EVOLUTION OF ALLERGEN RESPONSIVENESS

DURING DEVELOPMENT

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By

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ABSTRACT

Background: Early infancy is a critical period during which the interplay between host and environmental factors influences susceptibility to allergic sensitization, a process that can also be construed as a failure to induce tolerance. Indeed, allergic asthma emerges, in most instances, in early childhood although the specific intervals of protection or susceptibility remain to be elucidated. We found that exposure to a concentration of allergen, house dust mite (HDM), that normally induces robust airway inflammation in adult mice elicits negligible immune-inflammatory responses in infant mice.

Methods: We investigated immune-inflammatory responses in mice exposed to 25 μg of HDM intranasally for 10 consecutive days at different points in development (3, 4, 5 and 7 weeks of age). We delineated the immune cell profile in the lungs of naïve mice from birth to adulthood, focusing on markers of immune maturation and immunosuppression. Moreover, we studied the impact of T-regulatory cell (Treg) depletion with the use of α-CD25 antibodies administered intraperitoneally one day prior to the start of HDM exposures, and then again on day 6 of the above protocol.

Results: Our data show that there is a progressive acquisition of immune-inflammatory responsiveness to HDM in BALB/c mice as exposures are initiated later in development, evidenced by total cell number and eosinophilia in the BAL and serum HDM-specific IgG_1 levels. Additionally, there is an immunological shift that occurs in the infant lung during development in that the early immunosuppressive environment, defined by T-regulatory cells and immunosuppressive alveolar macrophages, subsides as the capacity to respond to ensuing immune challenges, defined by natural killer (NK) cell, dendritic

cell (DC) and alveolar macrophage (AM) maturation, increases. Specifically, in regards to the immunosuppressive lung environment during infancy, we identified higher baseline levels of $CD25^{+}Foxp3^{+}CD101^{+}$ and $CD25^{+}Foxp3^{high}$ Tregs, i.e. those with more potent suppressive ability. These populations also expand following HDM exposure in both adult and infant mice. Interestingly, 2 week-old infant mice depleted of Tregs or exposed to a very high dose of HDM (125 μg) overcome the natural immunosuppressive environment resulting in the acquisition of HDM responsiveness, as manifested by robust Th2 immune-inflammatory responses, comparable to that observed in 8 week-old adult mice.

Conclusion/Implications: Together, our data suggest that the hyporesponsiveness to HDM very early in life may be explained by two connected events: a) the inherent immunosuppressive environment in the lung, and b) the immaturity of the machinery for effective immune responses. Furthermore, we demonstrate that a disruption in the homeostatic immune balance in infancy, Treg depletion in this case, may lead to the imprinting of aberrant immune-inflammatory responses, like allergen sensitization. Thus, the inherent immunosuppressive environment in infancy may have long-term implications on allergen responsiveness.

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ABBREVIATIONS

AA – Allergic Asthma

AM – Alveolar Macrophage

ANOVA – Analysis of Variance

APC - Antigen-Presenting Cell

BAL – Bronchoalveolar Lavage

BALB/c - Bagg Albino c

BALF – Bronchoalveolar Lavage Fluid

BCG – Bacillus Calmette-Guérin

BSA – Bovine serum Albumin

CD – Cluster of Differentiation

CPI – Complete Protease Inhibitor

CTL – Cytotoxic T-Lymphocyte

CTLA-4 – Cytotoxic T-Lymphocyte-Associated Protein

CX3CR1 – CXC3 Rhemokine Receptor-1

DC – Dendritic Cell

Der p 1 – Dermatophagoides pteronissinus 1

DEREG - Depletion of Regulatory T cell

DT – Diphtheria Toxin

DTR – Diphtheria Toxin Receptor

ELISA – Enzyme-Linked Immunosorbent Assay

FACS – Fluorescence-Activated Cell Sorting

FBS – Fetal Bovine Serum

Fc – Fragment Crystallizable

FEF_{25-75} – Forced Expiratory Flow at 25, 50 or 75% of FVC

FEV_1 – Forced Expiratory Volume in 1 Second

FGL-2 – Fibrinogen-like Protein

FMO – Fluorescence Minus One

Foxp3 – Forkhead box p3

FVC – Forced Vital Capacity

H&E – Hematoxylin and Eosin

HBSS – Hank's Balanced Salt Solution

HDM – House Dust Mite

i.n. – Intranasal

i.p. – Intraperitoneal

ICOS – Inducible T-cell Costimulator

ICS – Intracellular Staining

IFN-γ – Interferon-gamma

IgE/G_1 – Immunoglobulin E/G_1

IL – Interleukin

IL-10R – Interleukin-10 Receptor

IPEX – Immune Dysregulation Polyendocrinopathy X-linked

iTreg – Inducible T-regulatory Cell

LAG-3 – Lymphocyte Activation Gene-3

LPS – Lipopolysaccharide

mDC – Myeloid Dendritic Cell

MFI – Mean Fluorescence Intensity

MHC II – Major Histocompatibility Complex II

NK – Natural Killer

NO – Nitric Oxide

nTreg – Natural T-regulatory Cell

OD – Optical Density

OVA – Ovalbumin

PBMC - Peripheral Blood Mononuclear Cell

PBS – Phosphate-Buffered Saline

PGE2 – Prostaglandin E2

RPMI – Roswell Park Memorial Institute

SEM – Standard Error of the Mean

SPF – Specific Pathogen Free

TCN – Total Cell Number

TCR – T-cell Receptor

TGF-β – Transforming Growth Factor

Th – T-helper

T_R – T-regulatory

Treg – T-regulatory cell

XLAAD - X-linked Autoimmunity Allergic Disregulation Syndome

CHAPTER 1
INTRODUCTION

1.1 Asthma – Overview of the Problem

Asthma is described as a chronic inflammatory lung disease characterized by reversible airflow obstruction [1]. The World Health Organization reports that asthma affects 235 million people worldwide, and ample evidence has shown a substantial increase in this number over the last four decades [1]. Currently, the prevalence of asthma in Canada stands at approximately 12% in children and over 6% in adults [2]. Recent evidence, however, suggests that the burgeoning asthma epidemic appears to be stabilizing and, in some cases, even decreasing. In this regard, Statistics Canada reports that the prevalence of asthma among children aged 2 to 7 has reached its lowest level in more than a decade at a rate of 10% [3]. Nevertheless, the majority of cases of asthma emerge in childhood and it remains the most prevalent chronic disease in children as well as the leading cause of school and work absenteeism [1].

Although non-allergic triggers, such as exercise and cold air, may induce asthma, two-thirds of all cases are allergic asthma (AA) [4]. AA is a complex immune-driven disease that occurs in susceptible individuals in response to allergen exposure [5]. Specifically, house dust mite (HDM) is the most pervasive indoor aeroallergen worldwide with sensitization rates estimated at 35% [4, 5]. Furthermore, sensitization to HDM is strongly associated with AA. Indeed, Arshad and colleagues (2001) previously demonstrated that 50% of HDM-sensitized children at age 4 also present with asthma [6].

Moreover, AA has a distinct immune profile characterized mainly by T-helper 2 (Th2) lymphocytes, eosinophils, mast cells and, in some cases, neutrophils [7]. Th2 cells,

in particular, play a vital role in orchestrating the immune response through the production of numerous cytokines, namely IL-4, IL-5, IL-9 and IL-13 [7]. These cytokines mediate numerous events, including but not limited to airway hyperresponsiveness, structural remodeling, eosinophil and mast cell recruitment and differentiation, as well as B-cell activation and immunoglobulin production [7]. Recent evidence also highlights the importance of dendritic cells (DCs) in inducing Th2 responses to HDM exposure in the respiratory tract [8]. As a counterpoint, the inflammatory response contributing to asthma pathogenesis is regulated, at least partly, by T-regulatory cells (Tregs) [9, 10]. Tregs play crucial roles in both the induction of tolerance to allergens as well as the control of the inflammatory response following allergen exposure [9]. Atopic asthmatics present with Tregs that have an impaired ability to suppress allergen-driven T-cell proliferation and cytokine production *in vitro* [11]. Therefore, there is an imbalance between suppression and activation in AA. The question still remains as to whether this is the result of impaired suppression by Tregs or over-activation of effector Th2 cells.

1.2 Allergic Asthma – Unresolved Issues

Despite remarkable progress in our understanding of the immunobiological basis of AA, there remain many unresolved problems. The need for disease-modifying agents, for instance, has been intensely studied in recent years since current treatments, such as corticosteroids and bronchodilators, target the symptoms but not the underlying disease [12]. These efforts have been relatively unsuccessful, as illustrated by anti-IL-5 monoclonal antibody trials [13, 14], though they have advanced our understanding of the

disease by challenging the conventional dogma of asthma as a homogenous disease. Indeed, an emerging idea is that new treatments will be discovered through the identification of distinct asthma phenotypes underlined by specific biological pathways [15-17].

Another important unresolved issue is related to the onset of the disease, and therein lies the focus of this thesis. Indeed, the origins of allergic asthma remain largely unknown [18]. The occurrence of allergic asthma in susceptible individuals has led to many studies on genetic predisposition; these studies have revealed human polymorphisms in over one hundred genes associated with different features of the asthmatic phenotype [19]. However, these studies are difficult to interpret given the poor reproducibility across populations [19, 20]. Moreover, statistical associations do not imply causality and, furthermore, correlative associations generally neglect potentially critical gene-environment interactions. Lastly, the dramatic increase in the prevalence of asthma over a relatively short period of time suggests the prominent contribution of factors other than genetics to the development of the disease [20].

