	-
10415019	---	---	AK030929	-	-	-	-	-	-	1.50
10421749	NM_001164503	A kinase (prka) anchor protein 11	Akap11	-	-	-	-1.52	-	-	-
10604094	NM_001033785	A kinase (prka) anchor protein 14	Akap14	-	-	-	-1.66	-	-	-
10449854	NM_017476	A kinase (prka) anchor protein 8like	Akap8l	-	-	-	-1.55	-	-	-
10479979	NM_138756	Ensmusg00000082953	AL929440.1	-	-	-	-1.80	-	-	-
10578515	NM_001039562	Ankyrin repeat domain 37	Ankrd37	-	-	-	-	-	-	1.52
10439009	NM_007470	Apolipoprotein d	Apod	-	-	-	1.66	-	-	-
10538753	NM_028035	Atonal homolog 1 (Drosophila)	Atoh1	-	-	-	-	-	-	1.63
10570201	NM_015804	ATPase, class vi, type 11a	Atp11a	-	-	-	-1.54	-	-	-
10516966	NM_001033308	cDNA sequence bc013712	BC013712	-	-	-	-	-	-	1.50
10500276	BC028528	cDNA sequence bc028528	BC028528	-	-	-	-1.67	-	-	-
10584691	NM_001081369	Coiledcoil domain containing 153	BC038167	-	-	-	-1.50	-	-	-
10521667	NM_009763	Bone marrow stromal cell antigen 1	Bst1	-	-	-	1.66	-	-	-
10388010	NM_007573	Complement component 1, q subcomponent binding protein	C1qbp	-	-	-	1.59	-	-	-
10404939	ENSMUST00000	Riken cDNA 2010001k21 gene	C78339	-	-	-	-	-	-	-1.62
10519855	NM_001110843	Calcium channel, voltage-dependent, alpha2/delta subunit 1	Cacna2d1	-	-	-	-1.59	-	-	-
10586118	NM_138304	Calmodulinlike 4	Calml4	-	-	-	-1.66	-	-	-
10509568	NM_025451	Calcium/calmodulindependent protein kinase ii inhibitor 1	Camk2n1	-	-	-	-	-	-	-1.56
10475378	NM_177054	Cancer susceptibility candidate 4	Casc4	-	-	-	-1.72	-	-	-
10536273	NM_145398	Cas1 domain containing 1	Casd1	-	-	-	-1.63	-	-	-

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10474437	NM_026613	Coiledcoil domain containing 34	Ccdc34	-	-	-	1.52	-	-	-
10596222	NM_145700	Chemokine (c-c motif) receptor-like 1	Ccrl1	-	-	-	-	-	-	-1.75
10435907	NM_021325	Cd200 receptor 1	Cd200r1	-	-	-	-	-	-	1.54
10482920	NM_025422	Cd302 antigen	Cd302	-	-	-	1.54	-	-	1.50
10588091	NM_023873	Centrosomal protein 70	Cep70	-	-	-	-1.98	-	-	-
10384956	NM_026527	Chac, cation transport regulator homolog 2 (e. Coli)	Chac2	-	-	-	-	-	-	1.67
10380398	NM_007689	Chondroadherin	Chad	-	-	-	-1.79	-	-	-
10347915	NM_001081746	Predicted gene 2635	Csprs	-	-	-	-	-	-	1.51
10582879	NM_033616	Component of sp100-rs	Csprs	-	-	-	-	-	-	1.50
10409876	NM_007796	Cytotoxic t lymphocyteassociated protein 2 alpha	Ctla2a	-	-	-	-1.62	-	-	-
10551282	NM_007812	Similar to cytochrome p450 2a5 (cypiia5) (coumarin 7-hydroxylase) (p450-15-coh) (p450-iia3.2)	Cyp2a5	-	-	-	-	-	-	-1.57
10345368	---	DNA segment, chr 1, erato doi 448, expressed	D1Ertd448e	-	-	-	-	-	-	-1.52
10595953	NM_177775	Family with sequence similarity 62 (c2 domain containing), member c	D9Ertd280e	-	-	-	-	-	-	-1.62
10502359	NM_011932	Dual adaptor for phosphotyrosine and 3phosphoinositides 1	Dapp1	-	-	-	-	-	-	1.61
10417829	NM_134081	DNAj (hsp40) homolog, subfamily c, member 9	Dnajc9	-	-	-	-	-	-	1.52
10511541	NM_001081201	Dpy19like 4 (c. Elegans)	Dpy19l4	-	-	-	-1.53	-	-	-
10452450	NM_001008973	Riken cDNA e130009j12 gene	E130009J12Rik	-	-	-	-1.65	-	-	-
10419154	NM_007894	Eosinophilassociated, ribonuclease a family, member 8	Ear8	-	-	-	-	-	-	-1.50
10518069	NM_025994	Ef hand domain containing 2	Efhd2	-	-	-	-	-	-	1.81
10501492	ENSMUST00000	Leptin receptor overlapping transcript	EG433643	-	-	-	-	-	-	-1.59
10520390	ENSMUST00000	Proline rich 8	EG545741	-	-	-	1.51	-	-	-
10403021		---	EG629908	-	-	3.34	6.67	-	7.74	12.34
10498972	NM_001146328	RNA binding motif protein 46	EG633285	-	-	-	1.51	-	-	-
10466843	---	similar to glyceraldehyde-3-phosphate dehydrogenase	EG667806|LOC634019	-	-	-	1.50	-	-	-
10566583	AK172683	Predicted gene 8995	EG668139	-	-	-	-	-	-	1.51
10362902		Glyceraldehyde3phosphate dehydrogenase	EG668192	-	-	-	1.59	-	-	-

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10523058	NM_001166589	Eukaryotic translation initiation factor 5a	Eif5a	-	-	-	1.53	-	-	1.67
10462111	---	Ensmusg00000054178	ENSMUSG00000054178	-	-	-	-1.61	-	-	-
10504755		Hypothetical protein loc641050	ENSMUSG00000056897	-	-	-	-	-	-	1.69
10542594	---	Ensmusg00000067389	ENSMUSG00000067389	-	-	-	-1.56	-	-	-
10503709	---	Riken cDNA d130062j21 gene	ENSMUSG00000073981	-	-	-	-2.02	-	-	-
10406452	---	---	ENSMUSG00000074792	-	-	-	-1.71	-	-	-
10344674	AK087278	Family with sequence similarity 150, member a	Fam150a	-	-	-	-	-	-	1.54
10518075	NM_177868	Forkheadassociated (fha) phosphopeptide binding domain 1	Fhad1	-	-	-	-1.64	-	-	-
10447649	NM_001081416	Fibronectin type iii domain containing 1	Fndc1	-	-	-	-1.59	-	-	-
10435641	NM_008047	Follistatinlike 1	Fstl1	-	-	-	-	-	-	1.66
10351035	NR_002840	Growth arrest specific 5	Gas5	-	-	-	1.76	-	-	1.53
10544583	NM_153175	GTPase, imap family member 6	Gimap6	-	-	-	-1.63	-	-	-
10492658	ENSMUST00000	---	Gm10291	-	-	-	-	-	-	1.51
10374764	NR_002890	Glyceraldehyde-3-phosphate dehydrogenase	Gm12070	-	-	-	-	-	-	1.51
10417667	---	Gene model 281, (NCBI)	Gm281	-	-	-	-1.69	-	-	-
10545182		---	Gm459	-	-	-	1.57	-	-	-
10361710	---	---	Gm5177	-	-	-	-	-	-	1.58
10601537	---	Similar to ubiquitin-conjugating enzyme e2n	Gm5943	-	-	-	-	-	-	1.64
10413977	XM_985917	Gene model 626, (NCBI)	Gm626	-	-	-	-1.51	-	-	-
10593490	NM_008084	glyceraldehyde-3-phosphate dehydro	Gapdh	-	-	-	-	-	-	1.54
10466735	NM_001114174	---	Gm967	-	-	-	-	-	-	-1.79
10360415	NM_011825	Gremlin 2 homolog, cysteine knot superfamily (xenopus laevis)	Grem2	-	-	-	-1.73	-	-	-
10577882	NM_029884	Heparanalphaglucosaminide nacetyltransferase	Hgsnat	-	-	-	-	-	-	-1.59
10368527	NM_025798	Histidine triad nucleotide binding protein 3	Hint3	-	-	-	-1.54	-	-	-
10404051	NM_175654	histone cluster 1, H4d	Hist1h4d	-	-	-	1.90	-	-	-
10575380	NM_172916	Hydrocephalus inducing	Hydin	-	-	-	-1.70	-	-	-
10346799	NM_017480	Inducible tcell costimulator	Icos	-	-	-	-	-	-	1.91
10436865	NM_008338	Interferon gamma receptor 2	Ifngr2	-	-	-	-	-	-	1.52

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10403034	---	Immunoglobulin heavy chain complex	Igh	-	-	8.64	12.32	-	14.49	19.42
10403054	ENSMUST0000(	Similar to monoclonal antibody heavy chain	IghIa	-	-	2.46	5.12	-	-	-
10403060	---	Immunoglobulin heavy variable v1-72	Ighv1-72	-	-	2.04	4.26	-	-	-
10545220	BC019474	Immunoglobulin kappa chain variable 12-41	Igk	-	-	-	-	-	6.87	9.11
10545239	---	Immunoglobulin kappa chain variable 8 (v8)-21	Igk-V8-21	-	-	-	3.35	-	-	-
10345752	NM_010555	Interleukin 1 receptor, type ii	Il1r2	-	-	-	-	-	-	2.14
10576692	NM_010568	Insulin receptor	Insr	-	-	-	-1.63	-	-	-
10548409	NM_001136068	Killer cell lectinlike receptor subfamily c, member 1	Klrc1	-	-	5.94	2.80	-	-	-
10438435	NM_177356	Lysosomalassociated membrane protein 3	Lamp3	-	-	-	-2.59	-	-	-
10360745	NM_133815	Lamin b receptor	Lbr	-	-	-	-	-	-	-1.50
10360542	NM_025511	Hypothetical loc100270747	LOC100270747	-	-	-	-1.54	-	-	-
10497372	DQ055451	Similar to sirp beta 1 like 1 protein	LOC381484	-	-	-	-	-	-	1.51
10506652	NM_001081272	Similar to low density lipoprotein receptor a domain containing 1	LOC546840	-	-	-	-1.59	-	-	-
10362751	XR_032514	Adenylate kinase domain containing	Akd1	-	-	-	-1.52	-	-	-
10595109	NM_001146048	Leucine rich repeat containing 1	Lrrc1	-	-	-	-1.51	-	-	-
10380403	NM_133807	Leucine rich repeat containing 59	Lrrc59	-	-	-	-	-	-	1.62
10429100	NM_019457	Leucine rich repeat containing 6 (testis)	Lrrc6	-	-	-	-1.51	-	-	-
10429573	NM_010741	Lymphocyte antigen 6 complex, locus c2	Ly6c2	-	-	-	1.53	-	-	-
10429560	NM_020498	Lymphocyte antigen 6 complex, locus i	Ly6i	-	-	6.53	3.61	-	2.65	4.61
10424113	NM_178920	Mal, tcell differentiation protein 2	Mal2	-	-	-	-1.51	-	-	-
10465244	NR_002847	Metastasis associated lung adenocarcinoma transcript 1 (non-coding RNA)	Malat1	-	-	-	-1.55	-	-	-
10463224	NM_183195	Marvel (membraneassociating) domain containing 1	Marveld1	-	-	-	-	-	-	-1.60
10400460	NM_145442	Map3k12 binding inhibitory protein 1	Mbip	-	-	-	-1.69	-	-	-
10452860	NM_133771	Mediator of cell motility 1	Memo1	-	-	-	-	-	-	1.68

Affy Transcript ID	Accession ID	Gene Name	Gene Symbol	Biological Function	Fold Change					
					HDM [EP]	Flu [EP]	Flu + HDM [EP]	HDM [LP]	Flu [LP]	Flu + HDM [LP]
10377774	NM_145137	Macrophage galactose nacetylgalactosamine specific lectin 2	Mgl2	-	-	-	-1.72	-	-	-
10380135	NR_029555	microRNA 142	Mir142	-	-	-	1.55	-	-	-
10435791	NR_030576	microRNA 568	Mir568	-	-	-	-1.70	-	-	-
10607774	NM_029730	Motile sperm domain containing 2	Mospd2	-	-	-	-1.72	-	-	-
10461587	XM_889011	Membrane-spanning 4-domains, subfamily a, member 4a	Ms4a4a	-	-	4.53	7.21	2.38	1.88	3.45
10461629	NM_025658	Membranespanning 4domains, subfamily a, member 4d	Ms4a4d	-	-	-	1.52	-	-	-
10466130	NM_022430	Membranespanning 4domains, subfamily a, member 8a	Ms4a8a	-	-	-	2.09	-	-	-
10367591	NM_026793	Myc target 1	Myct1	-	-	-1.86	-3.39	-	-	-
10381798	NM_010858	Myosin, light polypeptide 4	Myl4	-	-	-	-	-	-	-1.51
10501895	NM_021503	Myozenin 2	Myoz2	-	-	-	-1.73	-	-	-
10549647	NM_010746	Natural cytotoxicity triggering receptor 1	Ncr1	-	-	-	-1.80	-	-	-
10598087		Predicted gene 4076	ND6	-	-	-	-	-	-	-1.51
10490551	NM_021426	Na+/k+ transporting atpase interacting 4	Nkain4	-	-	-	-1.58	-	-	-1.69
10532390	NM_153570	Nucleolar complex associated 4 homolog (s. Cerevisiae)	Noc4l	-	-	-	1.74	-	-	-
10448748	NM_011956	Nucleotide binding protein 2	Nubp2	-	-	-	1.63	-	-	-
10375234	NM_026023	Nudc domain containing 2	Nudcd2	-	-	-	-	-	-	1.82
10469070	NM_016918	Nudix (nucleoside diphosphate linked moiety x)type motif 5	Nudt5	-	-	-	-	-	-	1.70
10483648	NM_025942	Obglike atpase 1	Ola1	-	-	-	1.56	-	-	1.82
10544133	NM_172893	Poly (adpribose) polymerase family, member 12	Parp12	-	-	-	1.63	-	-	-
10344707	NM_183028	Proteinlisoaspartate (daspartate) omethyltransferase domain containing 1	Pcmtd1	-	-	-	-1.52	-	-	-
10497646	NM_001165954	Polyhomeotic-like 3 (drosophila)	Phc3	-	-	-	-1.62	-	-	-
10344981	NM_053191	Peptidase inhibitor 15	Pi15	-	-	-	1.84	-	-	-
10473022	NM_019755	Proteolipid protein 2	Plp2	-	-	-	1.59	-	-	-
10606495	NM_181579	Premature ovarian failure 1b	Pof1b	-	-	-	-1.67	-	-	-
10355115	NM_025596	Preli domain containing 1	Prelid1	-	-	-	1.50	-	-	1.63
10520388	NM_028234	Proline rich 8	Prr8	-	-	-	-1.72	-	-	-
10607004	NM_016883	Proteasome (prosome, macropain) 26s subunit, nonatpase, 10	Psmd10	-	-	-	-	-	-	1.57
10578027	NM_026453	Mak16 homolog (s. Cerevisiae)	Rbm13	-	-	-	-	-	-	1.65
10436095	NM_020509	Resistin like alpha	Retnla	-	-	-	-	7.32	1.92	4.31

Affy Transcript ID	Accession ID	Gene Name	Gene Symbol	Biological Function	Fold Change					
					HDM [EP]	Flu [EP]	Flu + HDM [EP]	HDM [LP]	Flu [LP]	Flu + HDM [LP]
10436100	NM_181596	Resistin like gamma	Retnlg	-	-	-	1.77	-	-	-
10569057	NM_001172101	Ribonuclease/angiogenin inhibitor 1	Rnh1	-	-	-	1.73	-	-	-
10406250	NM_009084	Ribosomal protein l37a	Rpl37a	-	-	-	-	-	-	1.50
10589695	NM_153100	Receptor transporter protein 3	Rtp3	-	-	-	-	-	-	-1.60
10493995	NM_009112	S100 calcium binding protein a10 (calpactin)	S100a10	-	-	-	1.52	-	-	-
10428594	NM_177225	Sterile alpha motif domain containing 12	Samd12	-	-	-	-2.20	-	-	-
10440393	NM_023380	Sam domain, sh3 domain and nuclear localization signals, 1	Samsn1	-	-	-	-	-	-	1.87
10461642	NR_028560	small Cajal body-specific RNA 17	Scarna17	-	-	-	-1.53	-	-	-
10450904	NM_001039137	Short coiledcoil protein	Scoc	-	-	-	-	-	-	1.71
10355984	NM_009255	Serine (or cysteine) peptidase inhibitor, clade e, member 2	Serpine2	-	-	-	1.65	-	-	-
10459905	NM_053099	Set binding protein 1	Setbp1	-	-	-	-1.59	-	-	-
10347734	NM_001004173	Sphingosine1phosphate phosphotase 2	Sgpp2	-	-	-	-1.54	-	-	-
10561721	NM_001081028	Signalinduced	Sipa1l3	-	-	-	-1.62	-	-	-
10497349	NM_001002898	Signal-regulatory protein beta 1	Sirpb1a	-	-	-	-	-	-	1.53
10377372	NM_028048	Solute carrier family 25, member 35	Slc25a35	-	-	-	-1.55	-	-	-
10379650	AF099974	Schlafen 3	Slfn3	-	-	-	-1.75	-	-	2.60
10389143	NM_181545	Schlafen 8	Slfn8	-	-	-	-	-	-	1.64
10489463	NM_011414	Secretory leukocyte peptidase inhibitor	Slpi	-	-	-	2.27	-	-	-
10584580	NR_028275	small nucleolar RNA, C/D box 14E	SNORD14	-	-	-	1.50	-	-	-
10522445	NM_178387	Spermatogenesis associated 18	Spata18	-	-	-	-1.54	-	-	-
10427681	NM_177123	Sperm flagellar 2	Spef2	-	-	-	-1.51	-	-	-
10542740	NM_010656	Sarcospan	Sspn	-	-	-	-	-	-	-1.50
10389795	NM_011505	Syntaxin binding protein 4	Stxbp4	-	-	-	-2.15	-	-	-
10481147	NM_011512	Surfeit gene 4	Surf4	-	-	-	1.68	-	-	-
10545168	NM_020047	Tumorassociated calcium signal transducer 2	Tacstd2	-	-	-	1.69	-	-	1.56
10596900	NM_133986	Tcell leukemia translocation altered gene	Tcta	-	-	-	-	-	-	-1.58
10449661	NM_009363	Trefoil factor 2 (spasmolytic protein 1)	Tff2	-	-	-	-	3.35	-	1.52
10565609	NM_009381	Thyroid hormone responsive spot14 homolog (rattus)	Thrsp	-	1.92	-1.61	-3.44	-	-	-
10498273	NM_008536	Transmembrane 4 superfamily member 1	Tm4sf1	-	-	-	-1.68	-	-	-

Affy Transcript ID	Accession ID	Gene Name	Gene Symbol	Biological Function	Fold Change					
					HDM [EP]	Flu [EP]	Flu + HDM [EP]	HDM [LP]	Flu [LP]	Flu + HDM [LP]
10565514	NM_025460	Transmembrane protein 126a	Tmem126a	-	-	-	-	-	-	1.63
10553324	NM_026436	Transmembrane protein 86a	Tmem86a	-	-	-	1.52	-	-	-
10510580	NM_011612	Tumor necrosis factor receptor superfamily, member 9	Tnfrsf9	-	-	-	-	-	-	1.97
10538791	NM_001001495	Tnfaip3 interacting protein 3	Tnip3	-	-	-	-	-	3.55	5.56
10362420	NM_029726	Triadin	Trdn	-	-	-	-	-	-	1.64
10515282	NM_133681	Tetraspanin 1	Tspan1	-	-	-	-	-	-	1.61
10558961	NM_053082	Tetraspanin 4	Tspan4	-	-	-	1.87	-	-	-
10425808	NM_009775	Translocator protein	Tspo	-	-	-	1.84	-	-	-
10590212	NM_028735	Tetratricopeptide repeat domain 21a	Ttc21a	-	-	-	-1.53	-	-	-
10396064	NM_172054	Thioredoxin domain containing 9	Txndc9	-	-	-	-	-	-	1.53
10480714	NM_001033293	Udpnacteylglucosamine pyrophosphorylase 1like 1	Uap1l1	-	-	-	1.50	-	-	-
10478012	NM_011665	Ubiquitin-conjugating enzyme e2i	Ube2i	-	-	-	-	-	-	1.55
10364109	NM_009514	Preb lymphocyte gene 3	Vpreb3	-	-	-	-1.63	-	-	-
10419296	NM_172598	WD repeat and hmgbox dna binding protein 1	Wdhd1	-	-	-	-	-	-	1.54
10435862	NM_001033247	WD repeat domain 52	Wdr52	-	-	-	-1.71	-	-	-
10399632	---	RIKEN cDNA F630048H11 gene	F630048H11Rik	-	-	-	-1.52	-	-	-

Chapter 4

INFLUENZA A FACILITATES SENSITIZATION TO HOUSE DUST MITE IN INFANT MICE LEADING TO AN ASTHMA PHENOTYPE IN ADULTHOOD

Amal Al-Garawi[1], MA, Ramzi Fattouh[1], BSc, Fernando Botelho[1], PhD, Tina D. Walker[1], BSc, Susanna Goncharova[1], MD, Cheryl-Lynn Moore[1], BSc, Michiko Mori[2], BSc, Jonas S. Erjefalt[2], PhD, Derek K. Chu[1], BSc, Alison A. Humbles[3], PhD, Roland Kolbeck[3], PhD, Martin R. Stampfli[1], PhD, Paul M. O'Byrne[4], MD, Anthony J. Coyle[1,3], PhD, and Manel Jordana[1*], MD, PhD.

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In most instances, the development of allergic asthma has its origins in early life. To date, there are no studies that have investigated respiratory viral infection and *concurrent* allergen exposure in early life. Thus, we have developed an experimental platform that explores interactions between influenza A and HDM allergen within a relevant developmental time frame and determined the structural and functional consequences into adulthood. In this study we provide evidence that influenza A infection in neonatal mice facilitates long-standing allergic sensitization to HDM, precipitating the development of allergic asthma in adult mice. We demonstrate that infant mice, in contrast to adults, fail to respond to HDM, but not influenza A. Remarkably, HDM hyporesponsiveness is overcome when exposure occurs concurrently to influenza A infection; young mice now display robust allergen-specific immunity, allergic inflammation and lung remodeling. Notably, remodeling persists into early adulthood, after prolonged discontinuation of allergen exposure and resolved inflammation, and is associated with marked impairment in pulmonary function. Our data imply that severe viral infections during infancy can facilitate allergen responsiveness leading to an allergic asthma phenotype in adulthood.

ARTICLES

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Influenza A facilitates sensitization to house dust mite in infant mice leading to an asthma phenotype in adulthood

A Al-Garawi[1], R Fattouh[1], F Botelho[1], TD Walker[1], S Goncharova[1], C-L Moore[1], M Mori[2], JS Erjefalt[2], DK Chu[1], AA Humbles[3], R Kolbeck[3], MR Stampfli[1], PM O'Byrne[4], AJ Coyle[1,3] and M Jordana[1]

The origins of allergic asthma, particularly in infancy, remain obscure. Respiratory viral infections and allergen sensitization in early life have been associated with asthma in young children. However, a causal link has not been established. We investigated whether an influenza A infection in early life alters immune responses to house dust mite (HDM) and promotes an asthmatic phenotype later in life. Neonatal (8-day-old) mice were infected with influenza virus and 7 days later, exposed to HDM for 3 weeks. Unlike adults, neonatal mice exposed to HDM exhibited negligible immune responsiveness to HDM, but not to influenza A. HDM responsiveness in adults was associated with distinct Ly6c$^+$ CD11b$^+$ inflammatory dendritic cell and CD8α^+ plasmacytoid (pDC) populations that were absent in HDM-exposed infant mice, suggesting an important role in HDM-mediated inflammation. Remarkably, HDM hyporesponsiveness was overcome when exposure occurred concurrently with an acute influenza infection; young mice now displayed robust allergen-specific immunity, allergic inflammation, and lung remodeling. Remodeling persisted into early adulthood, even after prolonged discontinuation of allergen exposure and was associated with marked impairment of lung function. Our data demonstrate that allergen exposure coincident with acute viral infection in early life subverts constitutive allergen hyporesponsiveness and imprints an asthmatic phenotype in adulthood.

INTRODUCTION

Allergic asthma (AA), the most prevalent chronic inflammatory disease during childhood,[1] is characterized by airway inflammation, variable airflow obstruction, and reduced lung function, and is associated with structural remodeling of the airways. Despite remarkable progress in our understanding of the pathogenesis of this disease, elucidation of its origins, i.e., of the conditions under which AA emerges, remains elusive. Increasingly, environmental factors are believed to have preeminent roles in the emergence of allergic diseases, including asthma. In this context, acute respiratory viral infections are a major health threat in early life, and epidemiological studies have shown that by 2 years of age, most children will have been infected with at least one respiratory virus.[2,3] Importantly, those respiratory viral infections in early life that result in wheezy lower respiratory illness are associated with persistent wheezing, asthma, and reduced lung function at 6 years of age.[4,5] In addition, allergen sensitization to perennial allergens before the age of 2 years, but not later in childhood, is associated with a decrease in lung function and the presence of allergic airway disease in school-aged children.[6–8] What remains to be established is a direct causative link between viral infections and allergen sensitization in infancy with AA in later life.

That the majority of infants who wheeze do not develop AA suggests that tolerance, a state of homeostatic responsiveness, is the natural response to innocuous aeroallergens. Therefore, the generation of an immune-inflammatory response to aeroallergens assumes that tolerance was either prevented or subverted. Speculatively, profound perturbations of the lung microenvironment, especially during development, such as those caused by viral infections may prime the local immune environment to trigger immune-inflammatory responses to otherwise innocuous allergens.

[1]Division of Respiratory Diseases and Allergy, Center for Gene Therapeutics and Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada. [2]Department of Experimental Medical Science, Lund University, Lund, Sweden. [3]Department of Respiratory, Inflammation and Autoimmunity, MedImmune LLC, Gaithersburg, Maryland, USA. [4]Department of Medicine, McMaster University, Hamilton, Ontario, Canada. Correspondence: M Jordana (jordanam@mcmaster.ca)

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In this study, we investigated the interaction between viral infection and allergen sensitization in early life, and determined its structural and functional consequences into adulthood. We chose influenza A because the rate of influenza infection in pre-school children is estimated to be up to 40% annually with at least 1% resulting in hospitalization. Importantly, children younger than 6–12 months of age are at the highest risk of severe infection.[9] We also chose house dust mite (HDM) because it is the most pervasive common aeroallergen worldwide and permits studying mucosal immune-inflammatory responses in the absence of exogenous confounding adjuvants.

Our data show that, compared with adults, infant mice are immunologically hyporesponsive to a relatively high dose of HDM. In contrast, infant mice generate a robust immune-inflammatory response to influenza A infection. This influenza-induced environment facilitates allergen responsiveness, leading to airway inflammation, Th2 immunity, and evidence of both airway and lung parenchymal remodeling. Importantly, these structural changes persist into adulthood and are now associated with marked lung dysfunction even after a prolonged period of absence to allergen exposure and resolved inflammation.

RESULTS

HDM-mediated airway inflammation in early life

To determine the impact of aeroallergen exposure in early life, we exposed 2-week-old (infant) mice to 25 μg HDM for 3 weeks and compared the inflammatory response to an identical exposure in 8-week-old (adult) mice. Our data show, as reported previously, that adult mice responded with a robust inflammatory response including a substantial influx of eosinophils (43%) (data not shown); in sharp contrast, infant mice exhibited minimal lung inflammation, including eosinophilia (**Figure 1a–c**). In light of these data, we evaluated levels of transforming growth factor (TGF)-β1 and interleukin (IL)-10, cytokines with known anti-inflammatory and immune-regulatory properties, in the lungs of 2-, 5-week-old, and adult mice. Naive infant mice exhibited a 4–5-fold increase in the levels of active TGF-β1 over those observed in naive 5-week-old and adult mice. Similarly, IL-10 levels in naive 2-week-old mice diminished with increasing age (**Figure 1d** and **e**).

Next, we examined whether an ongoing influenza A infection altered responses to HDM. Groups of 8-day-old (neonatal) mice were either infected with influenza A virus (Flu) or given phosphate-buffered saline (PBS), and 7 days later at the peak of influenza-induced inflammation, were exposed to either HDM or saline (Sal) for 3 weeks (**Figure 2a**). Mice infected with influenza or exposed to HDM alone had only minimal inflammation at this time point, whereas mice exposed to HDM in the context of an influenza infection exhibited dramatically enhanced inflammation, similar to that observed in adult mice (**Figure 2b**). The increase in total inflammation was associated with significant increases in the numbers of mononuclear cells and eosinophils in the bronchoalveolar (BAL) and tissue (**Figure 2b** and **c**). In addition, we observed a modest 2.4-fold increase in the number of neutrophils following influenza A infection after co-exposure with HDM over that in mice infected with influenza only, but not in mice exposed to HDM alone. These local inflammatory changes were accompanied by changes in systemic immunity. Indeed, HDM-specific IgE, IgG_1, and IgG_{2a} levels were elevated only in mice exposed to HDM in the context of an influenza infection, whereas influenza-specific IgG responses were not altered by allergen exposure (**Figure 2d**).

Activation of the immune surveillance system in neonatal mice

To elucidate how influenza A infection may alter the immunoresponsiveness of neonatal mice to HDM exposure, we evaluated the impact on relevant components of the innate immune-sensing machinery. To this end, we examined the expression levels of several Toll-like receptors (TLRs) in the lungs of 8-day-old

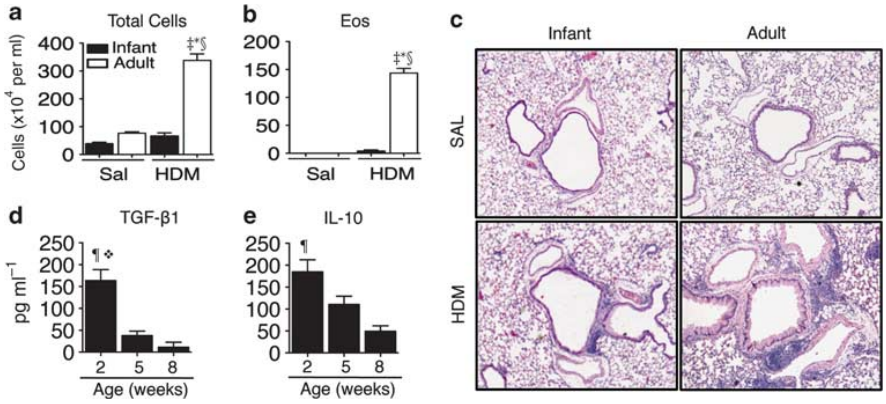

Figure 1 Impact of house dust mite (HDM) exposure in infant and adult mice. Infant and adult mice were exposed either to 3 weeks of 25 μg HDM or Sal and killed 72 h after the last HDM exposure. Cellular profile showing (**a**) total cell number and (**b**) absolute number in the bronchoalveolar lavage fluid (BAL). (**c**) Lung histopathology was evaluated by H&E to assess the degree of total lung inflammation. All images taken at ×50 total magnification as indicated. (**d**, **e**) Cytokine levels of active TGF-β and IL-10 in the lungs of naive mice at 2, 5, and 8 weeks of age. n=5–8 mice per group. One of two representative experiments is shown. Data represent mean±s.e.m. P<0.001 compared with *Sal (infant), ‡Sal (adult), and §HDM (infant), respectively. ■ (infant), □ (adult). P<0.01 compared with ✤5 and ¶8-week-old mice. H&E, hematoxylin and eosin; IL-10, interleukin-10; TGF-β, transforming growth factor-β.

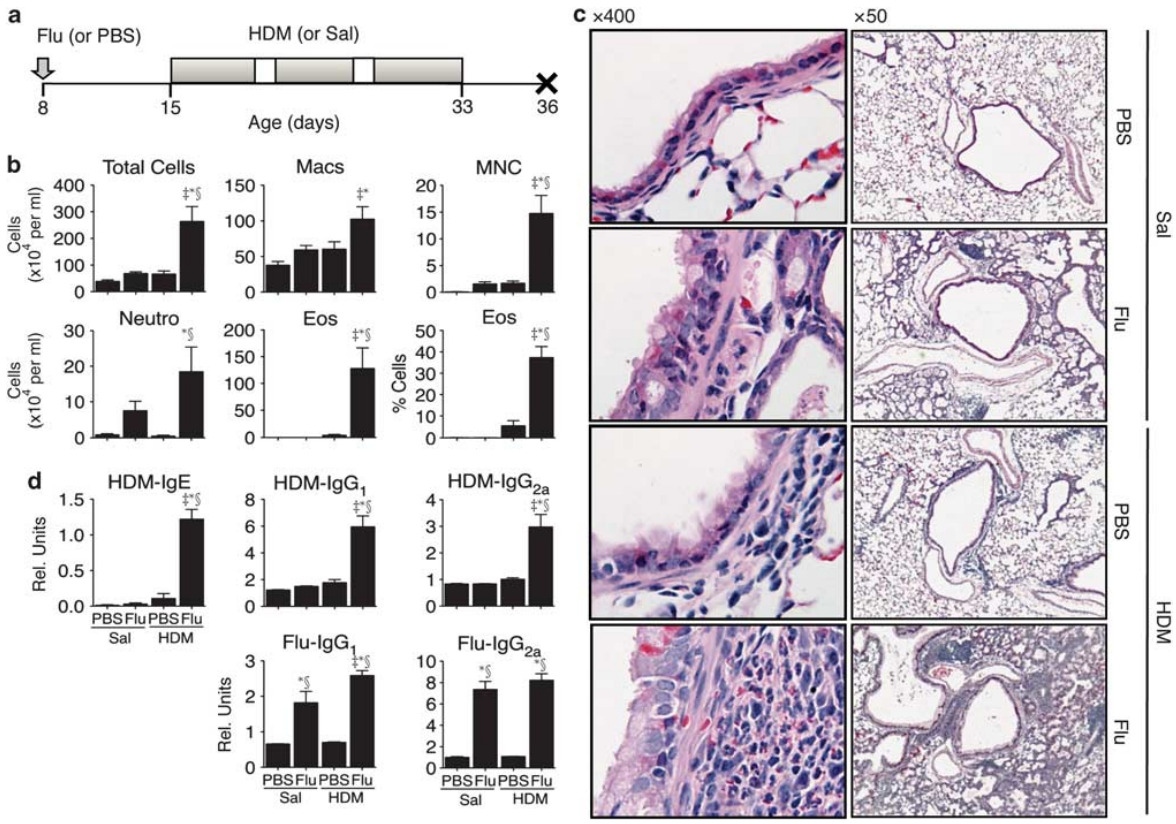

Figure 2 Impact of influenza A infection on subsequent house dust mite-induced inflammation in early life. (**a**) Eight-day-old mice were infected with influenza virus (Flu) or given PBS alone. Seven days later, PBS- and Flu-infected groups are exposed either to 3 weeks of HDM or Sal and killed 72 h after the last HDM exposure. (**b**) Cellular profile in BAL fluid showing the number of total cells, macrophages (Macs), mononuclear cells (MNC), neutrophils (Neutro), eosinophils (Eos), and the percentage of eosinophils. (**c**) Lung histopathology was evaluated by H&E to assess the degree of total lung inflammation and eosinophilia. All images taken at ×50 and ×400 total magnification as indicated. (**d**) Serum Ig levels measured by ELISA showing HDM-specific IgE, IgG1, and IgG2a and Flu-specific IgG1 and IgG2a. For all data, $n=8$–12 mice per group. One of two representative experiments is shown. Data represent mean±s.e.m. $P<0.001$ compared with *Sal, ‡Flu, and §HDM, respectively, except Flu-IgG1, $P<0.01$ compared with ‡Flu. ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; HDM, house dust mite; PBS, phosphate-buffered saline.

mice that were either infected with influenza A or exposed to 25 μg of HDM for 7 consecutive days. Our data show that only infection with influenza A led to significantly increased levels in TLR 2, 4, 7, and 9 (**Figure 3a**).

In addition to TLR expression, we evaluated the changes in antigen-presenting cell (APC) subtypes and T cells in the lungs of 8-day-old mice either infected with influenza virus, at day 7 post infection (p.i.), or exposed to 25 μg HDM for 7 consecutive days; we used this protocol to control for changes due to age. To identify various APC subtypes, 13-color flow cytometry was used. $CD45^+/CD3^-$ cells were first gated on B220 and CD11c expression and four populations, R1–R4, selected (**Figure 3b** and **Supplementary Figure 1a** online). On the basis of this initial gating strategy, we then classified APC subtypes in the lungs of neonatal mice into distinct subsets (outlined in **Supplementary Figure 1b–d** online) and identified B cells, $Ly6C^{hi}$ monocytes, and alveolar macrophages, as previously reported in adult mice.[10–12] We also identified conventional dendritic cells (DCs) which could be further subdivided, based on CD11b and CD103 expression, into $CD11b^-(CD103^+)$ and $CD11b^+(CD103^-)$ DCs, representing intraepithelial DCs and inflammatory DCs (iDC), respectively.[13] Furthermore, within the R1 gate ($B220^+CD11c^{int}$ population), we identified a heterogeneous mixture of cells that includes NK cells and two plasmacytoids (pDCs) subtypes, $CD11b^-(CD8\alpha^-)$ and a $CD11b^-(CD8\alpha^+)$ population representing an activated pDC subset[14–16] (**Supplementary Figure 2a–c** online). Moreover, within this gate, we observed the emergence of a distinct population of $Ly6C^+$ $CD11b^+(CD8\alpha^-)$ DCs, most similar in phenotype to iDCs (iDC-like)[17,18] (**Supplementary Figure 2c** online). Neonatal mice infected with influenza virus showed a dramatic increase in NK cells and monocytes, as well as significant increases in pDCs and B cells (**Figure 3c** and **Supplementary Figure 3** online). Moreover, influenza A infection led to statistically significant increased numbers in the $B220^{hi}$ iDC-like population, but not intraepithelial DCs and conventional iDCs.

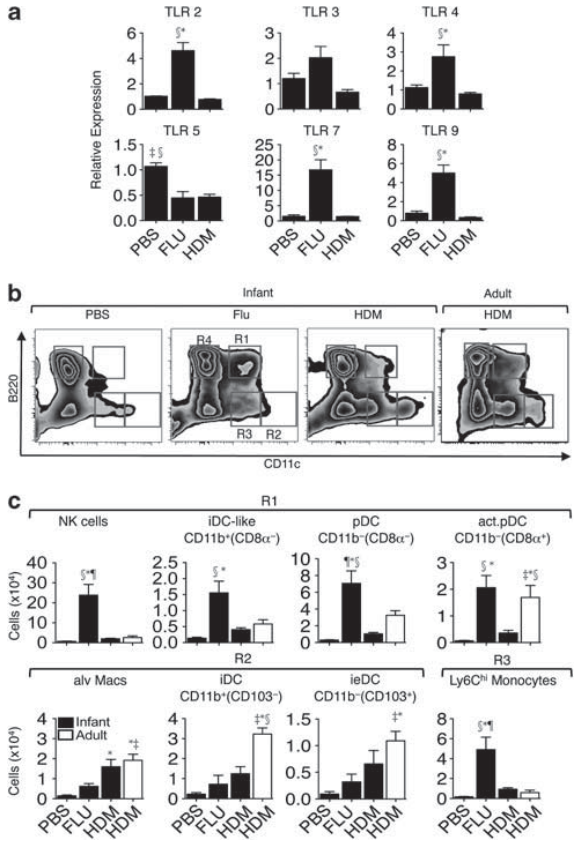

Figure 3 Lung immune status following influenza A infection or house dust mite exposure in neonatal mice. Groups of 8-day-old mice were either infected with influenza A, PBS treated, or exposed to HDM for 7 consecutive days, and adult mice exposed to HDM for 7 days. (**a**) Quantitative real-time PCR showing relative mRNA expression levels of TLR 2, 3, 4, 5, 7, and 9 in the lungs of neonatal mice infected with influenza A or exposed to HDM and compared with PBS-treated mice. $n=3$–4 mice per group. (**b**) Flow cytometric analysis of the APC compartment in whole lung digests at 7 days p.i. or 24 h after last HDM exposure using gating strategy shown in **Supplementary Figures 1** and **2** online. Representative Zebra plots showing B220 and CD11c expression and frequency of distinct immune cells populations present in the lungs of neonatal and adult mice. Gates were drawn and labeled R1–R4 as shown. (**c**) Number of NK cells, pDCs, act. pDC, and iDC-like populations, Ly6C$^+$ monocytes, alvMacs, ieDCs, and iDCs. Data representative of at least three independent experiments. $n=4$–6 mice per group. Data represent mean±s.e.m. $P<0.05$ compared with *PBS, ‡Flu, §HDM, and ¶HDM-adult, respectively. ieDC, intraepithelial DC; iDC, inflammatory DC; pDC, plasmacytoid DC; act. pDC, activated pDC; iDC-like, inflammatory DC-like; alvMacs, alveolar macrophages. ■ (infant), □ (adult). APC, antigen-presenting cell; HDM, house dust mite; PBS, phosphate-buffered saline; p.i., post infection; TLR, Toll-like receptor.

By comparison, exposure to HDM in both neonates and adult mice resulted in statistically significant increases in alveolar macrophages; however, only adult mice exhibited statistically significant increased numbers of intraepithelial DCs. Interestingly, adult, but not neonatal, HDM-exposed mice exhibited a significant increase in both iDCs and CD8α$^+$ pDCs, similar to that observed in influenza-infected neonatal mice.

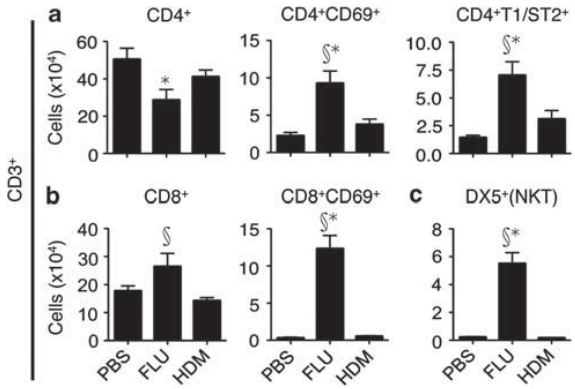

Figure 4 Impact on T-cell compartment in the lungs of neonatal mice following influenza A or house dust mite exposure. Groups of 8-day-old mice were either infected with influenza A, PBS treated, or exposed to HDM for 7 consecutive days and T cells evaluated by flow cytometry at day 7 p.i. or 24 h after last HDM exposure. (**a**, **b**) Absolute number of CD4$^+$ and CD8$^+$ T cells (CD3$^+$), their activation states (CD69$^+$), and expression of Th2 cell surface marker, T1/ST2. (**c**) Absolute number of DX5$^+$ (CD3$^+$) NK-T cells. For all data, $n=5$–6 mice per group. One of two representative experiments is shown. Data represent mean± s.e.m. $P<0.05$ compared with *PBS; ‡Flu; §HDM, respectively. H&E, hematoxylin and eosin; HDM, house dust mite; PBS, phosphate-buffered saline; p.i., post infection.