A prominent theory about the origins of allergic asthma has been the *Hygiene Hypothesis,* which purports that certain infections during childhood contribute to prevent the emergence of asthma, presumably by aborting the development of Th2 responses through the triggering of Th1 mechanisms [21]. However, there has been no conclusive demonstration of this hypothesis or experimental evidence to support it [22]. To further this point, the recent rise in Th1-mediated autoimmune diseases seems paradoxical if we approach the hypothesis solely from a Th1 versus Th2 perspective [23]. Thus, these

findings emphasize the importance of investigating mechanisms beyond the seemingly over-simplified Th1/Th2 dichotomy to explain the emergence of allergic diseases [9]. For instance, immune challenges, such as microbial exposures, may lead to the development of a more robust immune regulatory network that subsequently confers protection against allergic airway disease [24].

Ultimately, the impact that genetic and environmental factors have on the development of allergic disease may depend on specific developmental and/or temporal intervals that Guerra and Martinez (2008) define as 'windows of opportunity' [20]. For allergic asthma, it appears that infancy is one such critical window. Indeed, as outlined in the proceeding section, sensitization during infancy is highly indicative of the presence of asthma in later years, suggesting that developmental processes of the infant immune system may play a significant role in the generation of allergen responsiveness [20, 25, 26]. The identification of these processes is at the centre of this thesis.

1.3 The Importance of Early Childhood in Immune Programming

Early childhood is a period of significant immune programming i.e. sensitization versus tolerance [25, 27, 28]. Tolerance is an actively-acquired process whereby the immune system is educated to recognize and not respond to self or innocuous, non-self antigens [29]. Sensitization, on the other hand, occurs when this state of unresponsiveness is altered so that immune-inflammatory responses are mounted against such antigens [29]. Sensitization to otherwise innocuous aeroallergens is necessary for the development of AA [28, 30]. Rowe *et al.* (2007) have identified the postnatal period as critical to the priming of Th2 responses to HDM [25]. In a cohort of 200 infants with a high risk of

developing AA based on positive atopic family history, HDM-specific Th2 responses, namely IL-4 and 5 production in peripheral blood mononuclear cells (PBMCs) of children 6 months or older strongly correlated with sensitization outcomes (i.e. high HDM-specific IgE titres and positive skin prick test) at 2 years of age [25]. This, however, was not the case for HDM-induced Th2 responses in cord blood cells, which suggests that the early post-natal, and not pre-natal, period is critical for immune programming [25].

Furthermore, Illi *et al.* (2006) demonstrated that sensitization during infancy influences the asthmatic phenotype later in life [26]. Wheezing children sensitized to perennial allergens, including HDM, in the first three years of life were predisposed to airway hyper-responsiveness and reduced lung function at school age [26]. However, wheezing children emerging non-atopic from this critical three-year interval showed a clinical improvement with normalization of lung function at the onset of puberty [26]. Along with being a significant period of atopic sensitization, infancy can also be deemed as an important time for tolerance induction [27, 31]. Recalling the aforementioned Rowe *et al.* (2007) study, atopic children at two years of age had steadily increasing levels of total and HDM-specific IgE from birth [25]. A subpopulation of non-atopic children, however, had transient low-levels of IgE that peaked at 6 or 12 months and, then, subsided by 24 months [25]. This finding in non-atopic children is suggestive of the development of protective immunological tolerance. The transient, low level IgE response is consistent with observations in a rat model delineating IgE production during tolerance induction to ovalbumin [32]. These observations suggest that tolerogenic mechanisms are certainly active during infancy.

Additional evidence supporting infancy as an important period in the development of AA are findings from Morgan *et al.* (2005) proposing that lung function is established in infancy and *tracks* into the teenage years [33]. More specifically, children who were persistent or transient wheezers in the first 6 years of life had worsened lung function (measured by FEF_{25-75}, FEV_1, FEV_1:FVC ratio) at 16 years of age compared to children who never wheezed [33]. Interestingly, the lung function of children whose wheeze began after 6 years of age (late-onset wheezers) paralleled that of those who have never wheezed [33]. These findings highlight the first six years of life as vital to the establishment of lung function later in life.

Altogether, early infancy appears as a critical period during which the interplay between host and environmental factors influences susceptibility to allergic sensitization and establishes lung function [26, 27, 31, 33]. The identification of these factors is important, as they can serve as a basis for future preventative strategies.

1.4 The Infant Immune System

1.4.1 Maturity of the Infant Immune System

The infant immune system may be considered immature compared to that of the adult, which, in part, makes children are more susceptible to certain infections [34, 35]. Given the lack of previous environmental exposures and the progressive development of the adaptive immune repertoire, survival is initially dependent on antibodies from the mother transferred through the placenta and breast milk [36]. In addition, human cord blood and murine neonatal T-cells have, compared to adult cells, impaired *in vitro* proliferation and cytokine production (IL-2, IFN-γ) upon TCR stimulation [34, 37].

Although cord blood T-cells are phenotypically and functionally immature themselves, their deficiencies can also be attributed to impaired antigen-presenting cell function. Indeed, cord blood T-cell proliferative capacity and IFN- γ production is elevated to adult-like levels if they are cultured with adult, as opposed to neonatal APCs [38, 39]. Furthermore, Upham *et al.* (2002) demonstrate impaired number and/or function of peripheral blood DCs in childhood [40]. Specifically, monocytes from cord blood and the peripheral blood of 5 and 12 year-old children lack the appropriate maturational stimuli for DC differentiation to mount adult-like Th1 responses to lipopolysaccharide (LPS) or heat-killed *Staphlococcus aureus in vitro*, as evidenced by production of IL-12p70 [40].

This deficiency of the infant immune system is not, however, absolute – infant cells are able to mount robust immune responses under certain conditions [35]. Indeed, TCR-independent stimulation, phorbol ester and calcium ionophore treatment, produces equal proliferative and cytokine responses from infant and adult T-cells [34, 37]. Furthermore, the effectiveness of immunization during infancy supports the notion of immune competence in early life [36]. For example, although the infant immune response appears to be Th2-biased (high IL-4 and low IFN-γ production), infant humans immunized to Bacillus Calmette-Guérin (BCG) at birth mount a memory Th1 response similar to those immunized later in life [37, 41, 42]. Also, immunization of neonatal mice with protein antigens in complete Freund's adjuvant results in adult-like Th1 responses [43]. Moreover, infant mice can also mount robust, adult-like responses to threatening viral pathogens. Sarzotti *et al.* (1996) reported protective Th1 and CTL responses in neonatal mice following leukemia virus infection [44]. In addition, our laboratory has

demonstrated the ability of infant mice (8 day old) to mount robust immune-inflammatory responses to influenza A infection [45]. Thus, the body of evidence indicates that the infant immune system is capable of launching effective immune responses.

1.4.2 Immunosuppression in the Infant Immune System

The immune system is continuously faced with the challenge of protecting the organism from harmful intruders, while also regulating responses against these intruders so as to prevent excessive immune-inflammatory responses, i.e. immunopathology. In most cases, it is the immunopathology that does more harm than the intruder itself [46]. The need for this balance is especially pronounced in infancy since failure to achieve, or distortion of this equilibrium may imprint aberrant immune-inflammatory responses with long-lasting structural and functional impairment (i.e. allergic sensitization) [47].

The infant immune system, in particular, is rapidly confronted with a plethora of immune challenges, including commensal colonization and the developing self. Thus, powerful immunosuppression in early life may confer a survival advantage by protecting from immunopathological responses. Indeed, a *scurfy* mutation in the Foxp3 locus (mediates T-regulatory cell suppression (see: *1.4.3 T-regulatory cells*)) results in a lymphoproliferative disease that ultimately leads to death by day 15-24 of life [48]. Moreover, depletion of $Foxp3^+$ cells in newborn mice results in severe immunopathology, characterized by significantly enlarged spleens and lymph nodes, as well as severe inflammatory infiltrates in various organs, including peribronchial and perivascular inflammation in the lungs [49]. This immunopathology, however, is not observed in adult mice depleted of $Foxp3^+$ cells, which the authors deem to be as a result of the lack of a

secondary signal required for the development of autoimmunity, such lymphopenia-induced proliferation in newborn mice that leads to the activation of auto-reactive T-cells [49, 50]. The necessity for immunosuppression in early life speaks to the active induction of tolerance to both self and non-self antigens during this period of time [9, 10]. A prominent mechanism of this immunosuppression is mediated by regulatory T-cells (Tregs) [10].

1.4.3 T-regulatory Cells (Tregs)

It is clear that Tregs suppress inflammatory responses. They play a key role in preventing autoimmune and allergic diseases through the induction and maintenance of tolerance to self as well as innocuous non-self antigens, like allergens [51]. Tregs are classically identified as expressing CD4 and CD25 on the cell surface and the transcription factor forkhead box p3 (Foxp3) in the nucleus [52]. Indeed, Foxp3 has emerged as a prominent mediator of suppressive activity [53]. Humans with mutations in Foxp3 present with immune dysregulation polyendocrinopathy X-linked (IPEX) syndrome and X-linked autoimmunity allergic disregulation syndome (XLAAD). Along with the autoimmune manifestations, patients with IPEX/XLAAD syndrome typically have signs of allergy, namely high peripheral IgE levels, eczema and food allergy [54]. The incidence of asthma in this cohort is low, likely as a result of the difficulty of diagnosing asthma prior to six years of age, a period of time when IPEX/XLAAD manifestations are most prominent [9]. Nevertheless, the above mutations do suggest the importance of $Foxp3^+$ Tregs in controlling allergic inflammation

There are two main types of Tregs: the thymically-derived natural Treg (nTreg) with the classical $CD4^+CD25^{hi}Foxp3^+$ phenotype and the peripherally-induced adaptive Treg (iTreg) [9]. The iTreg is induced in the periphery from $CD4^+CD25^-$ cells following antigen stimulation and can be further subdivided into $CD4^+CD25^{hi}Foxp3^+$ Tregs, IL-10-expressing $CD4^+CD25^{hi}Foxp3^-$ T_R1 cells and TGF-β-expressing $CD4^+$ Th3 cells [9]. The latter two provide insights into the classical mechanisms, IL-10 and TGF-β, whereby Tregs mediate their immunosuppression. Recently, another $Foxp3^-$ iTreg has been identified, the iT_R35 cell, which mediates immunosuppression via IL-35, and not IL-10 and TGF-β [55].