When we examined T-cell populations, mice infected with influenza virus exhibited marked increases in CD8$^+$ but not in CD4$^+$T cells (**Figure 4a** and **b**). In addition, influenza-infected, but not HDM-exposed, mice exhibited significantly increased numbers of activated (CD69$^+$) CD4$^+$ and CD8$^+$ T cells 7 days p.i. Activated CD8$^+$ T cells were markedly increased (24-fold) over PBS-treated mice, whereas the numbers of activated CD4$^+$ T cells were doubled (2.4-fold increase). Furthermore, influenza infection led to an increase (2.3-fold) in T1/ST2$^+$ CD4$^+$ T cells, a cell surface marker specific for the induction of Th2 cells. Finally, we found that DX5$^+$CD3$^+$ cells (NK-T cells) were also dramatically increased in influenza-infected mice (25-fold) (**Figure 4c**). No significant changes on any of these T-cell subsets were observed in infant mice exposed to HDM.

Associated with the dramatic increase in APC and T-cell populations, evaluation of tissue histopathology (at 2 weeks of age) revealed substantial recruitment of inflammatory cells into the lung parenchyma in mice exposed to influenza A at 8 days of age (**Figure 5a**). On the basis of these observations, we next investigated the effector profile induced by an influenza infection in the lungs of neonatal mice. A wide array of cytokines was assessed in lung homogenates on days 3, 5, and 7 after infection of 8-day-old mice. Antiviral, pro-inflammatory, Th1, Th2, and Th17 cytokines, as well as additional cytokines associated with the promotion of Th2 responses (granulocyte macrophage colony-stimulating factor, TSLP, IL-33, IL-25) were all significantly increased with different kinetics during the examination interval (**Figure 5b**). Finally, we examined the levels of TGF-β1 following flu infection. Although we observed a trend for increased expression at 5 days p.i., this increase was not statistically significant (data not shown).

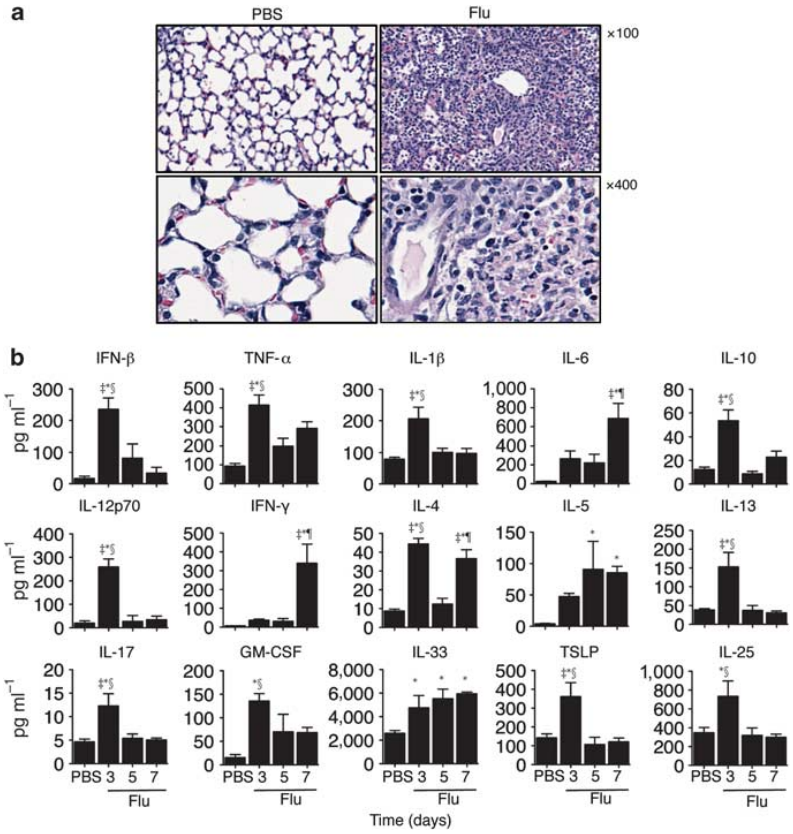

Figure 5 Lung histopathology and cytokine production following influenza A infection in neonatal mice. Groups of 8-day-old mice were either infected with influenza A, PBS treated, or exposed to HDM for 7 consecutive days. (**a**) Lung histopathology was evaluated by H&E to show the degree of immune cell recruitment into the lung parenchyma. All images taken at ×100 and ×400 total magnification as indicated. (**b**) The lungs were isolated at days 3, 5, and 7 p.i., following influenza A infection and kinetics of IFN-β, pro-inflammatory, Th1, Th2, and Th17 cytokines evaluated in lung homogenates. n=3–5 mice per group. Data represent mean±s.e.m. $P<0.05$ compared with *PBS, ¶d3, ‡d5, and §d7, respectively. H&E, hematoxylin and eosin; HDM, house dust mite; IFN-β, interferon-β; PBS, phosphate-buffered saline; p.i., post infection.

HDM-specific Th cytokine production in splenocytes following influenza A infection

To determine whether early-life influenza A infection facilitated subsequent HDM-specific T-cell responsiveness, we evaluated T-cell effector function in splenocytes by measuring the production of Th1-, Th2-, and Th17-associated cytokines after restimulation *in vitro* (**Figure 6**). Our data show significantly elevated levels of the Th2-associated cytokines IL-4, IL-5, and IL-13, as well as a 10-fold increase in IL-6 levels in mice concurrently exposed to influenza A and HDM in early life over HDM alone. Similar to Th2 cytokines, modest levels of IL-17 were detected in mice exposed to HDM only, which were significantly increased in mice exposed to influenza A and HDM. In contrast, interferon-γ levels were significantly elevated in both HDM- and HDM-influenza exposed mice with no significant differences between these two groups.

Impact of influenza A infection on structural remodeling in early life

To investigate whether concurrent influenza A infection and HDM exposure in early life promotes the development of airway structural changes, we evaluated goblet cell hyperplasia (mucus production), subepithelial collagen deposition, and smooth muscle thickness (**Figure 7a**). A significant increase in goblet cell hyperplasia (**Figure 7b**) and peribronchial collagen deposition (**Figure 7c**) was detected only in mice exposed to HDM in the context of an influenza infection. In contrast, we did not observe significant differences in peribronchiolar smooth muscle thickness among the treatment groups (**Figure 7d**). Along with these histological changes, we detected significant increases in the levels of the growth factors, TGF-β, $PDGF_{AA}$, and vascular endothelial growth factor only in those mice that had been infected with influenza and concurrently exposed to HDM (**Figure 7e**).

Impact of influenza A infection in early life on structural remodeling in adult life

To determine whether the airway remodeling changes observed after 3 weeks of HDM exposure (5-week-old mice) were dependent on continued allergen exposure, we suspended exposure for an additional 3 weeks (**Figure 8a**). At this point in time (8-week-old mice), tissue and BAL inflammation were resolved

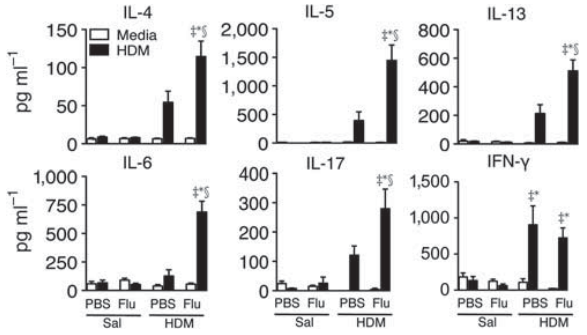

Figure 6 Impact of influenza A infection on house dust mite-specific Th-cytokine responses. Separate groups of 8-day-old mice were infected with influenza virus or given PBS alone. Seven days later, PBS- and Flu-infected groups are exposed either to 3 weeks of HDM or Sal and killed 72 h after the last HDM exposure. Splenocytes from individual mice were cultured in medium alone (□) or stimulated with HDM *in vitro* (■) and Th1-, Th2-, and Th17-associated cytokine production measured by ELISA. For all data, $n=4$–10 mice per group. Data represent mean± s.e.m. $P<0.05$ compared with *Sal, ‡Flu, and §HDM, respectively. ELISA, enzyme-linked immunosorbent assay; HDM, house dust mite; PBS, phosphate-buffered saline.

and the residual inflammation observed in influenza-infected mice was largely mononuclear in nature (**Supplementary Figure 4a** and **b** online). In contrast, HDM-specific immunoglobulin levels remained significantly elevated in mice that had been infected with influenza and exposed to HDM during infancy. As expected, influenza-specific immunoglobulins remained elevated (**Supplementary Figure 4c** online). Our data show that only animals infected with influenza virus and exposed to HDM early in life still exhibited significantly increased mucus production and subepithelial collagen deposition after 3 weeks of allergen discontinuance (**Figure 8b** and **c**). Whereas infant mice infected with influenza virus only did not exhibit increased collagen deposition at 5 weeks of age, a significant increase was observed in this parameter at 8 weeks of age (**Figure 8d**).

In light of the histopathological changes observed, we investigated whether remodeling extended to the parenchymal compartment. We detected an increased number of α-smooth muscle actin (α-SMA)-positive cells, likely representing myofibroblasts, only in animals exposed to HDM in the context of an influenza infection during infancy (**Figure 9a–c** and **e**). Combined staining of α-SMA and the pro-collagen peptide PINP revealed

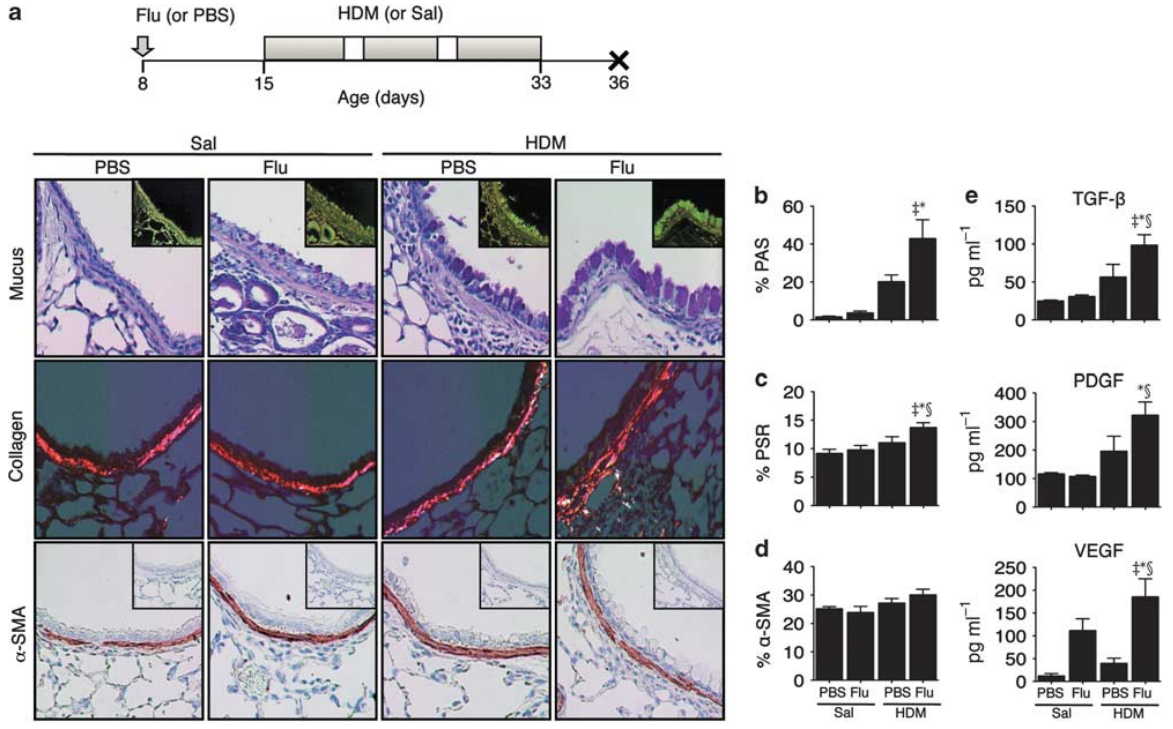

Figure 7 Impact of influenza A infection on airway remodeling in early life. (**a**) Separate groups of 8-day-old mice were infected with influenza virus or given PBS alone. Seven days later, PBS- and Flu-infected groups are exposed either to 3 weeks of HDM or Sal and killed 72 h after the last HDM exposure. Images are representative light photomicrographs of paraffin-embedded cross-sections of lung tissues obtained 72 h after the last HDM exposure. Histopathology was evaluated by (**b**) periodic-acid-Schiff (PAS) staining indicating mucus production of epithelial goblet cells (magenta; insets show color-inverted image used for morphometric analysis); (**c**) Picro Sirus Red (PSR) staining visualized under polarized light indicating subepithelial collagen deposition, and (**d**) Immunohistochemistry for α-smooth muscle actin (α-SMA), indicating contractile elements in the airway wall (brown; insets show nonspecific staining in the corresponding negative control section). (**e**) Remodeling associated growth factors TGF-β, $PDGF_{AA}$, and VEGF in BAL as evaluated by ELISA. All images were taken at ×200 total magnification. For all data, $n=8$–10 mice per group. One of two representative experiments is shown. Data represent mean±s.e.m. $P<0.05$ compared with *Sal, ‡Flu, and §HDM, respectively. ELISA, enzyme-linked immunosorbent assay; HDM, house dust mite; PBS, phosphate-buffered saline; TGF-β, transforming growth factor-β.

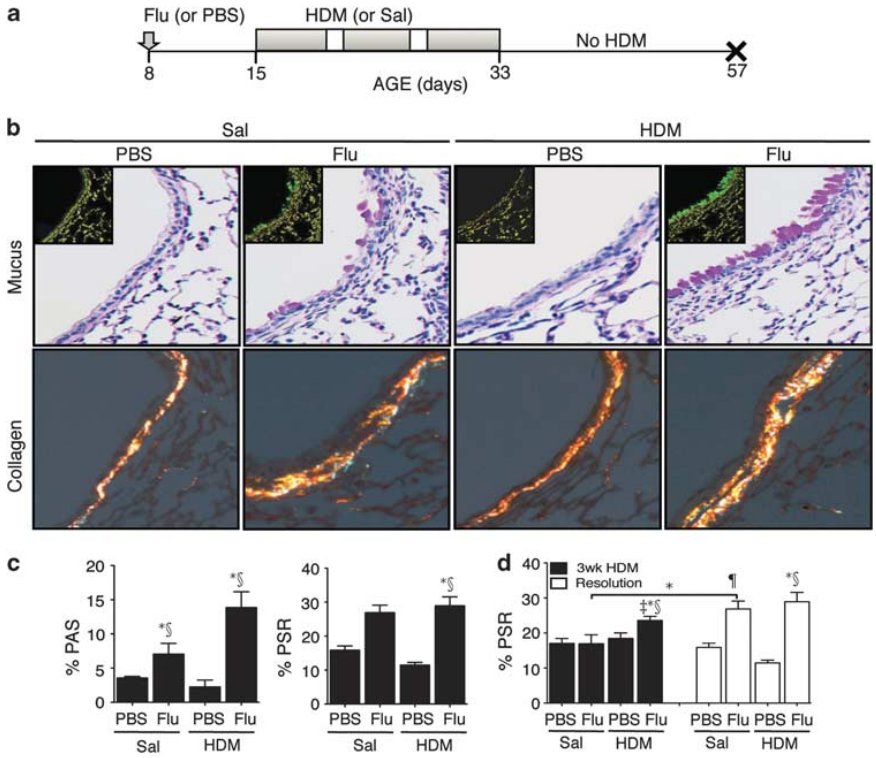

Figure 8 Impact of influenza A infection on airway remodeling in adulthood. (**a**) Separate groups of 8-day-old mice were infected with influenza virus or given PBS alone. Seven days later, PBS- and Flu-infected groups are exposed either to 3 weeks of HDM or Sal, then, after the last HDM exposure, mice were rested for 3 weeks and killed at 8 weeks of age. Images are representative light photomicrographs of paraffin-embedded cross-sections of lung tissue. Lung histopathology as evaluated by (**b**) periodic-acid-Schiff (PAS) staining indicating mucus production of epithelial goblet cells (magenta; insets show color-inverted image used for morphometric analysis) and Picro Sirus Red (PSR) staining visualized under polarized light indicating subepithelial collagen deposition. (**c**) Morphometric analysis of PAS and PSR tissues. (**d**) Comparison of PSR tissues after 3 weeks of HDM exposure or after additional 3 weeks cessation (resolution). All images were taken at ×200 total magnification. For all data, $n=8$–10 mice per group. Data represent mean±s.e.m. $P<0.05$ compared with *Sal, ‡Flu, and §HDM, respectively. HDM, house dust mite; PBS, phosphate-buffered saline.

ongoing collagen synthesis among α-SMA-positive cells in patchy regions of aggravated and cell-rich alveolar inflammation (**Figure 9d**).

Impact of influenza A infection in early life on lung mechanics in adulthood

Finally, we examined whether the changes in airway and parenchymal remodeling that persist into adulthood, led to altered lung function by evaluating respiratory mechanics, specifically airway resistance (R_N), tissue resistance (G), and tissue elastance (H_{TE}). Only mice infected with influenza A and exposed to HDM during infancy exhibited a marked functional impairment as measured by significant increases in R_N, G, and H_{TE} (**Figure 10a** and **b**).

DISCUSSION

A fundamental question regarding AA is the elucidation of its origins. Most cases of AA initiate during early childhood. Given the ubiquitous presence of allergen, it is unlikely that allergen exposure alone may explain its emergence in many instances. In this study, we have investigated the impact of concurrent viral infection and allergen exposure in early life and its impact on the development of allergic airway disease later in life. Prospective birth cohort studies have shown that respiratory viral infections and allergic sensitization in early life are independent risk factors for the development of asthma. Yet, a causal link between these two risk factors and AA has not been established. Respiratory viruses predominantly associated with severe lower respiratory illness and asthma in young children include Respiratory Syncytial Virus and Rhinovirus. However, advances in viral detection methods have identified additional viral types as etiological agents of severe lower respiratory illness's in infants, such as influenza A, human metapneumovirus, and adenovirus among others. Of these, influenza A virus is a significant cause of severe lower respiratory illness in young children that frequently leads to hospitalization.[19] At present, the role that severe influenza A-induced bronchiolitis in infants may have in the generation of asthma has not been clarified.

Several experimental studies have examined the impact of influenza A infections on allergic airway disease in adult mice with divergent outcomes.[20–22] These studies need to be taken with circumspection with regard to their relevance to the

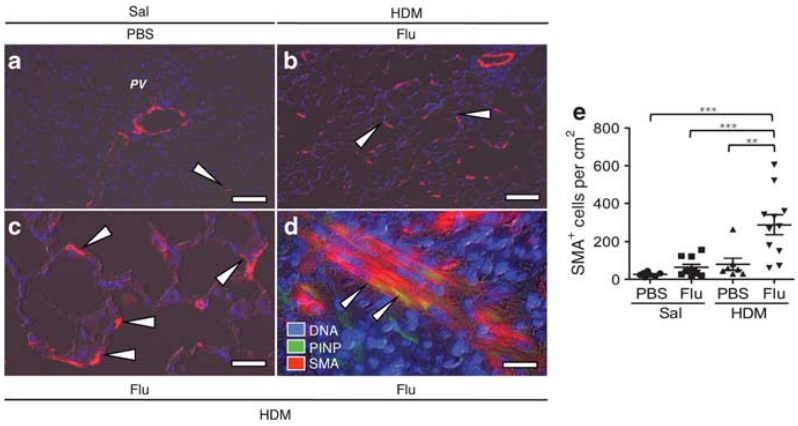

Figure 9 Impact of influenza A infection on parenchymal remodeling in adulthood. Groups of 8-day-old mice were infected with influenza virus or given PBS alone. Seven days later, PBS- and Flu-infected groups are exposed either to 3 weeks of HDM or Sal, then, after the last HDM exposure, mice were rested for 3 weeks and killed at 8 weeks of age. Pictures show immunofluorescence images of α-SMA$^+$ alveolar cells (arrow heads) in the lungs from (**a**) saline and (**b**) influenza and HDM-treated mice. (**c**) Higher magnification reveals the interstitial distribution of the alveolar α-SMA cells in influenza and HDM-treated mice. (**d**) Combined staining for α-SMA and the pro-collagen peptide PINP revealed ongoing collagen synthesis among α-SMA-positive cells in patchy regions of aggravated and cell-rich alveolar inflammation. (**e**) Quantification of α-SMA-positive alveolar cells parenchymal tissue of 8 week-old mice. Scale bars in panels **a** and **b** = 100 μm; panel **c** = 35 μm; panel **d** = 15 μm. n = 8–10 mice per group. Data represent mean± s.e.m. $^{**}P<0.01$; $^{***}P<0.001$. α-SMA, α-smooth muscle actin; HDM, house dust mite; PBS, phosphate-buffered saline; PV, pulmonary vessel.

neonatal period because the developing immune system is functionally different from that of adults.[23] In addition, two recent studies have examined whether a respiratory viral infection in early life would affect sensitization initiated in adulthood to the innocuous protein ovalbumin (OVA).[24,25] By design, these studies precluded examining whether an acute viral infection and concurrent aeroallergen exposure in early life leads to allergic sensitization and an asthmatic phenotype in adulthood. The study we report here models a very defined paradigm, which we believe is clinically very relevant. This paradigm encompasses several central features: (i) exposure to a naturally occurring aeroallergen exclusively through the respiratory mucosa, (ii) exposure to such aeroallergen concurrently with an ongoing respiratory viral infection, and (iii) focus on the initiation of these perturbations at a clinically relevant developmental time frame (approximately equivalent to the first 2 years of life in humans).