There has also been a concerted effort placed on identifying surface markers that better distinguish Tregs from conventional T-cells given the heterogeneity in suppressive function within the $CD4^+CD25^+$ subset, the expression of CD25 on activated T-cells, and the need for more precise cell surface markers for cell isolation purposes. CD127 (IL-7Rα), for example, is a transmembrane glycoprotein highly expressed on effector and memory T-cells [56]. Its expression inversely correlates with Foxp3 and Treg suppressive function [56]. Another recent example is CD101, expression of which on Tregs identifies those with significantly higher suppressive ability [57]. To date, CD101 has no known ligand and the mechanism through which CD101 on Tregs may mediate immunosuppression remains elusive.

1.4.4 Tregs in Infant Immune System

Tregs play important roles throughout development. During the fetal human stages, lymph nodes have high levels of $CD4^+CD25^{hi}$ Tregs that regulate T-cell responses

[58]. These fetal Tregs also promote tolerance to antigens transferred from the placenta to the fetus, and, as Mold *et al.* (2008) have recently demonstrated, play a critical role in suppressing fetal T-cell responses to maternal alloantigens [59, 60].

Furthermore, Tregs and Treg precursors from human cord blood have been shown to be more abundant and more suppressive than those isolated from adult peripheral blood [61]. Infant macaques have significantly higher levels of $CD4^+CD25^+CD127^-Foxp3^+$ Tregs in peripheral blood and lymph nodes than adult macaques [62]. These infant Tregs also exhibit significantly more potent *in vitro* suppressive activity [62]. Their critical role following birth is substantiated by the severe immunopathology that ensues in newborn, and not adult mice, if $Foxp3^+$ cells are depleted [49] (see *1.4.2 Immunosuppression in the infant immune system*). Furthermore, murine studies have identified an intrinsic mechanism whereby infant thymic and splenic $CD4^+CD25^-$ cells convert to a suppressive $CD4^+CD25^+Foxp3^+$ phenotype following TCR stimulation both *in vitro* and *in vivo* [63]. This conversion was significantly, 7-fold, less pronounced in adult cells [63]. These findings are all suggestive of a pervasive immunosuppressive environment in the infant.

1.4.5 Tregs – Implications with Respect to Sensitization and Tolerance

Impairment of this immunosuppressive environment in early infancy has been associated with a heightened risk of developing allergic disease. Haddeland *et al.* (2002) noted impaired $CD4^+CD25^{hi}$ Treg function from the cord blood of children with a hereditary risk of allergy following β-lactoglobulin stimulation in the presence of LPS [64]. This stimulation also generated significantly less $CD25^+$ T-cells from the cord blood of at-risk children [64]. Interestingly, Foxp3 expression was not assessed. In addition,

there is also evidence that Treg expression and function are also impaired in the cord blood of children born to atopic mothers [65]. Similarly, wheezy children, especially those with a hereditary risk of developing asthma, had lower absolute counts of $CD4^{+}CD25^{+/hi}$ Tregs in the peripheral blood [66]. Furthermore, a similar phenomenon is observed when a more specific subset of cord blood Tregs ($CD4^{+}CD25^{+}CD127^{lo/-}$) is correlated with the future development of allergy [67]. This subset of Tregs isolated from the cord blood of children who eventually develop egg allergy in the first year of life exhibit significantly lower suppression of IFN-γ production and effector responses compared to cord blood Tregs from children who do not become allergic [67]. The absolute numbers and level of Foxp3 expression of cord blood Tregs, however, did not differ between children who eventually become allergic and those who do not [67].

The imbalance between suppressive and effector cell function is also evident in established AA. $CD4^{+}CD25^{hi}$ Tregs isolated from the bronchoalveolar lavage fluid (BALF) of asthmatic children failed to suppress T-cell proliferation and cytokine/chemokine production, whereas those from non-asthmatic children exhibited potent suppressive ability [68]. Also, atopic, as compared with non-atopic, adults had significantly impaired Treg suppression [11]. This impairment was even more pronounced in allergic adults during high allergen season [11]. Furthermore, there is some controversy with respect to whether the absolute numbers of peripheral blood $CD4^{+}CD25^{hi}Foxp3^{+}$ Tregs are decreased in asthmatic over control subjects [68, 69]. Nevertheless, Provoost *et al.* (2009), who show no difference in absolute numbers,

demonstrate markedly reduced Foxp3 expression in $CD4^{+}CD25^{hi}$ Tregs in the peripheral blood of asthmatics [69].

Overall, Treg dysfunction is an important hallmark of allergic disease and its presence in early life appears predictive of future disease. In other words, the absence and/or dysfunction of Tregs in infancy facilitates sensitization to allergens. Indeed, Baru *et al.* (2010) observed, in an experimental setting, worsened allergic airway inflammation in adult mice if Tregs were depleted solely during the sensitization phase [70]. Further studies are especially needed in infant experimental models given the correlation of Treg dysfunction in infancy to allergic sensitization and inflammation later in life. Our recent observations about HDM responsiveness in infant mice have provided us with an ideal framework to investigate these issues [45] (see **1.5 Experimental Objectives and Hypothesis**). Moreover, our experimental model will also allow us to address the lack of data regarding the development of immune responses in the lung itself, as most studies, to date, have relied on analysis of human cord and peripheral blood cells.

It is plausible that the enhancement of Treg activity during infancy may promote tolerance induction. Experimental studies in adult mice have reported that repeated exposure to low doses of allergen promote the development of regulatory $CD4^{+}$ T-cells that, if adoptively transferred, prevent allergic sensitization in recipient mice [71]. Moreover, mice tolerized to ovalbumin during pregnancy are able to transfer tolerance to their pups through breast milk, a process mediated $CD4^{+}$ Tregs population and dependent on TGF-β [72]. Indeed, there is a belief amongst the research community that deliberate

exposure of humans to low-doses of allergen during prenatal and/or postnatal period may promote tolerance induction to allergens [9].

1.5 Experimental Objectives and Hypothesis

As outlined above, most cases of AA develop in the first years of life. Thus, in order to study the ontogeny of AA, it is imperative to consider the infancy period. Recent observations in our laboratory have provided a sound experimental platform from which to embark on future experimentation on this issue [45]. Specifically, we previously demonstrated that exposure of neonatal mice (2 week-old) to a concentration of HDM that normally elicits robust airway inflammation in adult (6-8 week-old) SPF mice, elicits negligible immune-inflammatory responses. However, this does not translate into universal hypo-responsiveness, since neonatal mice vigorously responded to a flu infection. In fact, we have provided evidence that HDM exposure in neonatal mice undergoing an acute flu infection leads to robust airway inflammation and Th2 immunity. These findings, therefore, suggest that responsiveness to HDM is (I) developmentally regulated and (II) selective in neonates as it depends on the antigen. From here, we set out to address the following objectives:

1.5.1 General Objective

To investigate the immune cell profile in the lung of mice over the course of development and examine how it may influence responsiveness to HDM.

1.5.2 Specific Objectives

I. To determine the evolution of HDM responsiveness over the course of development

II. To identify the evolution of specific components of the immune system from birth to adulthood, specifically focusing on markers of immune maturation and immunosuppression.

III. To identify mechanisms mediating hyporresponsiveness to HDM in infancy

1.5.3 Hypothesis

We hypothesize that there exists an immunosuppressive environment in the murine lung during infancy, a period of time critical to the development of tolerance to self and non-self antigens. As immunosuppression subsides over the course of development, HDM responsiveness is progressively acquired.

CHAPTER 2
MATERIALS AND METHODS

2.1 Animals

Female wild-type BALB/c mice (6-8 weeks old) were purchased from Charles River Laboratories. Male wild-type BALB/c mice (6-8 weeks old) were also ordered solely for breeding purposes. All mice were housed under specific pathogen-free conditions and maintained on a 12-hour light cycle, with food and water available to them *ad libitum*.

For breeding, 4 female mice were paired with 1 male in a single cage. Two weeks later, females were separated into individual cages prior to giving birth. Pups were weaned at 3 weeks of age, at which point the mother would be re-paired with a male. Breeding males and females were fed a high-fat diet: Teklad-S-2335 Mouse Breeder Diet (Harlan #7904). Once weaned, the offspring would be provided with a conventional diet: Teklad Irradiated Global 18% Protein Rodent Diet (Harlan #2918).

All experiments described in this study were approved by the Animal Research Ethics Board of McMaster University.

2.2 Sensitization Protocol

2.2.1 Allergen Administration

HDM extract from Greer Laboratories was re-suspended in sterile saline at concentrations of 2.5, 5.0 or 12.5 mg of protein/mL. 10 μL of the solution was administered intranasally to mice anaesthetized by gaseous isoflurane.

2.2.2 Protocols

I. Development of a short model of HDM-induced allergic airway inflammation

Adult BALB/c mice were exposed to saline, 1, 5, 10, 25, or 50 μg of HDM intranasally for 7 consecutive days. Mice exposed to 25 μg of HDM for 10 consecutive days served as a positive control. All groups were sacrificed ~24 hrs after the last HDM challenge.

II. *Evolution of HDM responsiveness in mice from birth to adulthood*

BALB/c mice were exposed to either saline or 25 μg HDM intranasally daily for 10 consecutive days and sacrificed ~24 hrs after the last HDM challenge. This protocol was initiated in separate groups of mice at different ages (2, 3, 4, 5 and 6-8 weeks).