We established a model of respiratory mucosal sensitization using HDM, the most pervasive indoor allergen worldwide, and which does not require the use of additional exogenous adjuvants. In accordance with our previous data,[26] exposure to a high dose of HDM for 3 weeks elicits, in adult mice, substantial airway inflammation and robust eosinophilia. In sharp contrast, infant mice exhibit minimal immuno-responsiveness to HDM as evidenced by reduced BAL and tissue inflammation, including minimal recruitment of eosinophils, absence of allergen-specific immunoglobulins, and minimal Th2 cytokine responses. Failure to respond to such an intrinsically allergenic material[27] suggests that allergen exposure in early life is, by itself, insufficient to generate allergen-specific sensitization and allergic airway inflammation. The homeostatic mechanisms mediating HDM hyporesponsiveness at this period of development likely encompass a complex network of regulatory immune pathways.[28,29] We provide evidence that, in the steady state, infant mice have elevated levels of TGF-β and IL-10 in the lungs, two cytokines with powerful regulatory and immunosuppressive activities.[30,31] Whether these are the only regulatory cytokines involved, the cellular source of these cytokines, the specific mechanisms of action, and the contribution from the mother to allergen hyporesponsiveness, through regulatory molecules present in the milk[32] are, among others, interconnected questions, the elucidation of which is beyond the scope of this study. Our research aimed to investigate whether a severe viral infection in infancy was able to overcome constitutive allergen hyporesponsiveness and the proximal and remote consequences of this effect. Our data clearly demonstrate that an acute infection with influenza A perturbs the lung in such a way as to enable allergen responsiveness as assessed by every parameter studied: airway inflammation, HDM-specific immunoglobulins, and systemic Th2 immunity. There is evidence that suggests that neonatal animals generate only mild inflammatory responses to inhaled antigens such as OVA and cockroach extracts,[33–35] and that co-exposure to air pollution,[33] endotoxin,[34] or bacterial and viral TLR ligands[35] significantly enhances inflammatory responses. Thus, our data are in line with the concept that sensitization to a pervasive allergen in early life and generation of asthma later on, depends on immunological perturbations brought about by concurrent exposures, such as severe viral infections.[5,36]

In contrast to the remarkable hyporesponsiveness to HDM, neonates respond, similar to adults, to a potentially life-threatening encounter such as influenza A infection. To gain insights into the immunological mechanisms underlying the divergent responses to HDM and influenza A, we investigated the status of the APC compartment. Our data show that influenza A infection led to recruitment and activation of NK cells, Ly6C^{hi}

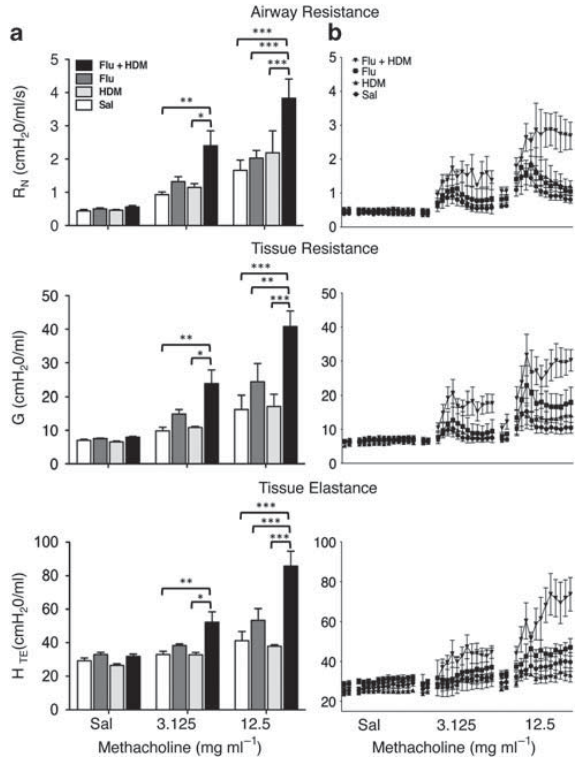

Figure 10 Impact of influenza A infection in early life on lung mechanics later in life. Groups of 8-day-old mice were infected with influenza virus or given PBS alone. Seven days later, PBS- and Flu-infected groups are exposed either to 3 weeks of HDM or Sal, then, after the last HDM exposure, mice were rested for 3 weeks and killed at 8 weeks of age and lung function evaluated. Airway responsiveness to increasing doses of methacholine was assessed for R_N, G, and H_{TE} and is shown (**a**) as maximum R_N, G, and H_{TE}, and, (**b**) as time course of 12 consecutive measurements. For all data, $n=8$–10 mice per group. Data represent mean±s.e.m. *$P<0.05$, **$P<0.01$, ***$P<0.001$; two-way ANOVA (Bonferroni's *post hoc* test). ANOVA, analysis of variance; HDM, house dust mite; PBS, phosphate-buffered saline.

monocytes and iDCs, as well as two pDCs subtypes; these events led to robust $CD4^+$ and $CD8^+$ T-cell responses. In comparison with influenza A, HDM exposure (in neonates) led to significant increases only in alveolar macrophages; however, these changes did not lead to significant increases in the number or activation state of $CD4^+$ and $CD8^+$ T cells. Interestingly, similar to influenza A, only adult, and not neonatal mice exposed to HDM exhibited significantly increased numbers of iDCs and $CD8\alpha^+$pDCs suggesting that these two cell populations may have a critical role in driving immune-inflammatory responses to HDM. Whereas, iDCs are recruited to the lung for the production of inflammatory cytokines,[18] and are necessary for HDM-mediated inflammation in adult mice,[37] $CD8\alpha^+$pDCs represent an activated pDC subtype involved in the generation of type I interferon and have been shown to emerge after microbial exposure.[14,16] Collectively, these data suggest that HDM exposure in neonatal mice had, with the exception of alveolar macrophages, a negligible impact on APC subsets in the lung, and further reveal that influenza infection in neonatal mice leads to the recruitment of specific APC subtypes such as iDCs and activated pDCs, which may be critical for HDM responsiveness.

To provide further mechanistic insights into the effects of influenza A infection that may facilitate allergen responsiveness, we investigated the expression of several members of the TLR family. Whereas HDM exposure in neonates did not increase TLR expression, influenza A infection did. Among those TLRs the expression of which was increased, TLR4 is particularly relevant as it has been directly implicated in HDM-mediated inflammation in adult mice.[38–40]

The efficient recognition of and rapid host response to influenza infection led to extensive infiltration of immune-inflammatory cells into the lung parenchyma resulting in acute bronchiolitis-like pathology resembling that observed in human infants infected with respiratory syncytial virus or influenza A virus.[41] As a result, the environment encountered by HDM in a lung undergoing a severe influenza infection is exceptionally rich in immune mediators. Although influenza A virus is considered an archetypic Th1-inducing signal, the effector profile that we demonstrate in neonates defies dichotomous categorizations (i.e., Th1 vs. Th2). In fact, influenza A induced an increased expression of both Th1 and Th2, as well as Th17 and a number of pro-inflammatory and anti-viral cytokines. Moreover, we observed increased production of cytokines capable of promoting Th2 immunity (granulocyte macrophage colony-stimulating factor, IL-25, IL-33, and TSLP).[42] Evidence of such a prolific effector response intimates that the viral-mediated acquisition of allergen responsiveness in the neonatal setting is unlikely mediated by a single molecular signal but, rather, by a community of cytokines.

From a mechanistic perspective, our data show that influenza A infection led to a heightened state of immune alertness, encompassing the activation of multiple cellular components and pathways, as well as the production of many immune mediators; we propose that this pervasive priming of the lung environment reduced the threshold necessary to trigger allergen responsiveness. These findings prompt a critical appraisal of a prevailing theory regarding the development of asthma. Indeed, according to the Hygiene Hypothesis, exposure to microbial agents in early life results in protective immunity against allergic disease.[43] Yet, we demonstrate that exposure to influenza A virus in early life overcomes constitutive allergen hyporesponsiveness and primes the lung environment to facilitate allergic responses. These findings expose the shortcomings of the Hygiene Hypothesis to account for the likely diverse consequences of distinctive microbial exposures in early life.

Structural abnormalities of the airway, collectively referred to as remodeling, are believed to contribute to airway dysfunction, the cardinal feature of asthma. The conventional paradigm is that remodeling is secondary to chronic inflammation. However, several features of remodeling have been observed in very young children with a diagnosis of asthma.[44] Moreover, Saglani *et al.*[45] have shown that changes in remodeling can be detected in children with persistent wheeze as young as 3 years of age. These findings have led to the suggestion that

inflammation and remodeling may be independent processes.[46] Yet, the link between remodeling and inflammation in young children remains tenuous, in part, because, in humans, it is difficult to track back with precision the individual's immune-inflammatory history.

Our study demonstrates that HDM exposure in very young mice leads to airway remodeling only when robust allergic inflammation, facilitated by a single severe event, such as that caused by an influenza infection, was elicited. Interestingly, these airway remodeling changes occurred at an accelerated pace as compared with adults,[47] and importantly, persisted after a prolonged period of cessation of allergen exposure. Translationally, our data advocate that influenza A infections, and, likely, other viral infections associated with severe bronchiolitis in early life may propel allergic airway remodeling, and that these structural changes could persist in young adults, even if inflammation is no longer present.

The pathology exhibited by mice exposed to HDM while undergoing an influenza A infection clearly extends beyond the airway and into the lung parenchyma. A closer examination of this compartment revealed a dramatic increase in the number of alveolar α-SMA$^+$ cells, similar to a finding reported in OVA-sensitized and OVA-challenged mice.[48] To the best of our knowledge, there are no studies in human asthma that have examined parenchymal remodeling. Interestingly, α-SMA$^+$ cells express muscarinic receptors and, hence are capable of responding to methacholine and contribute to airway hyperreactivity.[49,50] In this regard, we evaluated the long-term impact of concurrent viral and allergen exposure on the generation of airway hyperreactivity. Our data demonstrate that even after prolonged cessation of allergen exposure and, effectively, absent airway inflammation, airway resistance (R_N), tissue resistance (G), and tissue elastance (H), all indicators of airway closure in response to methacholine challenge,[51] were markedly elevated only in those, now adult, mice that had been exposed to HDM in the context of an influenza A infection during infancy. Thus, our findings underscore the long-lasting impact allergen exposure has on lung function at a time of a severe viral infection in early life. The precise links between inflammation, structural changes, and functional alterations remain obscure in both humans and experimental systems. Our data show that marked alterations in respiratory mechanics are evident when there is no longer inflammation but there is mucus metaplasia, an increase in parenchymal myofibroblasts, as well as increased subepithelial collagen deposition. With regard to the latter, such a feature is also detected in adult mice that were infected with influenza A (only) in early life; yet this is not associated with significant alterations in respiratory mechanics, suggesting that, at least in this system, increased subepithelial collagen deposition alone does not lead, by itself, to functional impairment.

Understanding the origins of AA remains elusive. Unfortunately, genetic studies to date have been plagued by inconsistencies and poor reproducibility across populations. As proposed by Guerra and Martinez,[52] meaningful progress requires an approach that integrates interactive influences from the environment, biological systems, and developmental processes.[53] Here, we have developed a model that encompasses: (i) the use of a relevant aeroallergen capable of inducing a response when delivered mucosally in the absence of additional adjuvants, (ii) an environmental disturbance of the system in the form of an influenza A infection at the time of allergen exposure, and (iii) a relevant developmental interval as, in most instances, AA develops in humans within the first few years of life. Our data demonstrate that a severe immune perturbation in early life, such as that caused by a severe influenza A infection, can subvert the responsiveness to an otherwise harmless allergen leading to the expression of an AA phenotype in adulthood. In a broader context, these findings intimate the notion that diverse microbial exposures may have distinctive consequences: protection from vs. promotion of allergic disease. We would speculate that the severity of the acute immune-inflammatory response, rather than the nature of the initiating immune signal, is the variable that determines a detrimental outcome. Importantly, our study suggests that interventions to reduce the lung inflammation caused by such events may be essential in preventing subsequent allergen sensitization and asthma in the youngest population.

METHODS

Animals. Fifteen-day pregnant (female) BALB/c mice were purchased from Charles River Laboratories (Ottawa, ON, Canada) and housed under specific pathogen-free conditions and maintained on a 12-h light–dark cycle with food and water *ad libitum*. Upon birth, mothers were housed with their litters in light-protected cages until completion of the study (or weaning at 4 weeks of age). All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University (Hamilton, ON, Canada).

Influenza A infection and sensitization protocols. Separate groups of 8-day-old BALB/c mice were infected without anesthesia with either influenza A/PR8 virus or PBS solution. Influenza type A virus strain A/PR/8/34 (H1N1) was prepared as described previously.[20] The viral stock suspension (10^9 PFU ml^{-1}) was diluted 6,000-fold and a sublethal dose of ~1 PFU administered intranasally in 15 µl PBS.

Allergen administration: HDM extract (Greer Laboratories, Lenoir, NC) was resuspended in sterile saline (Sal) at a concentration of 2.5 µg (protein) per µl and 10 µl (25 µg dose) was administered to lightly isofluorane-anesthetized 2- and 8-week-old mice by intranasal delivery. According to the manufacturer, the levels of endotoxin in HDM extracts range between 25 and 100 EU ml^{-1} extract; this corresponds to 0.25–1 EU per dose of allergen per day or 0.1–1 ng per 25 µg dose. These levels of lipopolysaccharide are significantly lower than the 100 ng dose of lipopolysaccharide required to promote Th2 responses in OVA models of allergic disease.[54]

Concurrent influenza A infection and allergen exposure in early life: Groups of 8-day-old mice were infected with influenza A or PBS and then 7 days later, groups of mice were exposed either to HDM or Sal, 5 days a week for a total of 3 weeks. The immune-inflammatory response and structural changes were evaluated 3 days after the last challenge (**Figure 2a**).

Airway inflammation and remodeling in adulthood: To investigate whether structural changes persisted, the protocol was recapitulated and, after the last allergen challenge, mice were allowed to rest for a period of 3 weeks (**Figure 8a**). The inflammatory and remodeling responses were re-evaluated and the impact on lung mechanics determined.

Immune activation after influenza A infection or allergen exposure: To examine the difference in immune activation in neonatal mice exposed to HDM or infected with influenza, groups of 8-day-old mice were infected with either flu virus or exposed to 7 doses of HDM or PBS

and an identical dose of HDM administered to adult mice. The immune-inflammatory response was evaluated in the lungs 24 h after the last challenge or at day 7 p.i.

Collection and measurement of specimens. BAL fluid, lungs, and blood were collected at the time of killing. BAL was performed as described previously.[20] In brief, the lungs were dissected and the trachea cannulated using a polyethylene tube (outer/inner diameter = 0.965/0.58 mm; Becton Dickinson, Sparks, MD). The lungs were lavaged twice with PBS (0.25 ml followed by 0.2 ml), and approximately 0.25–0.3 ml of the instilled fluid was retrieved consistently. Total cell counts were then determined using a hemocytometer. Each BAL sample was then centrifuged and the supernatants collected and stored at −20°C. Cell pellets were subsequently resuspended in PBS and cytospins were prepared by centrifugation (Shandon, Pittsburgh, PA) at 300 r.p.m. for 2 min. Protocol Hema 3 stain set (Fisher Scientific, Toronto, ON, Canada) was used to stain all smears. Differential cell counts of BAL were determined from at least 300 leukocytes using standard hemocytological criteria to classify the cells as neutrophils, eosinophils, or mononuclear cells. Peripheral blood was collected by retro-orbital bleeding, and serum was obtained and stored at −20°C. Harvested spleens were placed in sterile tubes containing sterile Hank's balanced salt solution. Where applicable, after BAL collection, the lungs were inflated with 10% formalin at constant pressure of 20 cm H_2O and then fixed in 10% formalin for 48–72 h until further processing. For some measurements, the lungs were dissected without previous BAL collection and placed in PBS at 4°C for tissue homogenate preparation or snap frozen in liquid nitrogen for RNA isolation and quantitative real-time PCR assay.

Histology and immunohistochemistry. After formalin fixation, the left lung was dissected and embedded in paraffin. Sections of 3-μm thickness were cut and stained with hematoxylin and eosin, Picro Sirius Red, or periodic acid-Schiff. Immunohistochemistry for α-SMA was also performed as described previously.[20,55] Images stained with hematoxylin and eosin were captured through Leica camera and microscope (Leica Microsystems, Richmond Hill, ON, Canada) with the magnification of the objective adjusted, as necessary, from ×5 to ×40. For all experiments, the eye piece remained constant at ×10, achieving total magnifications ranging from ×50 to ×400.

Morphometric analysis. Images for morphometric analysis were captured using OpenLab software (v3.0.3; Improvision, Guelph, ON, Canada) using Leica camera and microscope (Leica Microsystems). Image analysis was performed using a custom-computerized analysis system (Northern Eclipse software version 5; Empix Imaging, Mississauga, ON, Canada). Analysis of sections stained for α-SMA, Picro Sirius Red, and periodic acid-Schiff-stained sections were performed as described previously.[20,55] Distances of 20 and 40 μm (starting from below the airway epithelium and proceeding away from the lumen) were used for α-SMA and Picro Sirius Red images, respectively, whereas a distance of 30 μm (starting from below the airway epithelium and proceeding toward the lumen) was used for periodic acid-Schiff-stained images. All images were captured with the objective lens set to a magnification power of ×20 and the eye piece set to ×10, achieving a total magnification of ×200.

Preparation of lung tissue homogenate. Whole lungs were homogenized in 1.5 ml PBS supplemented with 1 COMPLETE protease inhibitor tablet (Roche, Laval, QC, Canada) per 10 ml of buffer. After homogenization, 150 μl of 10% Triton X-100 was added and samples were rocked at 4°C for 1 h. The supernatant was collected following a 15 min spin at 12,000 r.p.m. at 4°C and stored at −70°C.

RNA isolation and quantitative real-time PCR. Total RNA was extracted from frozen lung tissues using RNA-STAT60 reagent (Tel-Test, Friendwood, TX) as per the manufacturer's protocol. The extracted total RNA was further purified using RNeasy Mini Kit (Qiagen, Valencia, CA) and the quality assessed using the Agilent Bioanalyzer 2,100 (Agilent, Santa Clara, CA). Total RNA quantities were determined and 2 μg used for first-strand cDNA synthesis using TaqMan reverse transcriptase-PCR kit as per the manufacturer's protocol. Primer and probe sets for murine TLR 2, 3, 4 5, 7, and 9, and 18 sRNA (internal control) were chosen from Applied Biosystem's Pre-Developed TaqMan Assay reagents (Applied Biosystems, Foster City, CA) and mRNA expression profiles analyzed using ABI-7900HT. Each sample was normalized to the expression of 18sRNA. Relative expression levels were determined using the equation $2^{-\Delta\Delta Ct}$ (within Applied Biosystems Sequence Detection software version 2.2.1).

Lung cell isolation and flow cytometric analysis of lung cells. Total lung cells were isolated as described previously.[20] In brief, total lung cells were isolated by collagenase digestion (Collagenase type I; Life Technologies, Burlington, ON, Canada) washed twice in fluorescence-activated cell sorting buffer (PBS/0.5% bovine serum albumin), and then filtered through 40-μm cell strainer and stained with a panel of antibodies for analysis by 13-color flow cytometry. For each antibody combination, 2×10^6cells were incubated with monoclonal antibodies at 4°C for 30 min. Cells were then washed in fluorescence-activated cell sorting buffer, counted to obtain total cells, and data were collected using an LSRII flow cytometer (BD, Franklin Lakes, NJ). More than 300,000 events were collected for each group. Immune cells were analyzed using FlowJo software (Tree Star, Ashland, OR). The following antibodies were used for identification of intraepithelial DCs, iDCs, alveolar macrophages, pDC, act pDC, $Ly6C^{hi}$ monocytes, B cells, and NK cells: CD45-allophyocyanine-Cyanine(Cy)7, CD3-Pacific Blue, CD11c-fluorescein isothiocyanate, F4/80-phycoerythrin (PE)-Cy5 (all BD Bioscience, Mississauga, ON, Canada), major histocompatibility complex II-Alexa Fluor 700, CD11b-PE, Ly6c-Peridinin Chlorophyll Protein Complex-Cy5.5, DX5-PE-Cy7, and SiglecH-Alexa Fluor 647 (all eBioscience, San Diego, CA). CD8α-PE-Alexa Fluor 610, GR1-Pacific Orange, and CD4-Qdot605 (all Invitrogen, Carlsbad, CA) and mPDCA-1-allophyocyanine (Miltenyi Biotech, Auburn, CA). For some experiments, B220-Qdot800 (strepdavidin) or CD86-Qdot800 (strepdavidin) (BD Bioscience) and B220-Qdot655 or CD86-PE-Cy7 (eBioscience) were used interchangeably. T cells were identified using CD3-Pacific Blue, CD4-allophyocyanine, CD8-PE, CD69-PE-Cy7, CD45-allophyocyanine-Cy7 (all BD Bioscience), and T1/ST2- fluorescein isothiocyanate (MD Bioscience, St Paul, MN). All appropriate isotype controls and fluorescent minus one controls were used. Antibodies were titrated to determine optimal concentration. See **Supplementary Figures 1** and **2** online for gating strategy used to identify allophyocyanines and additional details on the methods used to make these measurements.

Splenocyte cultures. Splenocytes were isolated and resuspended in complete RPMI at a concentration of 8×10^6 cells per ml as described previously.[55] In brief, cells were cultured in medium alone or with the medium supplemented with HDM (31.25 μg ml^{-1}) in a flat-bottom, 96-well plate (Becton Dickinson, Mississauga, ON, Canada) in triplicate. After 5 days of culture, supernatants were harvested and triplicate samples were pooled for cytokine measurements.

Cytokine analysis and immunoglobulin measurements. Cytokines levels were measured in lung homogenates and supernatants of splenocyte cultures using Luminex 100 Total System (Luminex, Austin, TX) based on xMAPmultiplexing technology. 9-Plex cytokine kits containing microbeads with capture antibody and biotinylated reporter specific for mouse IL-1β, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, interferon-γ, and granulocyte macrophage colony-stimulating factor were purchased from Upstate (Charlottesville, VA), whereas type I interferon-β was measured using enzyme-linked immunosorbent assay (ELISA) kits purchased from PBL Biomedical Laboratories (Piscataway, NJ). IL-12p70 and IL-33 were measured using eBioscience ELISA kits. TSLP, IL-25, tumor necrosis factor-α, and TGF-β1 were measured using DuoSet ELISA kits (R&D Systems, Minneapolis, MN). Levels of growth factors in BAL were measured for TGF-β1 and vascular endothelial growth factor using DuoSet ELISA kits and for $PDGF_{AA}$ by Quantikine ELISA kits (R&D Systems) and used according to the manufacture's instructions. Levels of Flu IgG_1

and IgG_{2a} and HDM-specific IgE, IgG_1, and IgG_2 were measured by sandwich ELISA as described previously.[20,47] The formula used to calculate relative units = (OD reading – OD blank)×10.

Assessment of alveolar αSMA-positive cells. To detect alveolar α-SMA-positive cells, paraffin sections of 3 μm were immunostained for α-SMA. Sections were deparaffinized, rehydrated, and incubated for 1 h at room temperature with an alkaline phosphatase-conjugated monoclonal antibody against α-SMA (1:200, C5691, clone 1A4, Sigma-Aldrich, Stockholm, Sweden). Immunoreactivity was detected using a permanent red substrate kit (K0640, Dako, Glostrup, Denmark). Sections were counterstained with Mayer's hematoxylin, dehydrated in ethanol, and mounted in Pertex (HistoLab, Gothenburg, Sweden). High-resolution digital images of whole-lung tissue sections were generated using Aperio ScanScope slide scanner (Aperio Technologies, Vista, CA). The alveolar parenchyma, excluding small airways and pulmonary vessels, were delineated by freehand using image analysis (ImageScope software, Aperio Technologies), and the total number of $\alpha\text{-SMA}^+$ cells per cm^2 alveolar parenchyma was quantified on blinded sections. Double-staining immunofluorescence was performed for the identification of $\alpha\text{-SMA}^+$ cells co-expressing procollagen I (PINP) and α-SMA. After enzymatic retrieval with pepsin/HCl (0.4% pepsin in 0.01 M HCl) for 20 min in 37°C, paraffin sections were blocked with 10% normal goat serum (Sigma, Munich, Germany) for 20 min. Sections were then incubated overnight at 4°C with a rabbit polyclonal antibody against PINP (1:300, gift from J Risteli and J Karttunen, Oulu University, Finland), followed by incubation for 1 h at room temperature with Alexa 488-conjugated goat anti-rabbit secondary antibody (1:200, Invitrogen). A second staining for α-SMA was performed using a Cy3-conjuagted monoclonal antibody against α-SMA (1:1,000, C6198, Sigma-Aldrich). Cell nuclei were stained with Hoechst (H33342, Sigma-Aldrich) and sections mounted in Tris-buffered saline/glycerol. Negative controls were performed by isotype-matched control antibodies.

Airway responsiveness measurements. Airway responsiveness was assessed 30 days after the last exposure to HDM in response to increasing doses of nebulized methacholine (Sigma-Aldrich, Oakville, ON, Canada) using a previously described protocol.[26] In brief, mice were anesthetized with inhaled isoflurane (3% with $1\,l\,min^{-1}$ of O_2), paralyzed with pancuronium bromide (1 mg intraperitoneal), tracheotomized using a blunted 18-G needle, and mechanically ventilated using a small animal computer-controlled piston ventilator (flexiVent, SCIREQ, Montreal, QC, Canada). Mice received 200 breaths per minute and a tidal volume of 0.25 ml; the respiratory rate was slowed during nebulization (10 s) to provide 5 large breaths of aerosol at a tidal volume of 0.8 ml. The response to nebulized saline and increasing doses (3.125 and 12.5 mg ml) of methacholine was measured and the data fit with the constant phase model. Model parameters of airway resistance (Rn), tissue resistance (G), and tissue elastance (H) were calculated as described previously.[56] Model fits that resulted in a coefficient of determination < 0.08 were excluded.

Data analysis. Data were analyzed using GraphPad Prism (version 5.0; GraphPad, La Jolla, CA) and expressed as mean±s.e.m. Results were interpreted using either one-way analysis of variance and Tukey's *post hoc* test, or two-way analysis of variance and Bonferroni's *post hoc* text. Differences were considered statistically significant when $P < 0.05$.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declare no conflict of interest. AAH and RK are current employees of MedImmune, and AJC is a former employee of MedImmune, LLC.

REFERENCES

1. Asthma (WHO, 2008). Asthma. In Fact Sheet Nº 307 (WHO, 2011).
2. Sigurs, N. *et al.* Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. *Am. J. Respir. Crit. Care Med.* **171,** 137–141 (2005).
3. Simoes, E.A. Respiratory syncytial virus infection. *Lancet* **354,** 847–852 (1999).
4. Jackson, D.J. *et al.* Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. *Am. J. Respir. Crit. Care Med.* **178,** 667–672 (2008).
5. Kusel, M.M. *et al.* Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. *J. Allergy Clin. Immunol.* **119,** 1105–1110 (2007).
6. Martinez, F.D. *et al.* Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *N. Engl. J. Med.* **332,** 133–138 (1995).
7. Morgan, W.J. *et al.* Outcome of asthma and wheezing in the first 6 years of life: follow-up through adolescence. *Am. J. Respir. Crit. Care Med.* **172,** 1253–1258 (2005).
8. Illi, S. *et al.* Perennial allergen sensitisation early in life and chronic asthma in children: a birth cohort study. *Lancet* **368,** 763–770 (2006).
9. Prevention of influenza: recommendations for influenza immunization of children, 2007–2008. *Pediatrics* **121,** e1016–e1031 (2008).
10. Osterholzer, J.J. *et al.* Accumulation of CD11b+ lung dendritic cells in response to fungal infection results from the CCR2-mediated recruitment and differentiation of Ly-6Chigh monocytes. *J. Immunol.* **183,** 8044–8053 (2009).
11. Vermaelen, K. & Pauwels, R. Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow cytometry: methodology and new insights. *Cytometry A* **61,** 170–177 (2004).
12. GeurtsvanKessel, C.H. *et al.* Clearance of influenza virus from the lung depends on migratory langerin+CD11b- but not plasmacytoid dendritic cells. *J. Exp. Med.* **205,** 1621–1634 (2008).
13. Beaty, S.R., Rose, C.E. Jr. & Sung, S.S. Diverse and potent chemokine production by lung CD11bhigh dendritic cells in homeostasis and in allergic lung inflammation. *J. Immunol.* **178,** 1882–1895 (2007).
14. Swiecki, M. & Colonna, M. Unraveling the functions of plasmacytoid dendritic cells during viral infections, autoimmunity, and tolerance. *Immunol. Rev.* **234,** 142–162 (2010).
15. Blasius, A.L., Barchet, W., Cella, M. & Colonna, M. Development and function of murine B220+CD11c+NK1.1+ cells identify them as a subset of NK cells. *J. Exp. Med.* **204,** 2561–2568 (2007).
16. O'Keeffe, M. *et al.* Mouse plasmacytoid cells: long-lived cells, heterogeneous in surface phenotype and function, that differentiate into CD8(+) dendritic cells only after microbial stimulus. *J. Exp. Med.* **196,** 1307–1319 (2002).
17. GeurtsvanKessel, C.H. *et al.* Both conventional and interferon killer dendritic cells have antigen-presenting capacity during influenza virus infection. *PLoS One* **4,** e7187 (2009).
18. GeurtsvanKessel, C.H. & Lambrecht, B.N. Division of labor between dendritic cell subsets of the lung. *Mucosal Immunol.* **1,** 442–450 (2008).
19. Poehling, K.A. *et al.* The underrecognized burden of influenza in young children. *N. Engl. J. Med.* **355,** 31–40 (2006).
20. Al-Garawi, A.A. *et al.* Acute, but not resolved, influenza A infection enhances susceptibility to house dust mite-induced allergic disease. *J. Immunol.* **182,** 3095–3104 (2009).
21. Dahl, M.E., Dabbagh, K., Liggitt, D., Kim, S. & Lewis, D.B. Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nat. Immunol.* **5,** 337–343 (2004).
22. Wohlleben, G. *et al.* Influenza A virus infection inhibits the efficient recruitment of Th2 cells into the airways and the development of airway eosinophilia. *J. Immunol.* **170,** 4601–4611 (2003).
23. Adkins, B., Leclerc, C. & Marshall-Clarke, S. Neonatal adaptive immunity comes of age. *Nat. Rev. Immunol.* **4,** 553–564 (2004).

ARTICLES

24. You, D. *et al.* Exposure of neonates to respiratory syncytial virus is critical in determining subsequent airway response in adults. *Respir. Res.* **7,** 107 (2006).
25. Siegle, J.S. *et al.* Early-life viral infection and allergen exposure interact to induce an asthmatic phenotype in mice. *Respir. Res.* **11,** 14 (2010).
26. Llop-Guevara, A. *et al. In vivo*-to-in silico iterations to investigate aeroallergen-host interactions. *PLoS One* **3,** e2426 (2008).
27. Cates, E.C., Fattouh, R., Johnson, J.R., Llop-Guevara, A. & Jordana, M. Modeling responses to respiratory house dust mite exposure. *Contrib. Microbiol.* **14,** 42–67 (2007).
28. Holt, P.G., Strickland, D.H., Wikstrom, M.E. & Jahnsen, F.L. Regulation of immunological homeostasis in the respiratory tract. *Nat. Rev. Immunol.* **8,** 142–152 (2008).
29. Wissinger, E., Goulding, J. & Hussell, T. Immune homeostasis in the respiratory tract and its impact on heterologous infection. *Semin. Immunol.* **21,** 147–155 (2009).
30. Munger, J.S. *et al.* The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. *Cell* **96,** 319–328 (1999).
31. Saraiva, M. & O'Garra, A. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* **10,** 170–181 (2011).
32. Mosconi, E. *et al.* Breast milk immune complexes are potent inducers of oral tolerance in neonates and prevent asthma development. *Mucosal Immunol.* **3,** 461–474 (2010).
33. Hamada, K., Goldsmith, C.A., Goldman, A. & Kobzik, L. Resistance of very young mice to inhaled allergen sensitization is overcome by coexposure to an air-pollutant aerosol. *Am. J. Respir. Crit. Care Med.* **161,** 1285–1293 (2000).
34. Kulhankova, K. *et al.* Early-life co-administration of cockroach allergen and endotoxin augments pulmonary and systemic responses. *Clin. Exp. Allergy* **39,** 1069–1079 (2009).
35. Phipps, S. *et al.* Allergic sensitization is enhanced in early life through toll-like receptor 7 activation. *Clin. Exp. Allergy* **39,** 1920–1928 (2009).
36. Holt, P.G., Upham, J.W. & Sly, P.D. Contemporaneous maturation of immunologic and respiratory functions during early childhood: implications for development of asthma prevention strategies. *J. Allergy Clin. Immunol.* **116,** 16–24; quiz 25 (2005).
37. Hammad, H. *et al.* Inflammatory dendritic cells—not basophils—are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J. Exp. Med.* **207,** 2097–2111 (2010).
38. Hammad, H. *et al.* House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* **15,** 410–416 (2009).
39. Trompette, A. *et al.* Allergenicity resulting from functional mimicry of a Toll-like receptor complex protein. *Nature* **457,** 585–588 (2009).
40. Phipps, S. *et al.* Toll/IL-1 signaling is critical for house dust mite-specific helper T cell type 2 and type 17 [corrected] responses. *Am. J. Respir. Crit. Care Med.* **179,** 883–893 (2009).
41. Visscher, D.W. & Myers, J.L. Bronchiolitis: the pathologist's perspective. *Proc. Am. Thorac. Soc.* **3,** 41–47 (2006).
42. Paul, W.E. & Zhu, J. How are T(H)2-type immune responses initiated and amplified? *Nat. Rev. Immunol.* **10,** 225–235 (2010).
43. Strachan, D.P. Hay fever, hygiene, and household size. *B.M.J.* **299,** 1259–1260 (1989).
44. Barbato, A. *et al.* Airway inflammation in childhood asthma. *Am. J. Respir. Crit. Care Med.* **168,** 798–803 (2003).
45. Saglani, S. *et al.* Early detection of airway wall remodeling and eosinophilic inflammation in preschool wheezers. *Am. J. Respir. Crit. Care Med.* **176,** 858–864 (2007).
46. Bush, A. How early do airway inflammation and remodeling occur? *Allergol. Int.* **57,** 11–19 (2008).
47. Johnson, J.R. *et al.* Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am. J. Respir. Crit. Care Med.* **169,** 378–385 (2004).
48. Andersson, C.K. *et al.* Mice lacking 12/15-lipoxygenase have attenuated airway allergic inflammation and remodeling. *Am. J. Respir. Cell. Mol. Biol.* **39,** 648–656 (2008).
49. Kapanci, Y., Ribaux, C., Chaponnier, C. & Gabbiani, G. Cytoskeletal features of alveolar myofibroblasts and pericytes in normal human and rat lung. *J. Histochem. Cytochem.* **40,** 1955–1963 (1992).
50. Xisto, D.G. *et al.* Lung parenchyma remodeling in a murine model of chronic allergic inflammation. *Am. J. Respir. Crit. Care Med.* **171,** 829–837 (2005).
51. Lundblad, L.K. *et al.* Airway hyperresponsiveness in allergically inflamed mice: the role of airway closure. *Am. J. Respir. Crit. Care Med.* **175,** 768–774 (2007).
52. Martinez, F.D. Genes, environments, development and asthma: a reappraisal. *Eur. Respir. J.* **29,** 179–184 (2007).
53. Guerra, S. & Martinez, F.D. Asthma genetics: from linear to multifactorial approaches. *Annu. Rev. Med.* **59,** 327–341 (2008).
54. Eisenbarth, S.C. *et al.* Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J. Exp. Med.* **196,** 1645–1651 (2002).
55. Fattouh, R. *et al.* Transforming growth factor-beta regulates house dust mite-induced allergic airway inflammation but not airway remodeling. *Am. J. Respir. Crit. Care Med.* **177,** 593–603 (2008).
56. Hantos, Z., Daroczy, B., Suki, B., Nagy, S. & Fredberg, J.J. Input impedance and peripheral inhomogeneity of dog lungs. *J. Appl. Physiol.* **72,** 168–178 (1992).

SUPPLEMENTARY MATERIAL

INFLUENZA A FACILITATES SENSITIZATION TO HOUSE DUST MITE IN INFANT MICE LEADING TO AN ASTHMA PHENOTYPE IN ADULTHOOD

Amal Al-Garawi[1], Ramzi Fattouh[1], Fernando Botelho[1], Tina D. Walker[1], Susanna Goncharova[1], Cheryl-Lynn Moore[1], Michiko Mori[2], Jonas S. Erjefalt[2], Derek K. Chu[1], Alison A. Humbles[3], Roland Kolbeck[3], Martin R. Stampfli[1], Paul M. O'Byrne[4], Anthony J. Coyle[1,3] and Manel Jordana[1*].

[1]Division of Respiratory Diseases and Allergy, Center for Gene Therapeutics and Department of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada. [2]Department of Experimental Medical Science, Lund University, Lund, Sweden, [3]Department of Respiratory, Inflammation and Autoimmunity, MedImmune LLC., Gaithersburg, MD, USA, [4]Department of Medicine, McMaster University, Hamilton, ON, Canada.

Supplementary Figures

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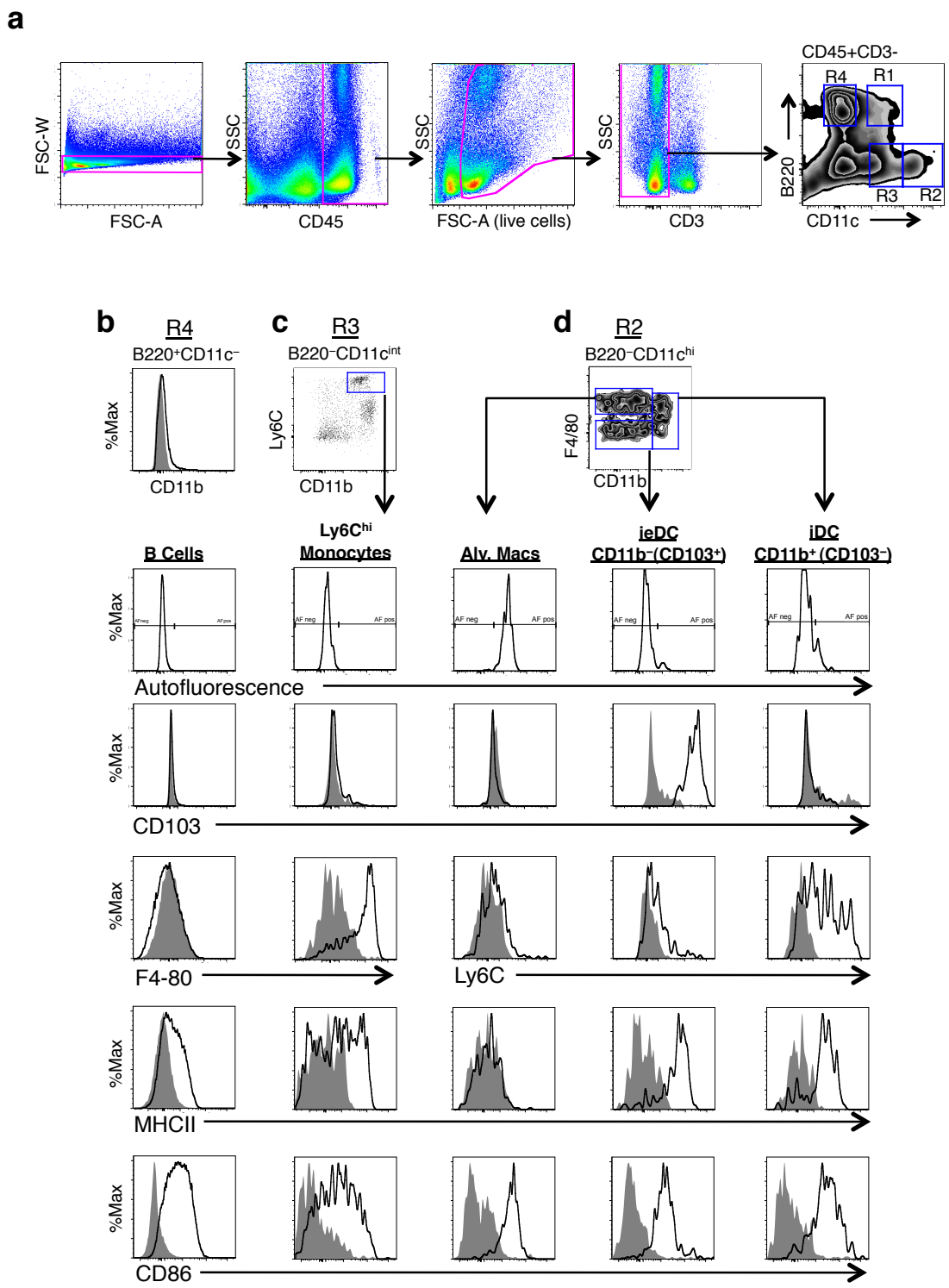
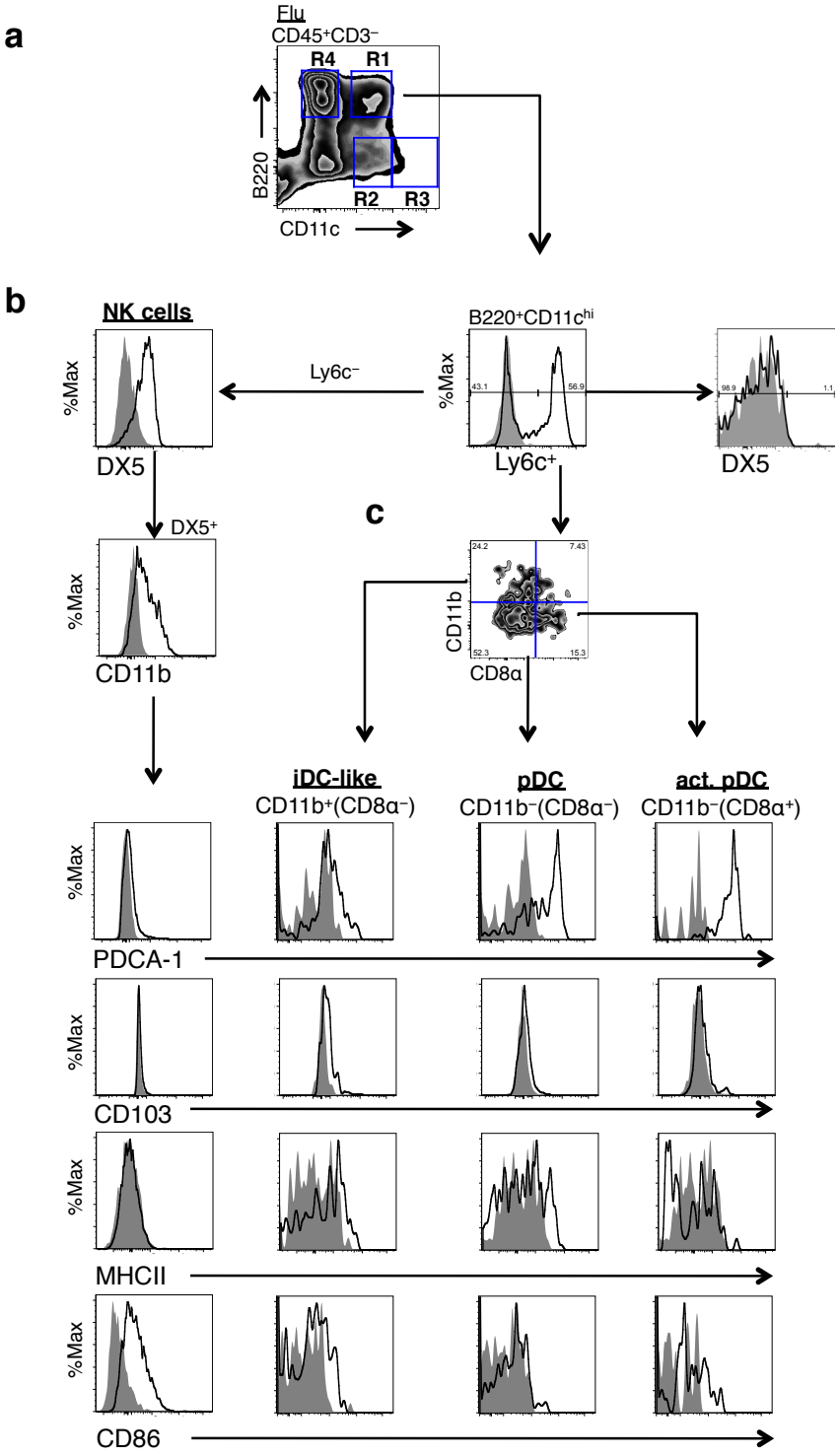
Figure 1