III. HDM Dose-Response in Neonatal Mice

2 week-old BALB/c mice were exposed to saline, 5 μg or 125 μg of HDM intranasally daily for 10 consecutive days and sacrificed ~24 hrs after the last HDM challenge. Adult mice (6-8 weeks old) exposed to 25 μg of HDM for 10 consecutive served as a positive control.

IV. Impact of Treg depletion on HDM-induced immune-inflammatory response

2 week-old and 6-8 week-old BALB/c mice were exposed to saline or 25 μg of HDM intranasally daily for 10 consecutive days and sacrificed ~24 hrs after the last HDM challenge. For depletion of $CD25^+$ cells, 100 μg of rat α-mouse-CD25 monoclonal antibody (PC61, Biolegend) or a control antibody (rat IgG, Biolegend) in 200 μL phosphate-buffered saline (PBS) was administered intraperitoneally one day prior to the start of HDM exposures, and then again on day 6 of the above protocol.

2.3 Collection and measurement of specimens

2.3.1 Blood Collection

Mice were anaesthetized and peripheral blood was collected via retroorbital bleeding using lime glass Pasteur pipettes (VWR). Approximately 500 μL of whole blood was collected per mouse into red-top collection tubes with clot activator (Terumo Capiject). Serum was obtained by incubation of whole blood at room temperature for at least 30 minutes and subsequent centrifugation at 13200 rpm for 10 minutes at 4°C. Supernatants (sera) were collected and stored at -20°C for future analysis.

2.3.2 Bronchoalveolar Lavage (BAL)

Mice were anaesthetized by gaseous isoflurane and their lungs removed from the thoracic cavity. The trachea was cannulated with a polyethylene tube (outer/inner diameter = 0.965/0.58mm) and lungs were subsequently washed with PBS + complete protease inhibitor (CPI) (Roche). First, ~ 250 μL PBS-CPI was instilled into the lung by means of the cannulated trachea. Next, the lungs were gently bounced 60 times against the table ensuring thorough dissemination of the PBS-CPI. BAL fluid was then recovered and stored in a 1.5 mL eppendorf tube. The above lavage process was repeated a second time with ~ 200 μL PBS-CPI. On average, 200-300 μL of total BAL fluid was recovered. Following collection, total cell counts were determined using a hemocytometer (Hausser Scientific). BAL samples were then centrifuged and supernatants stored at -20°C for future analysis. The remaining pellet was resuspended in an appropriate volume of PBS to bring the concentration of cells to 1.65 x 10^6 cells/mL. Next, BAL smears were prepared by loading 100 μL of the above cell suspension into a cytocentrifuge, and then spun at 300 rpm for 2 minutes. The smears were stained 24 hours later using the Protocol Hema 3 stain set (Fisher Scientific), and differential counts were determined by counting at least

500 leukocytes using standard hemocytological criteria to identify eosinophils, neutrophils, and mononuclear cells.

2.3.3 Preparation of Lung Tissue for Lung Homogenates or RNA analysis

Where applicable, following BAL, the four right lobes of the murine lung were isolated and placed in 500 μL of PBS-CPI in Falcon polysterene round-bottom tubes on ice. These samples were homogenized and then centrifuged at 13200 rpm for 10 minutes at 4°C. Supernatants of lung homogenates were collected and stored in 1.5 mL eppendorfs at -20°C for future analysis. Alternatively, the four right lobes were placed in 1 mL of RNA later (Ambion) and stored at -80°C for future RNA analysis.

2.3.4 Histological Analysis

Where applicable, following BAL, the single left lobe of the murine lung was prepared for histological analysis. First, the right bronchus was tied shut with string. The left lobe was then inflated with 10% formalin at a constant pressure of 20 cm H_2O. Once inflated, the trachea was also tied shut, and lobe was fixed in 4-5 mL of 10% formalin for 72 hours. Next, lobes were removed from the 10% formalin and cut cross-sectionally once immediately below the bronchial entry point, and then again approximately 5 mm below the first cut. Sections were sent to the McMaster University Centre for Gene Therapeutics Core Histology Research Services for embedding in paraffin, cross-sectioning and staining with hematoxylin and eosin (H&E).

2.4 Lung Cell Isolation

Where applicable, whole lungs were perfused through the right ventricle with 10 mL of Hank's balanced salt solution (HBSS). All 5 lobes of each lung were then dissected and

placed in a 1.5 mL eppendorf containing 500 µL of HBSS. For time-course studies of naïve mice, lungs were not lavaged prior to perfusion so as to provide a suitable comparison with lungs of fetal and newborn mice in which lavage is not feasible. Otherwise, BAL was performed before lungs were perfused. *Note:* lungs from fetal, newborn or 2 day-old mice were pooled at this point (n=3).

Once collected, lungs were cut into small pieces and transferred into 50 mL Falcon tubes. Samples were then agitated at 200 rpm for 1 hour at 37°C in 10 mL of 150 U/mL collagenase type I (Gibco) in HBSS. Next, lung suspensions were triturated through a 40 µm cell strainer (Becton Dickinson) using the plunger from a 3 mL syringe. The collagenase from the previous step was diluted with an additional 40 mL of HBSS to each triturated sample. Samples were then centrifuged at 1200 rpm for 10 minutes at 4°C and supernatants discarded. Next, red blood cells were lysed with 1 mL of ACK lysis buffer (0.5 M NH4Cl, 10 mM KHCO3, and 0.1 nM Na2EDTA, pH 7.2-7.4) for 1 minute. Lysis was stopped with the addition of 10 mL of Fluorescence-activated cell sorting (FACS) buffer (0.5% bovine serum albumin (BSA) in PBS). Samples were centrifuged afterwards, and washed once in 10 mL FACS buffer, followed by another centrifugation. Single cell lung suspensions were finally resuspended in 250-500 µL of FACS and filtered through 40µm cell strainer. Total cells were counted using a hemocytometer and Turks stain.

2.5 Splenocyte Isolation and Culture

Each spleen was collected in a 15 mL Falcon tube containing 5 mL of HBSS. To obtain single cells suspensions, whole spleens were triturated into HBSS through a 40 µm cell

strainer (Becton Dickinson) using the plunger from a 3 ml syringe. Samples were then centrifuged at 1200 rpm for 10 minutes at 4°C and supernatants discarded. Next, red blood cells were lysed with 1 mL of ACK lysis buffer (0.5 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 nM Na_2EDTA, pH 7.2-7.4) for 1 minute. Lysis was stopped with the addition of 10 mL of HBSS. Following another centrifugation, splenocytes were washed with 10 mL of FACS buffer if flow cytometric analysis ensued, or 10 mL Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.1% β-mercaptoethanol if culturing was to follow. Samples were then counted using a hemocytometer and Trypan Blue stain.

For culture, splenocytes were then resuspended in cRPMI at a concentration of 8 x 10^6 cells/mL. In a flat-bottom, 96-well plate (Becton Dickinson), samples were plated in triplicate: 100 µL of cell suspension + 100µl of cRPMI supplemented with HDM at a concentration of 31.25 µg/mL. A separate triplet of sample was plated in medium alone as a control. Splenocytes were culture for 5 days, after which supernatants were collected, triplicates pooled, and stored at -20°C for future analysis.

2.6 Flow Cytometric Analysis

2.6.1 Preparation of Flow Cytometry Staining Master Mixes and FMO Controls

Initially, antibodies were titrated to determine optimal staining concentrations in order to prevent over/under-staining. Tables 1 and 2 outline the colour combinations and dilutions that were typically used for the staining of lung/spleen APC and lymphocyte populations. The dilutions listed refer to the final concentration of antibody added to the well once the mix is added to the well (see *2.6.2 Cell Surface Staining*). Cell surface antibodies were

diluted in FACS buffer, and intracellular antibodies in 1x eBioscience Permeabilization Buffer (see: *2.6.3 Intracellular Foxp3 Staining* for preparation instructions).

Fluorescence minus one (FMO) controls were used to facilitate the precise analysis of positive and negative populations. For each FMO control, a mix was created containing all the antibodies in the same proportions as the staining mix; however, a different antibody was left out each time.

Marker	Fluorophore	Dilution	Supplier
CD45	APCCy7	1:400	BD Biosciences
CD11c	APC	1:200	BD Biosciences
CD11b	PeCy7	1:400	eBioscience
F4/80	eF450	1:100	eBioscience
Gr1	PacOrange	1:200	Invitrogen
MHC II	AF700	1:200	eBioscience
CD101	PE	1:100	eBioscience
Ly6C	PerCPCy5.5	1:100	eBioscience
B220; or CD49b; or CD103	Biotin-Qdot800	1:100 Biotin Ab 1:500 Qdot800	BD Biosciences
(open)	FITC		

Table 1 – Flow Cytometry stain for APC populations

Marker	Fluorophore	Dilution	Supplier
CD3	AF700	1:200	eBioscience
CD4	APC	1:400	eBioscience
CD8	eF450	1:100	eBioscience
CD69	PeCy7	1:200	eBioscience
CD25	Biotin-Qdot800	1:100 Biotin Ab 1:500 Qdot800	BD Biosciences
CD101	PE	1:100	eBioscience
CD62L	PerCPCy5.5	1:200	eBioscience
CD19	APCCy7	1:400	BD Biosciences
Foxp3	FITC	1:50	eBioscience

Table 2 – Flow Cytometry stain for Lymphocyte populations

2.6.2 Cell Surface Staining

Flow cytometric analysis of lung and spleen cells was performed. Once isolated, as per the protocols above, 1 – 2 x 10^6 cells from each sample were plated in round-bottom, 96-well plates. For the FMO and unstained controls, equal proportions of the remaining cells were pooled, and 1 – 2 x 10^6 cells plated for each control. The plate was subsequently spun at 1400 rpm for 10 minutes at 4°C. To minimize non-specific binding of flow antibodies, 50 µL of a 1:50 dilution of FcBlock (anti-CD16/32)(BD Biosciences) was added to each well and incubated on ice for 15 minutes. Antibody FMO and cell surface staining master mixes were prepared (outlined in *'Preparation of Flow Cytometry Staining Master Mixes and FMO Controls'*). 50 µL of the appropriate mix was added on top of the FcBlock, creating a final staining volume of 100 µL, and incubated on ice for 30 minutes avoiding direct exposure to light. Cells were washed once by adding 150 µL of FACS Buffer immediately following the 30 minute incubation, and then once more with 200 µL of FACS following centrifugation at 1400 rpm for 10 minutes at 4°C. Data was collected using an LSRII (BD Biosciences), and analysis was later performed with the FlowJo software (Tree Star and Stanford University).