Supplementary Figures Al-Garawi, A *et al.*

Figure 2



Supplementary Figures

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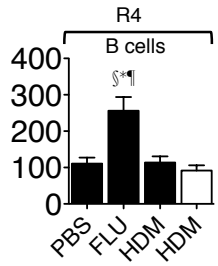
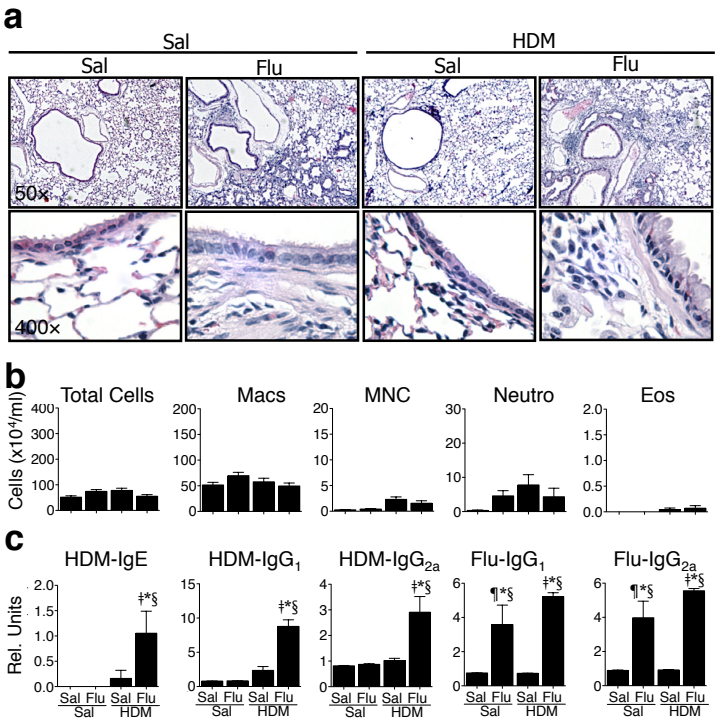
Figure 3



Supplementary Figures Al-Garawi, A *et al.*

Figure 4



SUPPLEMENTARY FIGURE LEGENDS

Figure 1. *Gating strategy and activation status of various APC populations:* Total lung cells were isolated at day 7 p.i. or 24 hr after the last HDM exposure and 13–color flow performed using an LSRII^{TM}. Cells were stained with the following cell surface markers: CD45, CD3, MHCII, B220, CD11b, CD11c, F4/80, GR1, CD103, Ly6c, DX5, CD3, CD4, and CD8. (**a**) First, to exclude doublets, cells were plotted as FSC-W vs. FSC-A and size exclusion applied to the population of interest. Then, cells were gated based on SSC vs. CD45 to identify leukocytes. The CD45^{+} cell population was plotted as SSC vs. FSC-A and an area representing live cells including, T cells and APCs selected. T cells were excluded based on CD3^{+} expression. The remaining CD3^{-} population was selected and immune cells plotted against B220 vs. CD11c expression. Differences in immune cell population were visualized using Zebra plot and populations of interest gated as shown. (**b**) B-Cells were identified as (B220^{hi}, CD11c^{-}, CD11b^{-}, CD103^{-}, F4/80^{-}, MHCII^{low}, CD86^{+} and no autofluoresence), (**c**) Ly6C^{hi}Monocytes (B220^{-}, CD11c^{int}, CD11b^{hi}, Ly6C^{hi}, F4/80^{+}, MHCII^{+}, CD86^{+}). The B220^{-} CD11c^{hi} population was differentiated between (**d**) alvMACS (CD11b^{-}, F4/80^{+}, MHCII^{low}, CD86^{+}, and high autofluorescence), iDC (CD11b^{+}, F4/80$^{low\text{-}high}$, CD103^{-}, Ly6C^{+}, MHCII^{+}, CD86^{+} and low autofluorescene) and ieDC (CD11b^{-}, F4/80^{-}, Ly6C^{-}, MHCII^{+}, CD86^{+} and low autofluresence). Solid gray line represents isotype control, solid line represents staining for marker of interest. alvMACS, alveolar macrophages; ieDC, intraepithelial DCs; iDC, inflammatory DCs. Figure shows analysis of immune cells in infant mice following HDM exposure as representative example.

Figure 2: *Gating strategy and Identification of NK cells, pDC , CD8α$^+$pDC and CD11b$^+$iDC populations:* (**a**) Immune cells were gated on B220 and CD11c as described in supplementary Figure 1. B220^{hi}CD11c^{int} cells were selected and evaluated for Ly6C and DX5 expression to distinguish (**b**) NK cells (B220^{hi}, CD11c^{int}, Ly6c^{-}, DX5$^+$, CD11b$^+$, PDCA-1^{-}) from a heterogeneous population of Ly6C$^+$ DX5^{-} cells. This gate was further characterized based on CD11b and CD8α and evaluated for PDCA-1 expression. (**c**) Three distinct populations could be identified: pDCs (B220^{hi}, CD11c^{int}, Ly6c^{hi}, DX5^{-}, CD11b^{-}, CD8α^{-}, PDCA-1$^+$), act. pDC (B220^{hi}, CD11c^{int}, Ly6c^{hi}, DX5^{-}, CD11b^{-}, CD8α$^+$ and PDCA-1$^+$) and a iDC-like population (B220^{hi}, CD11c^{int}, Ly6c^{hi}, DX5^{-}, CD11b$^+$, CD8α$^-$ and PDCA-1^{-}). Solid gray line represents isotype control, solid line represents staining for marker of interest. Figure shows analysis of immune cells following influenza A infection as representative example.

Figure 3: *Quantification of B-cells:* B-cells were identified as outlined in supplementary Figure 1b and total cells quantified. Data representative of at least three independent experiments. *n* = 4-6mice/group. Data represent mean ± SEM. *P*<0.05 compared with *PBS; ‡Flu; § HDM; and ¶HDM-adult, respectively.

Figure 4: *Immune Inflammatory response after cessation of allergen exposure.* Separate groups of 8-day old mice were infected with influenza virus or given PBS alone. Seven days later, PBS and flu-infected groups are exposed either to 3 weeks of HDM or Sal and sacrificed 3 weeks after the last HDM exposure. (**a**) Lung histopathology was evaluated by H&E to show degree of total lung inflammation and eosinophilia. (**b**) Cellular profile in BAL fluid showing number of total cells, macrophages (Macs), mononuclear cells (MNC), neutrophils (Neutro) and eosinophils (Eos). (**c**) Serum Ig levels showing HDM-specific IgE, IgG1 and IgG2a and flu-specific IgG1 and IgG2a. Images were taken at 50 and 200x original magnification. n= 8-10 mice/group. Data represent mean ± SEM. p<0.05 compared with *PBS ; ‡FLU; and § HDM, respectively.

Chapter 5

DISCUSSION

The incidence of asthma is on the rise. Indeed, the WHO estimates that over 100 million additional people will be diagnosed with this disease by 2025 (4). In the last century, much effort has centered on understanding the pathogenesis of asthma, yet the origins of the disease have remained elusive. That among allergic individuals, only a fraction develop asthma suggests that in addition to allergen sensitization, other factors contribute to the onset of disease. Yet, causative evidence supporting this notion is lacking.

This thesis aimed to examine the influence of respiratory viral infections to the development of asthma. Specifically, the studies described herein investigate the interaction between influenza A infection and a clinically relevant aeroallergen, HDM. The first study examines the consequences of a prior respiratory viral infection on the elaboration of an allergic phenotype in response to a subclinical dose of HDM in adult mice. These studies reveal that a severe, viral-induced immune perturbation alters the threshold of responsiveness to allergen exposure. In addition, it demonstrates that timing of allergen exposure in relation to a prior viral infection critically affects the outcome of the viral-allergen interaction. To provide greater insight into underlying immune mechanisms, we investigated influenza A-HDM interactions from a global perspective and at the genomic level. This research revealed the engagement of multiple immune pathways in this process (second study). Lastly, as allergic asthma most commonly develops early in life, we investigated such viral-allergen interactions

in infant mice; in particular, we examined whether they would imprint a long lasting asthma phenotype (third study).

Alteration of Immunological Thresholds to HDM Exposure

To address the impact that a influenza-A infection has on mucosal immune responsiveness to allergens, we utilized a well established mouse model of allergic airway disease based on the clinically relevant aeroallergen HDM. Our laboratory has shown that exposure to a sufficiently high dose of allergen over a sufficiently long period of time elicits all the cardinal features of allergic asthma (94, 99). Furthermore, we have reported extensive dose response studies (100). Directly pertinent to this thesis work, we performed a series of preliminary studies, to establish a concentration of HDM that elicits either no or only mild inflammation without discernable lung dysfunction. Thus, we used this *subclinical* dose to investigate the consequences of a prior influenza infection on the elaboration of an asthmatic phenotype. While it is difficult to predict the precise amount of allergen that humans inhale, it is reasonable to postulate that, in most instances, it does not reach a sufficient level to elicit dysfunction or clinical manifestation.

Our investigation revealed that sensitization and airway inflammation to a subclinical dose of HDM were amplified when allergen exposure occurred during an acute flu infection. Importantly, this resulted in the generation of marked lung dysfunction (i.e. AHR). In contrast, allergen exposure during the resolution phase

did not enhance allergic inflammation or had an impact on lung function. Thus, our data suggest that the enhanced immunologic and physiologic allergen responsiveness is dependent on the presence of the *pro-inflammatory* environment elicited by an acute influenza infection. These data are in accord with epidemiological observations proposing that the timing of allergen sensitization in the context of respiratory viral infections is critical in promoting the development of asthma (58, 71).

MECHANISMS UNDERLYING INFLUENZA-HDM INTERACTION

The mechanisms by which influenza A alters responsiveness to HDM exposure have not been established but are likely to be manifold. It is well established that Influenza A targets the bronchial epithelium. Indeed, an important consequence of influenza A infection is a compromised bronchial epithelial layer. In addition to apoptosis of infected cells, it has been proposed that respiratory viral infections have the ability to disrupt epithelial tight junctions leading to increased permeability (101). Hence, the loss in structural integrity of the epithelial barrier may facilitate the translocation of exogenous foreign antigens, such as HDM allergen, thus increasing the exposure level of underlying immune cells (102). In addition to a compromised mucosal barrier, disruption of the bronchial epithelium activates innate immune pathways that promote the generation of antigen specific adaptive immunity (103).

Activation of the innate immune response involves the production of type-I IFNs, a hallmark of influenza A infection. Type-I IFNs are powerful immune stimulators that regulate the expression of more than 100 IFN-stimulated gene products (ISGs) responsible for the generation of an anti-viral state aimed at reducing viral replication and limiting viral spread (104, 105). In addition to anti-viral responses, type-I IFNs facilitate the production of numerous pro-inflammatory cytokines and chemokines (106). In this regard, our study shows that influenza A infection lead to the production of both type-I IFN-α and IFN-β as well as increased levels of the pro-inflammatory cytokines TNF-α, IL-6 and IFN-γ. Collectively, these immune mediators are capable of orchestrating the recruitment and activation of additional immune-inflammatory cells to the site of infection (107). A number of studies, including our own, have shown, that that several leukocyte populations, including neutrophils, monocytes, B cells and various antigen presenting cell (APC) subtypes, such as myeloid (m) DCs and plasmacytoid (p) DCs, are recruited into the lung after influenza A infection.

A key function of the innate immune systems is to defend the host against invading pathogens; this is accomplished through the effective sensing of harmful entities. The recognition of foreign microbial products is mediated by a set of germline encoded pattern recognition receptors (PRRs) present on mucosal cells, such as ECs, macrophages and DCs (108). Multiple PRRs, such as Toll-like receptors (TLRs), C-type lectins receptors (CLRs), RIG-like receptors (RLR) and protease activated receptors (PARs), recognize various pathogen activated

molecular patterns (PAMPs) expressed by microbes (109, 110). In addition to PRRs, damage associated molecular pattern (DAMP) receptors recognize endogenous stress signals, such as intracellular molecules released as a result of tissue injury. These danger molecules, also known as DAMPs or "alarmins" (111, 112) bind to various DAMP receptors, such as complement receptors, purinergic receptors, high mobility group box 1 (HMGB1) receptors and heat shock protein (HSP) receptors and, similar to PRRs, initiate the inflammatory cascade through the recruitment of innate immune cells (111, 113). Importantly, the redundancy in sensory recognition molecules ensures an efficient response to a wide variety of harmful entities (114).

Using genome-wide transcriptional profiling, our data demonstrated the increased expression of numerous innate molecules, including TLRs, CLRs as well as various complement receptors that have been shown to be involved in the pathogenesis of allergic disease (115). Of particular interest, influenza increased the expression of a variety of CLR family members, most notably Dectin-1 and Dectin-2 that respond to complex carbohydrate structures, such as glycans present on aeroallergens such as *Dermatophagoides farinae (Df)* and *Dermatophagoides pteronyssinus (Dp)* (116). In addition to PRRs, influenza infection increased the expression of complement-associated proteins *C1q, C3* and *C3ar* that recognize endogenous and exogenous danger signals and are involved in the generation of anaphylatoxins (AT), C3a and C5a. AT are responsible for the recruitment and activation of a variety of leukocyte populations, including MCs, eosinophils and

basophils (115). Thus, these findings suggest that influenza A *primes* the lung microenvironment by increasing the expression of genes capable of sensing and responding to HDM. Arguably, this heightened state of alertness may decrease the threshold of allergen responsiveness. Indeed, our data demonstrate that exposure to HDM which led to only minor changes in gene expression reflective of mild inflammatory responses, in the context of a prior flu infection, now resulted in the increased expression of over 300 unique genes.

An important event elicited by influenza A infection is the recruitment of mDC and pDCs. It has been well established that DCs play key roles in promoting allergic inflammation in response to inhaled aeroallergens such as HDM (117, 118). However, the mechanisms by which viral-activated DCs may enhance allergic immune responses have not been fully elucidated. We found that influenza infection triggers the expression of the high affinity IgE receptor I (FcεRI) in the lung, and that this was associated with the expression of type-1 IFN and numerous interferon-stimulated genes (ISGs). Recent studies in mice have shown that infection by Sendaii viruses increase FcεR1 on lung DCs in a type-I IFN-dependent manner (119). Interestingly, *in vitro* receptor cross-linking of isolated FcεR1-bearing DCs, resulted in the production of CCL28 (MEC), a chemokine involved in the recruitment of both $CD4^+$ and $CD8^+$ T cells (119). Thus, these observations suggest a potential role of FcεRI-bearing DCs to contribute to the pathogenicity of asthma. For example, it has been proposed that the increased expression of FcεRI on pulmonary DCs during viral exacerbations

of asthma may promote increased allergen uptake (120). In this regard, FcεRI receptor bearing DCs were shown to be involved in allergen capture and migration to the mediastinal lymph nodes in a mouse model of allergic disease. Furthermore, depletion of DCs ameliorated HDM-mediated inflammation (117). Taken together our findings suggest, that the upregulation of FcεRI by influenza infection may contribute to the amplification of the inflammatory response to HDM exposure.

Influenza A infection leads to the early production of numerous cytokines, presumably by innate cells, that maybe associated with the development of Th2 responses. For example, numerous studies have supported a role for IL-6 in promoting the priming of Th2 cells (121, 122). However, the extent to which IL-6 may promote HDM-mediated inflammation has not been explored. Further to the studies included in this thesis, we have explored the role of IL-6 in HDM-responses and shown that blockade of IL-6 resulted in significantly reduced Th2-mediated inflammation and allergic sensitization (**Appendix I**). With regards to influenza A infection, our data show that influenza resulted in increased levels of IL-6. Consequently, it is conceivable that elevated levels of IL-6 induced by influenza A may amplify Th2-mediated allergic sensitization and inflammation.

In addition to IL-6, influenza A infection induces a prolific pro-inflammatory cytokine profile, as revealed both at the level of gene expression as well as protein production. A number of these cytokines have been shown to play

important roles in the initiation of Th2-mediated diseases, including allergic asthma (111, 123). Of particular interest is the expression of IL-33, a key epithelial-associated cytokine, that has been proposed to be involved in the development of Th2 immunity (124, 125).

Collectively, our data show that infection with influenza A shifts immune responsiveness to a subclinical dose of HDM. We propose that the pervasive pro-inflammatory environment induced by influenza A infection heightens the state of immune alertness and lowers the threshold needed for HDM to elicit overt inflammatory and functional responses.

INFLUENZA-HDM INTERACTIONS IN EARLY LIFE

In most instances, allergic asthma develops in early life. Given that respiratory viral infections are thought to be a risk factor for the development of asthma, it is surprising that very few experimental studies have investigated the interaction between viral infection and *concurrent* allergen exposure during this time frame. Such investigations are necessary because the immune system in early life is qualitatively and quantitatively different from that in adults (126). For example, there is evidence that the antigen-presenting cell (APC) compartment is incompletely developed at birth and early life, as indicated by the reduced expression of major histocompatibility complex (MHC)II and associated co-stimulatory molecules such as CD80, CD86 and CD40; thus, suggesting that the overall capacity to generate an adaptive immune response is diminished (127). In

addition, the T and B-cell repertoires are similarly under developed, further limiting the generation of a range of effector T cell populations. In contrast, the regulatory cell compartment appears to be particularly robust in the neonatal period, establishing an environment biased towards immuno-suppression (128-130). During this critical period of relative immaturity of the adaptive immune system, the more ancient innate immune system, along with transferred maternal immunoglobulins, comprise the primary defense mechanism against invading pathogens (96-98).

Despite a seemingly constitutive hyporesponsive immune system in early life, human infants are able to elicit strong Th1 immunity, under certain conditions. This is exemplified by the generation of protective immunity to Bacillus Calmette-Guerin vaccination (131). These observations underscore the notion that instead of pervasive hyporresponsiveness, the ability to generate immune responses depends on the nature/strength of the initiating immune signal. Thus, encounters with life threatening pathogens *vis-à-vis* ubiquitous environmental allergens is likely to elicit differential responsiveness. Speculatively then, immune perturbations elicited by one entity may alter immune responsiveness to a second entity. To investigate this paradigm, we developed an experimental platform to study the impact of influenza A infection in early life on allergen responsiveness, and inquired about the long-term consequences of such an interaction on the airway structural-functional phenotype.

Our study shows that neonatal mice were markedly hyporesponsive to doses of HDM that elicit maximal or near maximal allergic responses in adult mice. In sharp contrast, neonatal mice were able to generate a robust immune-inflammatory response to influenza A infection. Importantly, HDM exposure during the acute phase of the flu-induced inflammatory response resulted in the development of robust eosinophilic inflammation, airway remodeling and marked lung dysfunction that persisted into adulthood even in the absence of continued allergen exposure.

IMMUNE PERTURBATIONS IN EARLY LIFE

It is thought that maintenance of pulmonary homeostasis is largely dependent on the balance of inhibitory and activating processes (132). Influenza infection activates multiple pro-inflammatory pathways, that collectively may overcome immune regulatory mechanisms. Furthermore, following infection, we observed, in neonatal mice, the production of a vast array of cytokines, chemokines and growth factors, including thymic stromal lymphpoietin (TSLP), IL-25, IL-6, IL-33 and granulocyte-macrophage colony-stimulating factor (GM-CSF) that have been shown to be associated with the initiation of Th2 immunity (125). Arguably, then, influenza A infection conditions the lung microenvironment, to overcome immune homeostatic mechanisms and facilitate the emergence of Th2 responses.

As alluded to earlier, sensing of pathogens stimulates the recruitment and activation of APC subtypes into the pulmonary environment. Our data show that, influenza A infection resulted in the recruitment of various APC subtypes in infant mice, most notably inflammatory (i)DCs, as well as $CD103^{+}$intraepithelial DCs and pDCs. Although the role these APCs subtypes play in viral-mediated allergic disease has not been established, mouse models of asthma have shown that iDC and $CD103^{+}$ DC are necessary for the elicitation of HDM-mediated allergic inflammation (117, 118). Our findings are in accord with clinical studies that show severely reduced numbers of APCs in the lung of infants (under 1 year of age) who died suddenly, and that the numbers of mature DCs increased in those infants who died of respiratory infection (133). Thus, these findings illustrate the notion that the maturation of the lung immune system in early life is influenced by encounters with microbial pathogens.

It has been proposed that pDCs have important roles in the generation of immunologic tolerance to inhaled OVA allergen (134). In the study by deHeer *et al.*, depletion of $CD11c^{int}$ $Gr\text{-}1^{+}$ pDCs prior to intratracheal OVA adminstration resulted in enhance inflammation and OVA-specific immunity. Interestingly, the experimental approach used depleted $CD11c^{int}$ $Gr\text{-}1^{+}$ cells in the mediastinal lymphnodes, but not in the lungs of naïve mice (134). Thus, the precise role pDCs have in the development of allergic asthma has not been clarified. Our study shows the emergence of allergic inflammation despite an increased number of activated pDCs in the lungs of neonatal mice following influenza A infection.

pDCs are major producers of type-I IFNs and studies have shown that type-I IFNs are critical immune activators, facilitating the generation of adaptive immunity (135, 136). Hence, it is conceivable that viral induced type-I IFNs prime the immune environment prior to HDM exposure, thus overcoming allergen hyporesponsiveness, leading to allergic sensitization and inflammation.

As indicated earlier, PRRs, such as TLRs are critical innate sensory molecules for the detection of harmful entities. In addition to their involvement in initiating innate immune pathways, TLR genes possess promoter regions sensitive to the IFN response (137). Indeed, type-I IFNs engage in a positive feedback loop additionally enhancing the immune surveillance system by increasing TLR expression. Our data show that influenza A enhances the expression of the immune surveillance system of infant mice, increasing the expression of various TLRs, including TLR 2, 3, 4, 5, 7 and 9. Of these, TLR 4 is particularly interesting as it is known to play important roles in allergic asthma (138).

In sum our study furnishes evidence that immune perturbations in early life elicited by influenza A infection overcome immunologic homeostatic mechanisms thus enabling the infant lung immune system to respond to HDM. In particular, the production of immune activating cytokines, recruitment of various DC subtypes and increased expression of immune sensing machinery establish a lung microenvironment, conducive to the elicitation of allergen responses.

IMPRINTING OF A LONG LASTING ASTHMA PHENOTYPE

Arguably, the extended maturation of the respiratory tract after birth represents an interval of susceptibility to damage from exposure to environmental factors (139, 140). Therefore, severe immune perturbations of the lung environment during this period of time have the potential to alter normal lung growth and function with long-lasting consequences (139). Our studies show that HDM exposure in the context of an acute influenza A infection promoted accelerated structural remodeling of the airways and lung parenchyma. Clinical studies have documented airway remodeling in children as young as three years of age (141). This observation is somewhat in discordance with the prevailing notion that airway remodeling develops in response to repetitive inflammatory insults occurring over a prolonged period of time (i.e. many years). The observation that a respiratory flu infection may accelerate the development of allergic airway remodeling in young mice may provide one possible explanation to reconcile this apparent discrepancy. More importantly, the persistence of structural alterations and the presence of bronchial hyperreactivity even after prolonged absence of allergen exposure, supports the notion that immune perturbations due to severe respiratory infections in early life can imprint an asthmatic phenotype in adulthood.

A prevailing hypothesis, known as the "hit-and run" theory suggests that respiratory infection in early life initiate a series of events, culminating in a chronic asthma phenotype that includes both structural alterations as well as airway hyperreactivity long after viral clearance (142). Although respiratory viral

infections in early life are an independent risk factor for the development of asthma, clinical studies show that many children with viral-associated wheeze during infancy do not go on to develop asthma (58, 73). Indeed, the risk for developing persistent asthma is increased several fold in those who are *also* sensitized to allergen during the first year of life (58). Within this context, our study shows that respiratory viral infection alone is insufficient for the generation of long-lasting lung dysfunction, and that the interaction with allergen exposure in early life is necessary to imprint an asthmatic phenotype later in life. Importantly, the lung dysfunction is apparent, even in the absence of allergen exposure. Thus, these findings support a *"two hit"* theory by which the first hit (i.e. severe respiratory infection) is followed by the second hit (i.e. allergen exposure) (143).

The mechanisms that mediate structural remodeling are incompletely understood, and likely involve multiple interactions between epithelial cells and the underlying structural constituents of the submucosal layer (144). Importantly, the delineation of the relative contribution of specific remodeling alterations to airway/lung dysfunction is controversial and, ultimately, remains to be elucidated (145). With these limitations our data demonstrate the presence of marked airway hyperreactivity at a time where inflammation has completely resolved and both airway and parenchymal remodeling persist. It is tempting to suggest that the evolution of airway dysfunction paralleled the changes in structure. However, it is also possible, that airway hyperreactivity was established earlier, during the inflammatory phase, and is persistent. In this regard, observations in humans

show that lung function is set in early life and tracks into adult hood. Importantly, these studies suggest that a "window of opportunity" exists, in early life, during which severe immune perturbations can derail normal lung (146).

SUMMARY AND FUTURE DIRECTIONS

Allergic sensitization is an important risk factor for the development of allergic disease (71). In addition, respiratory viral infections are associated with an increased risk of asthma in young children. However, the relationship between allergen sensitization and viral infections in childhood, and its impact on the development of allergic asthma remains to be clarified. The studies described in this thesis show that influenza A primes the lung environment such that it lowers the threshold of responsiveness to allergens, thus facilitating the expression of an allergic phenotype. These studies highlight the idea that the *context* by which allergen is encountered critically determines the functional and structural outcome. Importantly, our findings demonstrate that a catastrophic immune event in infancy has profound consequences in adulthood, long after the encounter with the inciting triggers.

Influenza A infections have been typically assumed to generate archetypical Th1 immunity (147) capable of inhibiting or down-regulating Th2-mediated processes. However, our data show that influenza A infection generated a complex environment rich in immune mediators involving Th1-, Th2- and Th17-associated cytokines as well as an array of pro-inflammatory and antiviral

cytokines collectively capable of mediating the initiation and propagation of a variety of Th-mediated inflammatory responses. In this regard, a natural extension of the studies presented in this thesis is the elucidation of cellular and molecular pathways underlying the emergence of the influenza-induced allergic phenotype and, specifically, the role of relevant candidates such as type-I IFNs, IL-6 and IL-33 in this process. Likewise, uncovering whether specific respiratory viral strains are more likely than others to promote the development of allergic asthma should be a fertile future research direction.

While such questions are beyond the scope of the research presented herein, we surmise that the experimental models that we have established as part of this thesis work furnish a productive platform of inquiry. We suggest that understanding the basic mechanisms underlying the interactions between viruses and asthma especially during infancy is necessary to develop primary prevention strategies as well as focused therapeutics particularly in children.

REFERENCES

1. Asthma. In *Fact sheet N°307*, May 2011 ed. WHO.
2. Masoli, M., D. Fabian, S. Holt, and R. Beasley. 2004. The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy* 59:469-478.
3. Akinbami, L. J., J. E. Moorman, and X. Liu. 2011. Asthma prevalence, health care use, and mortality: United States, 2005-2009. *Natl Health Stat Report*:1-14.
4. 2012. Asthma Statistics. American Academy of Allergy Asthma & Immunology.
5. Weiss, S. T. 2012. New approaches to personalized medicine for asthma: where are we? *J Allergy Clin Immunol* 129:327-334.
6. Marketos, S., and C. Ballas. 1982. Bronchial asthma in medical literature of Greek antiquity. *Hist Sci Med* 17:35-39.
7. Stolkind, E. 1933. The History of Bronchial Asthma and Allergy. *Proc R Soc Med* 26:1120-1126.
8. Sakula, A. 1985. Henry Hyde Salter (1823-71): a biographical sketch. *Thorax* 40:887-888.
9. Diamant, Z., J. D. Boot, and J. C. Virchow. 2007. Summing up 100 years of asthma. *Respir Med* 101:378-388.
10. Persson, C. G. 1985. On the medical history of xanthines and other remedies for asthma: a tribute to HH Salter. *Thorax* 40:881-886.

11. Beaven, M. A. 2009. Our perception of the mast cell from Paul Ehrlich to now. *Eur J Immunol* 39:11-25.

12. Cohen, S., and M. Dworetzky. 2002. Pioneers and milestones. Francis M. Rackemann, MD. *J Allergy Clin Immunol* 109:179-184.

13. Kay, A. B. 2006. 100 years of 'Allergy': can von Pirquet's word be rescued? *Clin Exp Allergy* 36:555-559.

14. Meltzer, S. J., and J. Auer. 1910. The Respiratory Changes of Pressure at the Various Levels of the Posterior Mediastinum. *J Exp Med* 12:34-44.

15. Johansson, S. G., J. O. Hourihane, J. Bousquet, C. Bruijnzeel-Koomen, S. Dreborg, T. Haahtela, M. L. Kowalski, N. Mygind, J. Ring, P. van Cauwenberge, M. van Hage-Hamsten, and B. Wuthrich. 2001. A revised nomenclature for allergy. An EAACI position statement from the EAACI nomenclature task force. *Allergy* 56:813-824.

16. 1999. Asthma at a Glance In *National Center for Environmental Health (NCEH), U.S. CDC* U.S. CDC.

17. Pepys, J. 1973. Types of allergic reaction. *Clin Allergy* 3 Suppl:491-509.

18. Gould, H. J., and B. J. Sutton. 2008. IgE in allergy and asthma today. *Nat Rev Immunol* 8:205-217.

19. Koessler, H. L. H. a. K. K. 1922. The Pathology of Bronchial Asthma. *Archives of Internal Medicine* 30:689-760.

20. Ellis, A. 1908. The pathologic anatomy of bronchial asthma. *Am. J. Med. Sci.* 136:407-410.

21. Frigas, E., and G. J. Gleich. 1986. The eosinophil and the pathophysiology of asthma. *J Allergy Clin Immunol* 77:527-537.

22. Gowans, J. L., G. D. Mc, and D. M. Cowen. 1962. Initiation of immune responses by small lymphocytes. *Nature* 196:651-655.

23. Wasserman, S. I., E. J. Goetzl, and K. F. Austen. 1974. Preformed eosinophil chemotactic factor of anaphylaxis (ECF-A). *J Immunol* 112:351-358.

24. Janeway, C. A., Jr. 1999. The discovery of T cell help for B cell antibody formation: a perspective from the 30th anniversary of this discovery. *Immunol Cell Biol* 77:177-179.

25. Masopust, D., V. Vezys, E. J. Wherry, and R. Ahmed. 2007. A brief history of CD8 T cells. *Eur J Immunol* 37 Suppl 1:S103-110.

26. Moberg, C. L. An appreciation of Ralph Marvin Steinman (1943-2011). *J Exp Med* 208:2337-2342.

27. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136:2348-2357.

28. Lloyd, C. M., and E. M. Hessel. 2010. Functions of T cells in asthma: more than just T(H)2 cells. *Nat Rev Immunol* 10:838-848.

29. Proud, D., and R. Leigh. 2011. Epithelial cells and airway diseases. *Immunol Rev* 242:186-204.

30. Bals, R., and P. S. Hiemstra. 2004. Innate immunity in the lung: how epithelial cells fight against respiratory pathogens. *Eur Respir J* 23:327-333.

31. Bergeron, C., W. Al-Ramli, and Q. Hamid. 2009. Remodeling in asthma. *Proc Am Thorac Soc* 6:301-305.

32. Broide, D. H. 2008. Immunologic and inflammatory mechanisms that drive asthma progression to remodeling. *J Allergy Clin Immunol* 121:560-570; quiz 571-562.

33. Shakib, F., A. M. Ghaemmaghami, and H. F. Sewell. 2008. The molecular basis of allergenicity. *Trends Immunol* 29:633-642.

34. Spieksma, F. T., and P. H. Dieges. 2004. The history of the finding of the house dust mite. *J Allergy Clin Immunol* 113:573-576.

35. Voorhorst, R., and F. T. Spieksma. 1967. Correspondence: of mites and men. *J Allergy* 40:357-358.

36. Berrens, L. 1967. Correspondence: of mites and men. *J Allergy* 40:355-357.

37. Cates, E. C., R. Fattouh, J. R. Johnson, A. Llop-Guevara, and M. Jordana. 2007. Modeling responses to respiratory house dust mite exposure. *Contrib Microbiol* 14:42-67.

38. Jacquet, A. 2011. The role of the house dust mite-induced innate immunity in development of allergic response. *Int Arch Allergy Immunol* 155:95-105.

39. Wills-Karp, M., A. Nathan, K. Page, and C. L. Karp. 2009. New insights into innate immune mechanisms underlying allergenicity. *Mucosal Immunol* 3:104-110.

40. Gregory, L. G., and C. M. Lloyd. 2011. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol* 32:402-411.

41. Karp, C. L. 2010. Guilt by intimate association: what makes an allergen an allergen? *J Allergy Clin Immunol* 125:955-960; quiz 961-952.

42. Bartlett, N. W., G. R. McLean, Y. S. Chang, and S. L. Johnston. 2009. Genetics and epidemiology: asthma and infection. *Curr Opin Allergy Clin Immunol* 9:395-400.

43. Guerra, S., and F. D. Martinez. 2008. Asthma genetics: from linear to multifactorial approaches. *Annu Rev Med* 59:327-341.

44. Singh, A. M., P. E. Moore, J. E. Gern, R. F. Lemanske, Jr., and T. V. Hartert. 2007. Bronchiolitis to asthma: a review and call for studies of gene-virus interactions in asthma causation. *Am J Respir Crit Care Med* 175:108-119.

45. Russell, G. 2006. The childhood asthma epidemic. *Thorax* 61:276-278.

46. Holgate, S. T. 2004. The epidemic of asthma and allergy. *J R Soc Med* 97:103-110.

47. 1998. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. The International Study

of Asthma and Allergies in Childhood (ISAAC) Steering Committee. *Lancet* 351:1225-1232.

48. 2011. Sir John Floyer' s A Treatise of the Asthma (1698). 1-7.

49. Ho, S. M. 2010. Environmental epigenetics of asthma: an update. *J Allergy Clin Immunol* 126:453-465.

50. Gerrard, J. W., C. A. Geddes, P. L. Reggin, C. D. Gerrard, and S. Horne. 1976. Serum IgE levels in white and metis communities in Saskatchewan. *Ann Allergy* 37:91-100.

51. Strachan, D. P. 1989. Hay fever, hygiene, and household size. *BMJ* 299:1259-1260.

52. Cookson, W. O., and M. F. Moffatt. 1997. Asthma: an epidemic in the absence of infection? *Science* 275:41-42.

53. Strachan, D. P. 2000. Family size, infection and atopy: the first decade of the "hygiene hypothesis". *Thorax* 55 Suppl 1:S2-10.

54. Bisgaard, H., M. N. Hermansen, K. Bonnelykke, J. Stokholm, F. Baty, N. L. Skytt, J. Aniscenko, T. Kebadze, and S. L. Johnston. 2010. Association of bacteria and viruses with wheezy episodes in young children: prospective birth cohort study. *BMJ* 341:c4978.

55. Ball, T. M., J. A. Castro-Rodriguez, K. A. Griffith, C. J. Holberg, F. D. Martinez, and A. L. Wright. 2000. Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. *N Engl J Med* 343:538-543.

56. Illi, S., E. von Mutius, S. Lau, R. Bergmann, B. Niggemann, C. Sommerfeld, and U. Wahn. 2001. Early childhood infectious diseases and the development of asthma up to school age: a birth cohort study. *BMJ* 322:390-395.

57. Strachan, D. P., E. M. Taylor, and R. G. Carpenter. 1996. Family structure, neonatal infection, and hay fever in adolescence. *Arch Dis Child* 74:422-426.

58. Kusel, M. M., N. H. de Klerk, T. Kebadze, V. Vohma, P. G. Holt, S. L. Johnston, and P. D. Sly. 2007. Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. *J Allergy Clin Immunol* 119:1105-1110.

59. Sigurs, N., R. Bjarnason, F. Sigurbergsson, and B. Kjellman. 2000. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am J Respir Crit Care Med* 161:1501-1507.

60. Sigurs, N., P. M. Gustafsson, R. Bjarnason, F. Lundberg, S. Schmidt, F. Sigurbergsson, and B. Kjellman. 2005. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. *Am J Respir Crit Care Med* 171:137-141.

61. Douwes, J., and N. Pearce. 2008. Commentary: The end of the hygiene hypothesis? *Int J Epidemiol* 37:570-572.

62. Armann, J., and E. von Mutius. 2010. Do bacteria have a role in asthma development? *Eur Respir J* 36:469-471.

63. Sevin, C. M., and R. S. Peebles, Jr. 2010. Infections and asthma: new insights into old ideas. *Clin Exp Allergy* 40:1142-1154.

64. Johnston, N. W., S. L. Johnston, G. R. Norman, J. Dai, and M. R. Sears. 2006. The September epidemic of asthma hospitalization: school children as disease vectors. *J Allergy Clin Immunol* 117:557-562.

65. Sears, M. R., and N. W. Johnston. 2007. Understanding the September asthma epidemic. *J Allergy Clin Immunol* 120:526-529.

66. Johnston, S. L., P. K. Pattemore, G. Sanderson, S. Smith, F. Lampe, L. Josephs, P. Symington, S. O'Toole, S. H. Myint, D. A. Tyrrell, and et al. 1995. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* 310:1225-1229.

67. Heymann, P. W., H. T. Carper, D. D. Murphy, T. A. Platts-Mills, J. Patrie, A. P. McLaughlin, E. A. Erwin, M. S. Shaker, M. Hellems, J. Peerzada, F. G. Hayden, T. K. Hatley, and R. Chamberlain. 2004. Viral infections in relation to age, atopy, and season of admission among children hospitalized for wheezing. *J Allergy Clin Immunol* 114:239-247.

68. Holt, P. G., D. H. Strickland, M. E. Wikstrom, and F. L. Jahnsen. 2008. Regulation of immunological homeostasis in the respiratory tract. *Nat Rev Immunol* 8:142-152.

69. Martinez, F. D., A. L. Wright, L. M. Taussig, C. J. Holberg, M. Halonen, and W. J. Morgan. 1995. Asthma and wheezing in the first six years of life. The Group Health Medical Associates. *N Engl J Med* 332:133-138.

70. Morgan, W. J., D. A. Stern, D. L. Sherrill, S. Guerra, C. J. Holberg, T. W. Guilbert, L. M. Taussig, A. L. Wright, and F. D. Martinez. 2005. Outcome of asthma and wheezing in the first 6 years of life: follow-up through adolescence. *Am J Respir Crit Care Med* 172:1253-1258.

71. Illi, S., E. von Mutius, S. Lau, B. Niggemann, C. Gruber, and U. Wahn. 2006. Perennial allergen sensitisation early in life and chronic asthma in children: a birth cohort study. *Lancet* 368:763-770.

72. Simoes, E. A. 1999. Respiratory syncytial virus infection. *Lancet* 354:847-852.

73. Jackson, D. J., R. E. Gangnon, M. D. Evans, K. A. Roberg, E. L. Anderson, T. E. Pappas, M. C. Printz, W. M. Lee, P. A. Shult, E. Reisdorf, K. T. Carlson-Dakes, L. P. Salazar, D. F. DaSilva, C. J. Tisler, J. E. Gern, and R. F. Lemanske, Jr. 2008. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. *Am J Respir Crit Care Med* 178:667-672.

74. Lemanske, R. F., Jr., D. J. Jackson, R. E. Gangnon, M. D. Evans, Z. Li, P. A. Shult, C. J. Kirk, E. Reisdorf, K. A. Roberg, E. L. Anderson, K. T. Carlson-Dakes, K. J. Adler, S. Gilbertson-White, T. E. Pappas, D. F. Dasilva, C. J. Tisler, and J. E. Gern. 2005. Rhinovirus illnesses during

infancy predict subsequent childhood wheezing. *J Allergy Clin Immunol* 116:571-577.

75. 2003. Influenza In *Fact sheet N°211*, March 2003 ed. WHO.