Note: If a biotinylated antibody is present within the cell surface staining mix, there is an additional incubation step following the washes associated with the initial stain: 50 µL of a 1:500 dilution of Qdot800-Streptavidin (Invitrogen) is added to the cells and incubated on ice for 30 minutes. This step is also followed by two washes with 200 µL of FACS buffer prior to collecting data on the LSRII.

2.6.3 Intracellular Foxp3 Staining

For intracellular Foxp3 staining, we used the Foxp3-Staining Buffer Set supplied by eBioscience and optimized a protocol for staining in a 96-well plate using the one provided by eBioscience for staining in tubes as a template. $1 – 2 \times 10^6$ cells were first stained for surface markers (see *2.6.2 Cell Surface Staining*). Next, 200 µL of the eBioscience Fixation/Permeabilization solution (Preparation: 1 part Fixation Solution for every 3 parts Dilutent Solution) was added and incubated on ice for 1 hour. Following centrifugation at 1400 rpm for 10 minutes at 4°C, cells were washed with 200 µL of 1x eBioscience Permeabilization Buffer (Preparation: 1 in 10 dilution of 10x Permeabilization Buffer in ddH_2O) and centrifuged again. Next, Foxp3 antibody intracellular staining mix was prepared (Preparation: 1 in 50 dilution of Foxp3-FITC in 1x eBioscience Permeabilization Buffer) (see *2.6.1 Preparation of Flow Cytometry Staining Master Mixes and FMO Controls'*) and 50 µL was added to the cell pellets and incubated on ice for 30 minutes (*Note*: FcBlock step prior to intracellular staining is optional provided that blocking was performed during staining of cell surface markers). Cells were washed once by adding 150 µL of FACS Buffer immediately following the 30 minute incubation, and then once more with 200 µL of FACS following centrifugation at 1400 rpm for 10 minutes at 4°C. At this point, cells are ready for data collection and analysis (see *2.6.2 Cell Surface Staining*).

2.7 Cytokine Immunoglobulin (Ig) Measurements

2.7.1 Serum IgG_1

Levels of IgG_1 were measured by an enzyme-linked immunosorbent assay (ELISA). First,

Maxi-Sorp plates were coated with 5 μg HDM in carbonate-bicarbonate buffer overnight at 4°C. Next, plates were washed with 300 μL PBS-0.05% Tween 20 five times; all subsequent washes refer to this strategy. Wells were then blocked with PBS-1% BSA for 2 hours at room temperature. Following another series of washes, serum samples (diluted 1:20, 200, 2000, and 20,000 in 0.3% BSA-PBS) were added to each well in a volume of 50 μL/well and incubated overnight at 4°C. The next day, wells were washed again, and incubated with 0.25 μg/mL of rat anti-mouse IgG_1 for 2 hours at room temperature. After washing, a 1:1000 dilution of alkaline-phosphatase streptavidin was added in a volume of 50 μL/well and incubated for 1 hour at room temperature. Next, samples were washed for a final time, and the colour reaction was developed with the addition of 50 μL/well of *p*-nitrophenyl phosphate in 10% v/v diethanolamine buffer. The reaction was stopped with 25 μL/well of 2N NaOH and the optical density (OD) was read at a wavelength of 405 nm.

2.7.2 Cytokine Measurements in Lung Homogenates and BAL

DuoSet ELISA kits for mouse interleukin (IL)-10 and active TGF-β1 (R&D Systems) were used according to the manufacturer's instructions. Total TGF-β was measured in lung homogenates by activating latent TGF-β1 with 1N HCl.

2.8 Data Analysis

Data were analyzed using GraphPad Prism version 4.0 and expressed as mean ±SEM. Results were interpreted using either a t-test or a one-way analysis of variance (ANOVA) with a Tukey's *post hoc* test. Differences were considered statistically significant when P values were less than 0.05.

CHAPTER 3
RESULTS

3.1 Development of a Short Model of HDM-induced Allergic Airway Inflammation

Given the relatively brief period of murine development (6-8 weeks) we sought to establish a model requiring sufficiently short HDM exposures to identify changes in the evolution of HDM responsiveness during development in BALB/c mice. The HDM dose-response relationship in adult BALB/c mice has been well characterized by our group in a model where HDM is administered for 10 consecutive days. Responsiveness increases with dose, reaching a plateau at about 25 μg HDM [73]. First, we performed a similar series of experiments examining the response of adult mice to 1, 5, 10, 25 or 50 μg HDM but, this time, over 7 consecutive days.

Adult mice receiving doses of 1, 5, and 10 μg of HDM over 7 days did not show any statistically significant response in the BAL, based on total cell number (TCN) and eosinophilia, as compared to saline-treated controls (Fig. 1A). In contrast, mice receiving higher doses, 25 and 50 μg, over 7 days exhibited significant BAL inflammation. However, the extent of inflammation was significantly lower than that observed after 10 days of exposure (Fig. 1A). Bone marrow was also collected to detect incipient responses in mice exposed to 1, 5, 10, 25, and 50 μg of HDM over 7 days. However, analysis did not reveal any significant differences in total cell number nor eosinophilia (data not shown). We also evaluated serum levels of HDM-specific IgG_1 (Fig. 1B). Moreover, HDM-IgG_1 was not detected in the sera of mice exposed to 7 days of HDM, irrespective of dose. These data indicate that 7 days of HDM exposure is not enough to mount a

significant humoral response in adult mice. HDM-specific IgE was not quantified since it only begins to be detectable after 20 days.

In addition, flow cytometry analysis of lung APC and lymphocyte populations was performed on mice exposed to either saline or 25 μg of HDM for 7 or 10 days. For the sake of brevity, Figure 1C summarizes the results with check marks denoting statistical significance over saline-exposed mice. In both HDM groups, there is significant immune activation based on the expression of CD69 on T-cells, as well as significant expansion and activation of macrophage and dendritic cell subsets. However, the extent of activation of T-cells and APC subsets was consistently higher in the 10-day group. Furthermore, there are significantly higher levels of B-cell ($Cd11c^{+}CD11b^{-}$ $B220^{+}Gr1^{-}$) expansion in the 10-day over the 7-day group, which parallels the aforementioned lack of HDM-IgG_1 in sera in the 7-day group (Fig. 1C).

Collectively, these data indicate that 7 consecutive days of exposure to 25 or 50 μg of HDM results in a significant immune-inflammatory response, albeit less robust than that observed following 10 days of exposure, with no measurable serum IgG_1 levels. Therefore, we determined that the 10-day model would provide the best option to investigate the evolution of allergen responsiveness over the course of development.

3.2 Evolution of HDM Responsiveness in Mice During Development

In light of previous data from our laboratory demonstrating that 2 week-old mice exposed for three weeks to 25 μg of HDM are hyporesponsive [45], we sought to delineate the evolution of responsiveness over the entire murine developmental period. Thus, we initiated a protocol of HDM exposure (25 μg daily for 10 days) at different time-points in

development. Our results demonstrate that within the first three to four weeks of life, mice are indeed hyporesponsive to HDM (Fig 2A). There is a critical interval, at approximately 4 weeks of age, where HDM responsiveness emerges, as evidenced by total cell number and eosinophilia, reaching full responsiveness at 6-8 weeks of age. Additionally, this progressive increase in responsiveness is also apparent in the analysis of serum IgG_1 levels (Fig 2B).

3.3 Immune Cell Profile in the Lung Over the Course of Development

To understand the immunological basis of HDM responsiveness and identify potential mechanism(s) underlying hyporresponsiveness in infant mice, we used flow cytometric analysis to delineate the immune cell profile in the lung of naïve and HDM-exposed mice over the course of development. This analysis yielded distinct, novel observations with respect to changes in the expression of maturation and immunosuppression markers in immune cells.

3.3.1 Natural Killer (NK) Cells

It has been shown that NK cells acquire CD11b expression as they mature [74]. Differentiation follows at least four distinct stages ($CD11b^{low}CD27^{low} \rightarrow CD11b^{low}CD27^{high} \rightarrow CD11b^{high}CD27^{high} \rightarrow CD11b^{high}CD27^{low}$), recently reported by Chiosonne *et al.* (2009) by examining NK cell phenotype over time in the bone marrow, spleen, liver, lymph nodes and peripheral blood following *in vivo* depletion [75]. This developmental program also appeared to be associated with an increase in NK cell effector function [74-76]. Our data reveal the progressive emergence of a mature $CD11b^{+}$ NK cell population ($CD3^{-}CD49b^{+}$) in the naïve lung over the course of development. As

shown in Figure 3A, approximately 45% of NK cells in the lung of newborn and two week-old mice are $CD11b^{+}$; this number progressively increases through development reaching a steady state of about 80-85% in adulthood (6-8 weeks). Preliminary evidence further reveals a higher percentage of $CD11b^{low}CD27^{+}$ NK cells (early stage of maturation) in the lungs of 2-week old as compared to 6-8 week old mice.

The pattern of NK cell maturation during the developmental period parallels the generation of HDM responsiveness. These data suggest that the state of maturation of NK cells in early infancy may contribute to allergen responsiveness. Furthermore, although the *percentage* of NK cells within $CD45^{+}$ subset does not change following HDM-exposure, evaluation of absolute numbers demonstrates that there is an expansion of NK cells in adult, not infant, mice following 25 μg HDM over 10 days (Fig. 3B). Further interpretation of this rise in total NK cells should be restricted, as it is merely the product of greater total lung cell counts in HDM-exposed adult mice because of their ability to mount a response.

3.3.2 Antigen-Presenting Cells (APCs) – Alveolar Macrophages and Dendritic Cells

We based our APC gating strategy on a recent publication by Bedoret *et al.* (2009), which identified three major APC populations from a CD11c-F4/80 FACS plot (Fig. 4B) [77]. However, our additional studies of the $CD11c^{-}F4/80^{+}$ population provide a different interpretation. While Bedoret *et al.* (2009) claim that this population represents interstitial (tissue) macrophages based on their strong phagocytic activity and high level of MHC II and CD68 expression, we demonstrate, through FACS sorting, that this population displays morphologic characteristics typical of eosinophils, and a very low

expression of MHC II (Fig. 4C) [77]. Since we cannot clearly identify a population of tissue macrophages in the lung, at this point, we will limit our temporal analysis of APCs to alveolar macrophages and dendritic cells.

We further characterized the populations in the CD11c-F4/80 FACS plot and noted autofluorescence in the FITC channel by alveolar macrophages along with low CD11b expression (Fig. 4B). Dendritic cells, on the other hand were $FITC^-$ with a spectrum of CD11b expression. These features are consistent with descriptions in the literature [78, 79]. Furthermore, we used expression of MHC II on APCs as a marker of immune maturation, specifically representing antigen presenting capacity, and CD101 as a potential marker of immunosuppression. CD101 is a type I membrane protein expression with no known ligand [80, 81]. Its expression on murine Tregs denotes enhanced suppressive activity. Specifically, splenic $CD101^+$Tregs are considerably more immunosuppressive than $CD101^-$Tregs both *in vivo and in vitro* [57]. Additionally, CD101 ligation on monocytes/macrophages elicits significant IL-10 production [82], and its triggering on human cutaneous DC results in IL-10-mediated inhibition of T cell proliferation [83].

3.3.3 Alveolar Macrophages (AM)

Alveolar macrophages, identified as $CD11c^+$ $F4/80^+CD11b^{low}$ plus FITC autofluorescence, make up a dominant proportion of immune cells in the naïve lung. The fetal and 6-12 hour newborn lung, however, contain very low levels of alveolar macrophages. Figure 5 clearly shows that there is rapid acquisition of cells with an alveolar macrophage phenotype within the first 2 to 3 days of life that is maintained over

the course of development. Notably, that these cells are $CD11b^{lo}$ and $Ly6C^{-}$ suggests that they are not monocytes (see **4.9 The role of regulatory T-cells and alveolar macrophages in tolerance induction** for a more detailed discussion). Our results also indicate that there is an age-dependent inverse relationship in the proportion of cells expressing CD101 and MHC II on alveolar macrophages, but not dendritic cells (Fig. 6). Specifically, $CD101^{+}$ AMs were very prominent within the first 3 days of life; indeed, approximately 70-80% of all alveolar macrophages were $CD101^{+}$; this number decreased to 30-40% by week 1 and reached steady-state levels of 2-6% by week 2. Conversely, MHC II^{+} alveolar macrophages were sparse in the days after birth and considerably increased over two weeks (Fig. 6). We have confirmed this observation with flow cytometry analysis of the BAL (data not shown). From the perspective of APCs, these findings suggest that lung maturation is characterized by the progressive acquisition of the capacity to respond to ensuing immune challenges as well as the steady waning of the robust immunosuppressive environment present very early in life.

In adult mice, the alveolar macrophage population expands following HDM exposure (Fig. 7A). Interestingly, MHC II expression on alveolar macrophages increases only in adult mice, while CD101 expression remained unchanged regardless of age and exposure (Fig. 7B).

3.3.4 Dendritic Cells

The small, resident DC population, defined as $CD11c^{+}F4/80^{-}CD11b^{+/-}FITC^{-}$, in the naïve lung (1-2% of all CD45+ cells) remains relatively constant over the course of development in terms of relative percentage, MHC II and CD101 expression (Fig. 5, 6).

However, there is a marked difference in expansion following HDM exposure between infant (2 week-old) and adult mice. Specifically, we observed approximately an 8-fold higher expansion of a subset of DCs ($CD11c^{+/hi}CD11b^{hi}MHCII^{hi}$) in adult, as compared to infant, mice (Fig. 7C). This finding intimates a potential degree of immaturity amongst infant lung DCs in response to HDM. Further, the $CD11c^{hi}$ subset is thought to be a rich source of pro-inflammatory chemokines suggesting that the lack of these cells may contribute to the absence of inflammation in infant mice [79].

3.3.5 T-cells

We found that the number of $CD3^{+}CD4^{+}$ T cells is very low in the fetal as well as the newborn lung, and increases substantially 48-72 hrs. after birth reaching a level at week 1 that remains constant to adulthood (Fig. 8A). The level of activated T-cells ($CD3^{+}CD4^{+}CD69^{+}$) remains comparable throughout development, as does the level of Tregs ($CD3^{+}CD4^{+}CD25^{+}Foxp3^{+}$) (data not shown; Fig. 8A). However, for Tregs specifically, there is significantly higher expression of Foxp3 on a per cell basis (MFI) in the infant lung (MFI ≈ 3000 at week 1; ≈ 1800 at week 3; ≈ 1400 at week 6-8) (Fig. 8B). In addition, there is a higher percentage of $CD101^{+}$ Tregs in the early stages of development, intimating the presence of more potent Tregs in infancy (Fig. 8A) [57]. Approximately, 40% of Tregs are $CD101^{+}$ at week one, a percentage that decreases progressively in subsequent weeks eventually reaching baseline levels of 8.5% in adulthood. The $CD101^{+}$ Tregs were also $CD62L^{+}$, a subset that Fernandez *et al.* (2008) describe to be more potently suppressive *in vivo* (data not shown) [57].

As shown in Figure 9C, HDM exposure leads to a marked increase in $CD4^+CD25^+FoxP3^+CD101^+$ Tregs in both the responsive adult and hyporesponsive infant lungs, insinuating their role in control of HDM-induced responses. As a point of comparison, we were unable to detect $CD3^+CD8^+FoxP3^+$ Tregs in neither naïve nor HDM-exposed lungs at any point in development (data not shown). Interestingly, despite the presence of more potent Tregs in the infant lung, the *percentage* of $CD3^+CD4^+$ T-cells that express CD69, an early activation marker, remains comparable following HDM-exposure in both the infant and adult mouse (Fig. 9B), which suggests that if Tregs are mediating infant HDM hyporesponsiveness, perhaps they are influencing DC expansion (Fig. 7C) moreso than T-cell proliferation. Equally possible is an inherent deficiency or immaturity in infant DC function. *Note:* We are unable to present our results in the form of absolute numbers because, overall, the total lung inflammation is substantially lower in infant mice. Thus, by normalizing the above percentages to total lung cell counts, one fails to appreciate the fact that proportionally the number of activated T-cells is similar in the HDM-exposed infant and adult.

In summary, $CD3^+CD4^+$ T-cells appear in the days following birth with a higher proportion of the more suppressive $CD101^+$ and $Foxp3^{high}$ Tregs in early life. Following HDM exposure, there are similar proportions of Tregs and activated T-cells in both infant and adult mice, despite their differences in overall inflammation.

Taken together, these findings about various cell types over the course of development suggest that there is a major immunological shift that occurs in the lung during infancy where the pervasive immunosuppressive environment in early life steadily

subsides while the capacity to respond to ensuing immune challenges progressively increases.

3.4 Impact of Treg Depletion on HDM-induced Immune-Inflammatory Responses

To functionally investigate the impact of an immunosuppressive environment on HDM responsiveness, we first depleted $CD4^+CD25^+FoxP3^+$ Tregs in adult mice using a αCD25 monoclonal antibody (PC61). Over the course of 10 consecutive days of 25 μg HDM exposure, we administered 100 μg of the depleting antibody or isotype control intraperitoneally one day prior to the start of exposures and, then, again on day 6. This resulted in the 85-90% depletion of $CD4^+CD25^+FoxP3^+$ Tregs in saline-exposed mice and 65-70% depletion in HDM-exposed mice as assessed 24 hours after the last HDM exposure. As shown in figure 10, HDM-exposed adult mice depleted of Tregs exhibited significantly more pulmonary inflammation than their counterparts with an intact Treg compartment. Absolute numbers of eosinophils were also higher in the Treg-depleted mice, although there was no difference in the percentage of eosinophils in the BAL (Fig. 10B/C). There were also increases in the systemic immune response, as mice depleted of Tregs had significantly higher serum titres of HDM-specific IgG_1 (Fig. 10D). We also examined whether Treg depletion had an impact on T-cell and DC activation through flow cytometric analysis. We observed increases in the absolute numbers of activated T-cells ($CD3^+CD4^+CD69^+$) and a subset of myeloid DCs ($CD11c^{+/hi}CD11b^+MHCII^{+/hi}$) in Treg-depleted mice. These increases, however, did not reach statistical significance, which we will further test by increasing our n-value in future experiments (Fig. 10E/F). Overall, these results are consistent with previous data reporting that natural Tregs play a

role in controlling the extent of HDM-induced inflammation.

We, then, carried out the same Treg depletion experiments in two-week old mice exposed to 10 consecutive days of 25 μg HDM. In this instance, Treg depletion dramatically reversed the hyporesponsiveness of infant mice to HDM. PC61-treated, HDM-exposed infant mice demonstrated levels of pulmonary inflammation (BAL TCN, eosinophilia and total lung cells) comparable to untreated, HDM-exposed adult controls (Fig. 11A/B). This dramatic reversal of responsiveness was also evident systemically, as depicted through serum HDM-specific IgG_1 levels (Fig. 11C). Given the previous description of more "immunosuppressive" Tregs in infancy (see: *3.3.5 T-cells* and *1.4.4 Tregs in infant immune system*), these results suggest that Tregs play a pivotal role in mediating HDM hyporesponsiveness in infant mice.