76. Wilson Smith, C. H. A., P.P. Laidlaw. 1933. A virus obtained from influenza patients. *Lancet*:66-68.

77. Baccam, P., C. Beauchemin, C. A. Macken, F. G. Hayden, and A. S. Perelson. 2006. Kinetics of influenza A virus infection in humans. *J Virol* 80:7590-7599.

78. S.J. Flint, L. E., VR Racaniello, AM Skalka. 2004. *Principles of Virology, Molecular Biology, Pathogenesis and Control of Animal Viruses*. ASM Press.

79. Kash, J. C., C. F. Basler, A. Garcia-Sastre, V. Carter, R. Billharz, D. E. Swayne, R. M. Przygodzki, J. K. Taubenberger, M. G. Katze, and T. M. Tumpey. 2004. Global host immune response: pathogenesis and transcriptional profiling of type A influenza viruses expressing the hemagglutinin and neuraminidase genes from the 1918 pandemic virus. *J Virol* 78:9499-9511.

80. Cheung, T. K., and L. L. Poon. 2007. Biology of influenza a virus. *Ann N Y Acad Sci* 1102:1-25.

81. Medina, R. A., and A. Garcia-Sastre. 2011. Influenza A viruses: new research developments. *Nat Rev Microbiol* 9:590-603.

82. Jansen, A. G., E. A. Sanders, A. W. Hoes, A. M. van Loon, and E. Hak. 2007. Influenza- and respiratory syncytial virus-associated mortality and hospitalisations. *Eur Respir J* 30:1158-1166.

83. Poehling, K. A., K. M. Edwards, G. A. Weinberg, P. Szilagyi, M. A. Staat, M. K. Iwane, C. B. Bridges, C. G. Grijalva, Y. Zhu, D. I. Bernstein, G. Herrera, D. Erdman, C. B. Hall, R. Seither, and M. R. Griffin. 2006. The underrecognized burden of influenza in young children. *N Engl J Med* 355:31-40.

84. Louie, J. K., R. Schechter, S. Honarmand, H. F. Guevara, T. R. Shoemaker, N. Y. Madrigal, C. J. Woodfill, H. D. Backer, and C. A. Glaser. 2006. Severe pediatric influenza in California, 2003-2005: implications for immunization recommendations. *Pediatrics* 117:e610-618.

85. Saglani, S., and A. Bush. 2008. Asthma, atopy, and airway inflammation: what does it mean in practice? *Am J Respir Crit Care Med* 178:437-438.

86. Brimnes, M. K., L. Bonifaz, R. M. Steinman, and T. M. Moran. 2003. Influenza virus-induced dendritic cell maturation is associated with the induction of strong T cell immunity to a coadministered, normally nonimmunogenic protein. *J Exp Med* 198:133-144.

87. Sakamoto, M., S. Ida, and T. Takishima. 1984. Effect of influenza virus infection on allergic sensitization to aerosolized ovalbumin in mice. *J Immunol* 132:2614-2617.

88. Suzuki, S., Y. Suzuki, N. Yamamoto, Y. Matsumoto, A. Shirai, and T. Okubo. 1998. Influenza A virus infection increases IgE production and airway responsiveness in aerosolized antigen-exposed mice. *J Allergy Clin Immunol* 102:732-740.

89. Tsitoura, D. C., S. Kim, K. Dabbagh, G. Berry, D. B. Lewis, and D. T. Umetsu. 2000. Respiratory infection with influenza A virus interferes with the induction of tolerance to aeroallergens. *J Immunol* 165:3484-3491.

90. Yamamoto, N., S. Suzuki, A. Shirai, M. Suzuki, M. Nakazawa, Y. Nagashima, and T. Okubo. 2000. Dendritic cells are associated with augmentation of antigen sensitization by influenza A virus infection in mice. *Eur J Immunol* 30:316-326.

91. Yamamoto, N., S. Suzuki, Y. Suzuki, A. Shirai, M. Nakazawa, M. Suzuki, T. Takamasu, Y. Nagashima, M. Minami, and Y. Ishigatsubo. 2001. Immune response induced by airway sensitization after influenza A virus infection depends on timing of antigen exposure in mice. *J Virol* 75:499-505.

92. Dahl, M. E., K. Dabbagh, D. Liggitt, S. Kim, and D. B. Lewis. 2004. Viral-induced T helper type 1 responses enhance allergic disease by effects on lung dendritic cells. *Nat Immunol* 5:337-343.

93. Wohlleben, G., J. Muller, U. Tatsch, C. Hambrecht, U. Herz, H. Renz, E. Schmitt, H. Moll, and K. J. Erb. 2003. Influenza A virus infection inhibits

the efficient recruitment of Th2 cells into the airways and the development of airway eosinophilia. *J Immunol* 170:4601-4611.

94. Cates, E. C., R. Fattouh, J. Wattie, M. D. Inman, S. Goncharova, A. J. Coyle, J. C. Gutierrez-Ramos, and M. Jordana. 2004. Intranasal exposure of mice to house dust mite elicits allergic airway inflammation via a GM-CSF-mediated mechanism. *J Immunol* 173:6384-6392.

95. Matzinger, P. 2007. Friendly and dangerous signals: is the tissue in control? *Nat Immunol* 8:11-13.

96. Levy, O. 2007. Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat Rev Immunol* 7:379-390.

97. Levy, O., K. A. Zarember, R. M. Roy, C. Cywes, P. J. Godowski, and M. R. Wessels. 2004. Selective impairment of TLR-mediated innate immunity in human newborns: neonatal blood plasma reduces monocyte TNF-alpha induction by bacterial lipopeptides, lipopolysaccharide, and imiquimod, but preserves the response to R-848. *J Immunol* 173:4627-4634.

98. Zaghouani, H., C. M. Hoeman, and B. Adkins. 2009. Neonatal immunity: faulty T-helpers and the shortcomings of dendritic cells. *Trends Immunol* 30:585-591.

99. Johnson, J. R., F. K. Swirski, B. U. Gajewska, R. E. Wiley, R. Fattouh, S. R. Pacitto, J. K. Wong, M. R. Stampfli, and M. Jordana. 2007. Divergent

immune responses to house dust mite lead to distinct structural-functional phenotypes. *Am J Physiol Lung Cell Mol Physiol* 293:L730-739.

100. Llop-Guevara, A., M. Colangelo, D. K. Chu, C. L. Moore, N. A. Stieber, T. D. Walker, S. Goncharova, A. J. Coyle, L. K. Lundblad, P. M. O'Byrne, M. Lovric, and M. Jordana. 2008. In vivo-to-in silico iterations to investigate aeroallergen-host interactions. *PLoS One* 3:e2426.

101. Rezaee, F., N. Meednu, J. A. Emo, B. Saatian, T. J. Chapman, N. G. Naydenov, A. De Benedetto, L. A. Beck, A. I. Ivanov, and S. N. Georas. 2011. Polyinosinic:polycytidylic acid induces protein kinase D-dependent disassembly of apical junctions and barrier dysfunction in airway epithelial cells. *J Allergy Clin Immunol* 128:1216-1224 e1211.

102. Busse, W. W., R. F. Lemanske, Jr., and J. E. Gern. 2010. Role of viral respiratory infections in asthma and asthma exacerbations. *Lancet* 376:826-834.

103. Lloyd, C. M., and S. Saglani. 2010. Asthma and allergy: the emerging epithelium. *Nat Med* 16:273-274.

104. Ehrhardt, C., R. Seyer, E. R. Hrincius, T. Eierhoff, T. Wolff, and S. Ludwig. 2009. Interplay between influenza A virus and the innate immune signaling. *Microbes Infect* 12:81-87.

105. Garcia-Sastre, A., and C. A. Biron. 2006. Type 1 interferons and the virus-host relationship: a lesson in detente. *Science* 312:879-882.

106. Seo, S. U., H. J. Kwon, H. J. Ko, Y. H. Byun, B. L. Seong, S. Uematsu, S. Akira, and M. N. Kweon. 2011. Type I interferon signaling regulates Ly6C(hi) monocytes and neutrophils during acute viral pneumonia in mice. *PLoS Pathog* 7:e1001304.

107. Sanders, C. J., P. C. Doherty, and P. G. Thomas. 2010. Respiratory epithelial cells in innate immunity to influenza virus infection. *Cell Tissue Res* 343:13-21.

108. Lavelle, E. C., C. Murphy, L. A. O'Neill, and E. M. Creagh. 2009. The role of TLRs, NLRs, and RLRs in mucosal innate immunity and homeostasis. *Mucosal Immunol* 3:17-28.

109. Iwasaki, A. 2007. Mucosal dendritic cells. *Annu Rev Immunol* 25:381-418.

110. Granucci, F., I. Zanoni, S. Feau, and P. Ricciardi-Castagnoli. 2003. Dendritic cell regulation of immune responses: a new role for interleukin 2 at the intersection of innate and adaptive immunity. *Embo J* 22:2546-2551.

111. Willart, M. A., and H. Hammad. 2010. Alarming dendritic cells for allergic sensitization. *Allergol Int* 59:95-103.

112. Meylan, E., J. Tschopp, and M. Karin. 2006. Intracellular pattern recognition receptors in the host response. *Nature* 442:39-44.

113. Willart, M. A., and B. N. Lambrecht. 2009. The danger within: endogenous danger signals, atopy and asthma. *Clin Exp Allergy* 39:12-19.

114. Nish, S., and R. Medzhitov. 2011. Host defense pathways: role of redundancy and compensation in infectious disease phenotypes. *Immunity* 34:629-636.

115. Wills-Karp, M. 2007. Complement activation pathways: a bridge between innate and adaptive immune responses in asthma. *Proc Am Thorac Soc* 4:247-251.

116. Barrett, N. A., A. Maekawa, O. M. Rahman, K. F. Austen, and Y. Kanaoka. 2009. Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. *J Immunol* 182:1119-1128.

117. Hammad, H., M. Plantinga, K. Deswarte, P. Pouliot, M. A. Willart, M. Kool, F. Muskens, and B. N. Lambrecht. 2010. Inflammatory dendritic cells--not basophils--are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. *J Exp Med* 207:2097-2111.

118. Nakano, H., M. E. Free, G. S. Whitehead, S. Maruoka, R. H. Wilson, K. Nakano, and D. N. Cook. 2011. Pulmonary CD103(+) dendritic cells prime Th2 responses to inhaled allergens. *Mucosal Immunol* 5:53-65.

119. Grayson, M. H., D. Cheung, M. M. Rohlfing, R. Kitchens, D. E. Spiegel, J. Tucker, J. T. Battaile, Y. Alevy, L. Yan, E. Agapov, E. Y. Kim, and M. J. Holtzman. 2007. Induction of high-affinity IgE receptor on lung dendritic cells during viral infection leads to mucous cell metaplasia. *J Exp Med* 204:2759-2769.

120. Subrata, L. S., J. Bizzintino, E. Mamessier, A. Bosco, K. L. McKenna, M. E. Wikstrom, J. Goldblatt, P. D. Sly, B. J. Hales, W. R. Thomas, I. A. Laing, P. N. LeSouef, and P. G. Holt. 2009. Interactions between innate antiviral and atopic immunoinflammatory pathways precipitate and sustain asthma exacerbations in children. *J Immunol* 183:2793-2800.

121. Diehl, S., and M. Rincon. 2002. The two faces of IL-6 on Th1/Th2 differentiation. *Mol Immunol* 39:531-536.

122. Dodge, I. L., M. W. Carr, M. Cernadas, and M. B. Brenner. 2003. IL-6 production by pulmonary dendritic cells impedes Th1 immune responses. *J Immunol* 170:4457-4464.

123. Prefontaine, D., S. Lajoie-Kadoch, S. Foley, S. Audusseau, R. Olivenstein, A. J. Halayko, C. Lemiere, J. G. Martin, and Q. Hamid. 2009. Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *J Immunol* 183:5094-5103.

124. Smithgall, M. D., M. R. Comeau, B. R. Yoon, D. Kaufman, R. Armitage, and D. E. Smith. 2008. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. *Int Immunol* 20:1019-1030.

125. Paul, W. E., and J. Zhu. 2010. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol* 10:225-235.

126. Adkins, B., C. Leclerc, and S. Marshall-Clarke. 2004. Neonatal adaptive immunity comes of age. *Nat Rev Immunol* 4:553-564.

127. Willems, F., S. Vollstedt, and M. Suter. 2009. Phenotype and function of neonatal DC. *Eur J Immunol* 39:26-35.

128. Hinz, D., M. Bauer, S. Roder, S. Olek, J. Huehn, U. Sack, M. Borte, J. C. Simon, I. Lehmann, and G. Herberth. 2011. Cord blood Tregs with stable FOXP3 expression are influenced by prenatal environment and associated with atopic dermatitis at the age of one year. *Allergy* 67:380-389.

129. Schaub, B., J. Liu, S. Hoppler, I. Schleich, J. Huehn, S. Olek, G. Wieczorek, S. Illi, and E. von Mutius. 2009. Maternal farm exposure modulates neonatal immune mechanisms through regulatory T cells. *J Allergy Clin Immunol* 123:774-782 e775.

130. Fernandez, M. A., F. K. Puttur, Y. M. Wang, W. Howden, S. I. Alexander, and C. A. Jones. 2008. T regulatory cells contribute to the attenuated primary CD8+ and CD4+ T cell responses to herpes simplex virus type 2 in neonatal mice. *J Immunol* 180:1556-1564.

131. Marchant, A., T. Goetghebuer, M. O. Ota, I. Wolfe, S. J. Ceesay, D. De Groote, T. Corrah, S. Bennett, J. Wheeler, K. Huygen, P. Aaby, K. P. McAdam, and M. J. Newport. 1999. Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guerin vaccination. *J Immunol* 163:2249-2255.

132. Wissinger, E., J. Goulding, and T. Hussell. 2009. Immune homeostasis in the respiratory tract and its impact on heterologous infection. *Semin Immunol* 21:147-155.

133. Tschernig, T., A. S. Debertin, F. Paulsen, W. J. Kleemann, and R. Pabst. 2001. Dendritic cells in the mucosa of the human trachea are not regularly found in the first year of life. *Thorax* 56:427-431.

134. de Heer, H. J., H. Hammad, T. Soullie, D. Hijdra, N. Vos, M. A. Willart, H. C. Hoogsteden, and B. N. Lambrecht. 2004. Essential role of lung plasmacytoid dendritic cells in preventing asthmatic reactions to harmless inhaled antigen. *J Exp Med* 200:89-98.

135. Mattei, F., G. Schiavoni, and D. F. Tough. 2010. Regulation of immune cell homeostasis by type I interferons. *Cytokine Growth Factor Rev* 21:227-236.

136. Le Bon, A., and D. F. Tough. 2002. Links between innate and adaptive immunity via type I interferon. *Curr Opin Immunol* 14:432-436.

137. Khoo, J. J., S. Forster, and A. Mansell. 2011. Toll-like receptors as interferon-regulated genes and their role in disease. *J Interferon Cytokine Res* 31:13-25.

138. Hammad, H., M. Chieppa, F. Perros, M. A. Willart, R. N. Germain, and B. N. Lambrecht. 2009. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat Med* 15:410-416.

139. Kajekar, R. 2007. Environmental factors and developmental outcomes in the lung. *Pharmacol Ther* 114:129-145.

140. Narayanan, M., J. Owers-Bradley, C. S. Beardsmore, M. Mada, I. Ball, R. Garipov, K. S. Panesar, C. E. Kuehni, B. D. Spycher, S. E. Williams, and

M. Silverman. 2011. Alveolarization continues during childhood and adolescence: new evidence from helium-3 magnetic resonance. *Am J Respir Crit Care Med* 185:186-191.

141. Saglani, S., D. N. Payne, J. Zhu, Z. Wang, A. G. Nicholson, A. Bush, and P. K. Jeffery. 2007. Early detection of airway wall remodeling and eosinophilic inflammation in preschool wheezers. *Am J Respir Crit Care Med* 176:858-864.

142. Holtzman, M. J., L. P. Shornick, M. H. Grayson, E. Y. Kim, J. W. Tyner, A. C. Patel, E. Agapov, and Y. Zhang. 2004. "Hit-and-run" effects of paramyxoviruses as a basis for chronic respiratory disease. *Pediatr Infect Dis J* 23:S235-245.

143. Holt, P. G., and D. H. Strickland. 2010. Interactions between innate and adaptive immunity in asthma pathogenesis: new perspectives from studies on acute exacerbations. *J Allergy Clin Immunol* 125:963-972; quiz 973-964.

144. Holgate, S. T. 2010. A look at the pathogenesis of asthma: the need for a change in direction. *Discov Med* 9:439-447.

145. James, A. L., and S. Wenzel. 2007. Clinical relevance of airway remodelling in airway diseases. *Eur Respir J* 30:134-155.

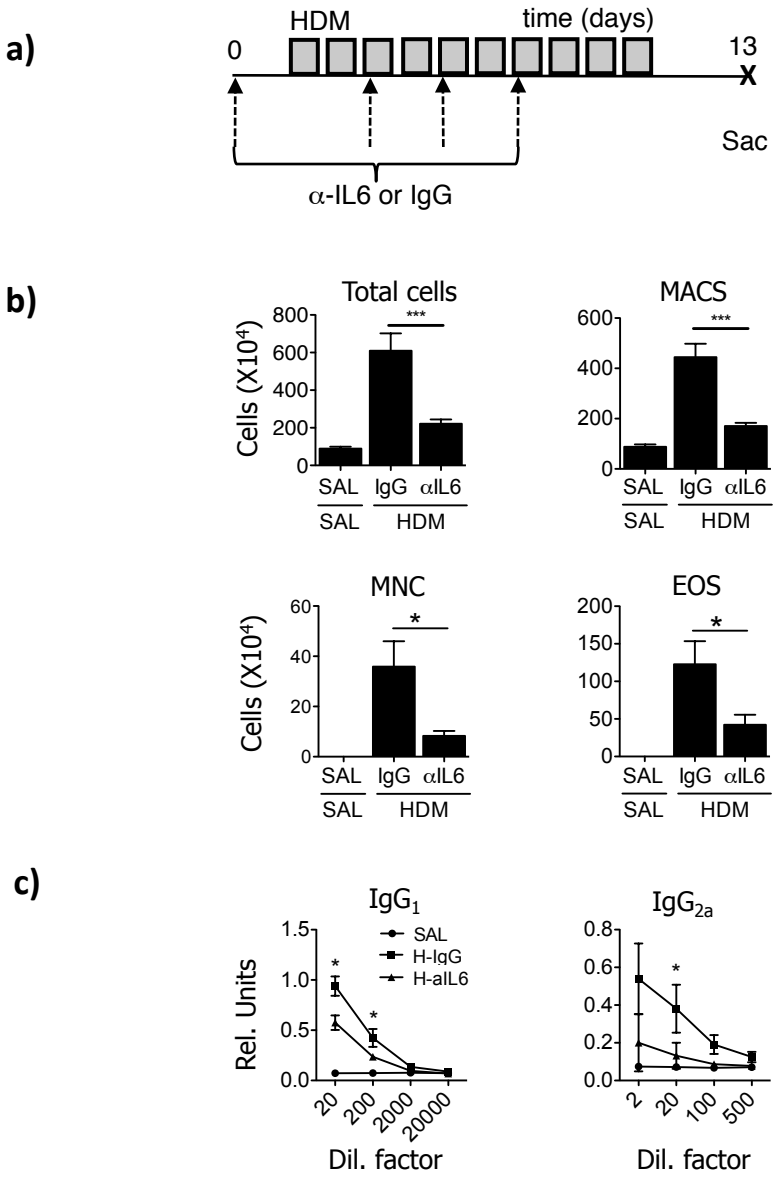
146. Martinez, F. D. 2009. The origins of asthma and chronic obstructive pulmonary disease in early life. *Proc Am Thorac Soc* 6:272-277.

147. Cella, M., F. Facchetti, A. Lanzavecchia, and M. Colonna. 2000. Plasmacytoid dendritic cells activated by influenza virus and CD40L drive a potent TH1 polarization. *Nat Immunol* 1:305-310.

APPENDIX I

IMPACT OF IL-6 BLOCKADE ON HDM-INDUCED INFLAMMATION

Appendix 1


a)
HDM
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0
13
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α-IL6 or IgG
b)
Total cells
MACS
MNC
EOS
Cells (X10⁴)
SAL
IgG
αIL6
SAL
HDM
c)
IgG1
IgG2a
SAL
H-IgG
H-aIL6
Rel. Units
Dil. factor

Appendix 1

Figure 1: *Impact of IL-6 blockade on HDM-induced inflammation.*
a) Schematic diagram depicting 10-day HDM protocol. Groups of mice were exposed to either saline (SAL) or to 25μg HDM for 10 days and sacrificed 72 hrs later. At day 0, 3, 5 and 7, prior to HDM administration, separate groups of mice were injected with either 50μg anti-IL6 (α-IL6) or IgG antibody in 200μL saline via intrapertitoneal route. **b**) Cellular profile in BAL fluid showing number of total cells, macrophages (MACS), mononuclear cells (MNC), and eosinophils (EOS). **c**) Serum Ig levels measured by ELISA at indicated dilutions showing HDM-specific IgG1 and IgG2a. n = 5 mice/group. One of two representative experiments is shown. Data represent mean ± SEM. ***P<0.001, *P<0.05 compared with HDM(α-IL6).

APPENDIX II

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McMaster University
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1280 Main Street West
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Email: algarawi@mcmaster.ca

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April 16, 2012

Amal Al-Garawi
McMaster University
Center for Gene Therapeutics
1280 Main Street West
Hamilton, ON L8S4K1
Canada
Email: algarawi@mcmaster.ca

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