3.5 HDM Dose-Response in Infant Mice

We carried out further dose-response studies in infant mice (2 week-old) using two doses of HDM: 5 μg and 125 μg. Thus, either five-fold higher or lower than the dose, 25 μg, we used to this point in infant mice. Our results, shown in Figure 12, provided a better understanding of HDM responsiveness in the neonatal system. Firstly, lowering the dose from 25 to 5 μg resulted in no immune inflammatory response in infant mice, as depicted through serum IgG_1 analysis and BAL inflammation. In contrast, we observed a marked inflammatory and immunoglobulin response in infant mice exposed to a very high dose of HDM (125 μg). Furthermore, there were no differences in neutrophilia between adult mice and the high-dose infants, and lung cell isolation demonstrated a significant increase in the total cell number of the adult and high-dose infant groups (Fig.

12A; data not shown). These results rule out the possibility that allergen responsiveness follows a bell-shaped pattern, which postulates that in environments of high allergen exposure, there is a low risk of sensitization [84]. Moreover, they demonstrate that the neonatal immune system has the capacity to respond to a signal of exceptional strength.

CHAPTER 4
DISCUSSION

4.1 Conclusions

It is thought that the homeostatic response to aeroallergen exposure is tolerance [31, 85] and, thus, that the development of allergic asthma is either a failure to induce tolerance or a disruption of established tolerance. In this context, most cases of allergic asthma occur during the first 3 years of life [25-27]. However, the specific intervals of protection or susceptibility remain to be elucidated [20, 28]. In order to address these issues the principal goal of this project was to delineate allergen responsiveness and its immunological basis during development. Our findings demonstrate the progressive acquisition of HDM responsiveness in BALB/c mice from birth to adulthood. We also observed profound immunological shifts in that the prominent immunosuppressive environment of the neonatal lung, as indicated by regulatory T-cells and alveolar macrophages among, possibly, other cell types, steadily subsides while the capacity to respond to ensuing immune challenges progressively increases, as evidenced by NK cell and DC maturation and the acquisition of MHC II expression on alveolar macrophages. Moreover, exposure to a very high dose of HDM or depletion of Tregs overcomes the natural immunosuppressive environment resulting in the acquisition of HDM responsiveness, as manifested by robust immune-inflammatory responses. The mechanisms whereby this immunosuppressive environment mediates HDM hyporesponsiveness in infant mice, as well as the implications that infant allergen exposure have on subsequent adult responses, remain as future directions of study.

4.2 The Influence of Immune Maturation During Development on Allergen Responsiveness

In part, our findings agree with the prevailing notion that the infant immune system is immunologically immature relative to the adult [34, 35, 40]. We report that NK cells in the lung acquire CD11b expression over the course of development, indicating the presence of a developmental NK cell maturation process ($CD11b^{low} \rightarrow CD11b^{high}$). Indeed, $CD11b^{high}$ (or Mac-1^{high}) NK cells have greater cytotoxic ability and also produce significantly higher amounts of IFN-γ than $CD11b^{low}$ NK cells following poly(I:C) treatment both *in vivo* and *in vitro* [74]. The maturation process has recently been more specifically defined: $CD11b^{low}CD27^{low} \rightarrow CD11b^{low}CD27^{high} \rightarrow CD11b^{high}CD27^{high} \rightarrow CD11b^{high}CD27^{low}$ [75, 76]. While we require further CD27 phenotypic classification of lung NK cells, preliminary evidence suggests a higher proportion of $CD11b^{low}CD27^{high}$ NK cells earlier in life, with CD27 expression subsiding and CD11b increasing throughout development. Chiossone *et al.* (2009) have noted a similar maturation process in the murine spleen in relation to age, although the focus of this study was on NK cells from 3 to 50 weeks of life [75].

The emergence of $CD11b^{+}$ NK cells in the lung in parallel with the acquisition of HDM responsiveness over the course of development inspires future experimentation on whether the immaturity of infant NK cells is, at least partially, mediating the hyporesponsiveness of infant mice to HDM. In adult mice, the percentage of NK cells within the $CD45^{+}$ leukocyte population is similar in both saline and HDM-exposed mice. The absolute number, however, is significantly higher in the HDM group; an increase that is not observed in HDM-exposed infant mice. This difference between total NK cells in

HDM-exposed infant and adult mice should be cautiously interpreted as it is the direct product of the significantly high total lung cell inflammation in adult mice. Thus, to fully understand the potential role NK cells play in our HDM model, the next steps would be to deplete them in HDM-exposed adult mice and/or transfer adult NK cells into HDM-exposed infant mice. Based on previous evidence in adult OVA/alum intraperitoneal sensitization models, we can speculate that NK cells play a crucial role in the initiation of Th2 responses, as Korsgren *et al.* (1999) have reported that NK cell depletion during the sensitization phase dramatically decreases allergic airway inflammation upon challenge [86, 87]. Furthermore, the NK cell cytokine profile is altered in allergic asthma, as atopic asthmatics have slightly higher levels of IL-4^+, as compared with IFN-γ^+, NK cells following *ex vivo* activation of human PBMCs [87-89]. Thus, another worthwhile point of investigation is to study whether the maturity of NK cells influences the subsequent cytokine profile following HDM exposure.

Along with NK cells, we have also made observations of potential immaturity in infant APC populations. A subset of mDCs expands in adult, and not infant, mice following HDM exposure (Fig. 7C). Recently, the Lambrecht group demonstrated that $CD11c^+$ DCs are necessary and sufficient for the induction of Th2 immunity in adult mice [8]. An important point, however, is that CD11c-specific depletion and isolation strategies were used in these studies without accounting for the high expression of CD11c on alveolar macrophages. Nevertheless, given the lack of mDC expansion in HDM-exposed infants, it would be interesting to investigate whether the presence of adult DCs in the neonatal system would influence HDM responsiveness. Indeed, the impaired proliferation

and cytokine production of cord blood T-cells is reversed if cord blood APCs are replaced with adult peripheral blood APCs [38, 39]. Therefore, DC immaturity may also contribute to infant hyporesponsiveness to HDM.

Overall, while we provide evidence of immune immaturity in the infant mouse lung, this is not absolute since it can be overcome with a signal of sufficient immunological strength. This is substantiated by our observations in Figure 12 demonstrating the ability of infant mice to mount robust inflammatory responses if exposed to a sufficiently high dose of HDM: 125 μg, which is 5 times the dose (25 μg) where responsiveness in adult mice plateaus. Furthermore, infant mice mount robust responses to 25 μg of HDM, a dose to which they are hyporesponsive, if exposed in the context of a flu infection i.e. sufficient immunological strength [45].

4.3 Tregs in the Regulation of Allergic Responses in Adult Mice

A number of experimental studies have examined the role of regulatory T-cells in allergic airway disease [90-94]. The overall understanding is that Tregs play an important role in controlling the extent of induced inflammation. We have confirmed this in our model of acute HDM inflammation, where we observe enhanced airway inflammation and serum IgG_1 levels following α-CD25 treatment in adult BALB/C mice exposed to 25 μg of HDM for ten consecutive days. Similar observations have been reported in other strains of mice, such as C57BL/6 and C3H/HeJ mice, though it appears that the role of Tregs in A/J mice is less pronounced given the modest effects that $CD25^+$ depletion on eosinophilia and the lack of any effect on airway hyperresponsiveness, IgE levels and Th2 cytokine synthesis [90, 91].

4.4 Tregs in the Regulation of Allergic Responses in Neonatal/Infant Mice

The paradigm in the neonatal setting is distinct from that investigated in adult animal studies in that the steady-state is unresponsiveness to HDM. To our knowledge, there is no precedent for describing the potency of Tregs in the murine infant lung, nor for studying the effect of their depletion on subsequent allergen responsiveness. Our data show that there is potentially greater suppressive activity in the lung of naïve infant mice, compared to adults, based on higher Foxp3 MFI and CD101 expression. On $CD4^+CD25^+Foxp3^+$ cells, this suppressive phenotype steadily subsides as the mouse matures into adulthood. These findings are consistent with evidence of enhanced Treg potency in human fetal tissues and cord blood [58, 61]. Recently, Wang *et al.* (2010) described an enhanced tendency of neonatal murine $CD4^+Foxp3^-$ T-cells from the spleen and thymus to acquire a $Foxp3^+$ phenotype and regulatory function following TCR stimulation [95]. For instance, where 70% of infant $CD4^+Foxp3^-$ cells become $Foxp3^+$ following TCR stimulation, <10% of adult cells exhibit the same phenotype [95]. Our data demonstrate that Tregs expand comparably in both HDM-exposed infant and adult mice; in adults presumably to control the extent of HDM responsiveness. Moreover, the expansion of Tregs in infants despite minimal overall responsiveness to HDM may be the result of a similar intrinsic "default" mechanism of T-cells from the infant lung to become Tregs following TCR stimulation, in this case induced by exposure to allergen [63].

Treg depletion in infant mice reverses allergen responsiveness resulting in immune-inflammatory responses analogous to those of adults. These findings highlight the importance of the regulatory T-cell compartment in infancy in shaping HDM

responsiveness, and are particularly relevant given recent evidence suggesting functional impairment of Tregs from the cord blood of infants with a higher risk of developing allergy [64, 65].

4.5 Methodological Considerations Regarding Treg Depletion

The strategy we used to deplete Tregs, α-CD25 treatment, has two limitations: (I) the potential depletion of 'activated' T-cells along with Tregs; and (II) the incomplete depletion of all regulatory cell subsets given the existence of $CD25^{-}Foxp3^{+/-}$ cells. The importance of potentially deleting 'activated' T-cells stems from the fact that allergic asthma is recognized as a T-cell driven disease [7]. Indeed, our murine model is absolutely CD4 T-cell-dependent (data not shown). Consequently, depletion of 'activated' T-cells would, in fact, prevent the generation of the subsequent immune inflammatory response, since approximately 30% of the $CD69^{+}$ T-cells from a HDM-exposed adult mouse are in fact $CD25^{+}$ (data not shown). However, our data demonstrate that there is even a slight increase in the absolute numbers of activated $CD69^{+}$ T-cells in α-CD25-treated HDM-exposed mice over HDM-exposed rat IgG controls (Fig. 10E). Moreover, we show that infant mice treated with α-CD25 antibodies actually gain allergen responsiveness suggesting that the physiological effects of depleting $CD25^{+}$ Tregs are so dramatic that they override the potential effects of $CD25^{-}$ Tregs and only have a minor effect on activated T-cells. Nevertheless, since other Treg depletion strategies exist, we feel that it is necessary to employ them to confirm our observations.

In this regard, Baru *et al.* (2010) recently used a Foxp3-diphtheria toxin receptor (DTR) system to demonstrate the importance of $Foxp3^{+}$ Tregs during the sensitization

phase in preventing exacerbated immune responses upon challenge in an OVA-induced model of airway inflammation [70]. To our knowledge, the transgenic "depletion of regulatory T cell" (DEREG) mice from this study have not been used in a HDM model in neither adult nor infant mice. While this seems the logical next step in our studies, this method is not exempt of drawbacks. Indeed, activated T-cells transiently express Foxp3 [63, 96]; moreover, there is evidence of regulatory T-cell subsets that are $Foxp3^-$ [9]. In addition, we must consider that our experimental protocol would require repeated administrations of DT; therefore, the tolerability of infant mice to its effects is a serious concern. The above concerns notwithstanding, there is evidence that the Foxp3-DTR system may be more effective in depleting Tregs since newborn DEREG mice depleted of $Foxp3^+$ Tregs using DT injections on days 1 and 7 of life developed a scurfy-like autoimmune phenotype as a result of a breakdown of peripheral tolerance, whereas newborns depleted of $CD25^+$ cells using α-CD25 antibodies showed little, or no autoimmune disease [49]. Adult mice showed no disease regardless of depletion strategy [49, 70]. The scurfy-like syndrome in Foxp3-DTR newborn mice does not deter us from using the strategy to test allergen responsiveness in infant mice since we begin our protocol later in development, at 2 weeks of age. Ultimately, we will deal with the specificity issues of the currently available Treg depletion strategies through intracellular cytokine analysis, *in vitro* proliferation assays and *in vivo* adoptive transfers (see below).

4.6 Further Studies to Investigate T-cell Mediated Immunosuppression in the Infant Lung

Nevertheless, α-CD25 treatment, or partial Treg depletion, results in exacerbated responses to HDM in adult mice and a dramatic reversal of HDM hyporesponsiveness in infants. In parallel with *in vitro* proliferation assays, we plan to perform adoptive transfers of $CD4^+CD25^+$ cells from infants and adults into adult mice exposed to HDM to compare their *in vivo* suppressive abilities. It has been reported that the intravenous or intratracheal transfer of 5×10^5 naïve or antigen-specific $CD4^+CD25^+$ cells from adult mice abolishes certain parameters of the asthmatic phenotype [92-94]. Thus, in order to increase the resolution between the effects elicited by infant and adult Tregs, we will conduct detailed dose-response studies. The expectation is that we will require transferring less cells. This will increase the feasibility of these studies particularly as it pertains to isolating such cells from the lungs of infant mice. This approach is further substantiated by the fact that such a small number of Tregs can have such a large physiological effect (see Fig. 8/10/11). We have performed flow cytometric analysis on the spleen in hopes of identifying a richer source of Tregs for adoptive transfers. To our surprise, there were even fewer Tregs in the spleen (data not shown). Thus, we speculate that Treg maturation is accelerated at mucosal sites, like the lung, as compared with non-mucosal sites, like the spleen.

We have also identified a functional subset of $CD4^+CD25^+Foxp3^+$ Tregs that express CD101, which is particularly abundant in the infant lung. There is evidence that $CD101^+$ Tregs are significantly more immunosuppressive than those lacking CD101 [57]. We have determined that there is a progressive decline in CD101 expression on Tregs over the course of development that correlates with the acquisition of responsiveness to

HDM. $CD101^+$ Tregs have previously been described in a murine model of graft-versus-host disease, but not in Th2-driven responses [57]. Recent evidence suggests the $CD101^+$ Tregs from human peripheral blood do not exhibit the same superior suppressive phenotype of those initially described and isolated from the murine spleen [82]. Nevertheless, given the plasticity of cells depending on their environment, nothing, as of yet, can be concluded about the function of $CD101^+$ Tregs isolated from the murine or human respiratory tract. We are limited with respect to CD101 neutralizing and knock-out reagents, especially since CD101 is expressed on a number of other cell types, such as monocyte/macrophages and granulocytes [80, 81]. We have, thus, opted for assessing the impact of $CD101^+$ Tregs on T-cell responses to HDM *in vitro* prior to embarking on adoptive transfers such as those outlined in the previous paragraph. Moreover, we will further characterize this subset and others, including cytotoxic T-lymphocyte-associated protein (CTLA-4) as a negative regulator of DC activation, CD127 as a marker inversely correlated with suppressive function, and inducible T-cell costimulator (ICOS) as a marker of IL-10 production [56, 97, 98].

4.7 Mechanisms by which Tregs may Mediate Allergen Hyporesponsiveness

There are a number of potential mechanisms by which Tregs may be mediating HDM hyporesponsiveness in infant mice. The literature identifies IL-10 and TGF-β as classical soluble mediators of Treg immunosuppression [99]. In adult mice, Kearley *et al.* (2005) demonstrated suppression of Th2 responses by adoptively transferred $CD4^+CD25^+$ cells dependent on IL-10 in an OVA model [93]. Moreover, Chen *et al.* (2003) reported the ability, also in adult mice, of TGF-β-induced Tregs to prevent HDM-induced allergic

pathogenesis, specifically through the dampening of inflammation and mucin production [92]. Both IL-10 and TGF-β from Tregs have also been identified to play critical roles in clinically effective HDM immunotherapy [100-102]. In adult murine models, the role of IL-10 appears controversial depending on allergen and protocol used. For instance, concurrent blockade of IL10R reverses the suppressive effects of the transfer of $CD4^+CD25^+$ cells in OVA-induced airway inflammation [93], while having no effect in a model of Der p 1-induced inflammation [91]. We will investigate the effect of IL-10R blockade *in vivo* on HDM responsiveness in infant mice to assess whether Tregs mediate allergen immunosuppression via IL-10. We have elected to not use IL-10-/- mice to avoid potential confounding variables that this deletion may have on murine morphogenesis [103]. Along with IL-10 and TGF-β, we hope to study other Treg-derived immunsuppressive cytokines, namely IL-35 and fibrinogen-like protein (FGL-2). IL-35 is a recently identified Treg-specific cytokine with potent suppressive abilities both *in vitro* and *in vivo* [55, 104]. It has not been described in the context of allergy and asthma. At present, there are no commercially available reagents for IL-35 but we are establishing collaborations to access these reagents, as with those for FGL-2, which is another Treg-derived cytokine that inhibits DC maturation and activation, among other immunosuppressive effects such as the induction B-cell apoptosis [99, 105]. Ultimately, we aim to elucidate the interplay between IL-10, TGF-β, IL-35 and FGL-2 in the generation of Tregs and their immunosuppressive activities, particularly in the context of our infant HDM model.

Tregs can regulate inflammation by controlling the proliferation, activation and maturation of many cell types, including T-cells, APCs and NK cells [99]. Although we have yet to directly study the effects of infant versus adult Tregs on T-cells *in vitro*, our data show there is a similar percentage of activated T-cells ($CD69^{+}$) in the hyporesponsive infant and responsive adult following HDM exposure *in vivo*. This finding insinuates that the main target of Tregs in this system is not T-cell activation. However, Tregs could be influencing T-cell cytokine production. Indeed, Kearley *et al.* (2005) have suggested that Tregs induce the production of IL-10 from effector T-cells which, unlike IL-10 from Tregs, is imperative for the suppression of Th2-driven responses [93]. Further experimentation is needed to test this hypothesis in our infant HDM model, including the optimization of an intracellular cytokine staining (ICS) protocol to map the sources of various immunosuppressive cytokines.

Tregs may also, at least partially, suppress allergen responsiveness in infant mice through controlling DC activation and maturation [99]. We have found that, following HDM exposure, a subset of myeloid DCs (mDCs) ($CD11c^{+/hi}CD11b^{hi}$MHC II^{hi}) expands in adult, but not infant, mice. The $CD11c^{hi}$ subset is thought to be a rich source of pro-inflammatory chemokines, suggesting that the deficiency of these cells may contribute to the absence of inflammation in infant mice [79]. In this context, Lewkowich *et al.* (2005) have demonstrated enhanced mDC activation following HDM exposure in adult mice depleted of Tregs [90]. To better understand this potential mechanism of immunosuppression in our infant model, we must broaden the scope of DC activation markers to include CD80/86 along with MHC II. From there, we have a number of

mechanisms to explore, which include, but are not limited to: FGL-2 secretion (described above) [105], CTLA-4 or lymphocyte activation gene-3 (LAG-3) ligation with CD80/86 and MHC II respectively, among other mechanisms. Briefly, LAG-3 is a CD4 homolog that binds MHC II on APCs with a higher affinity and reduces MHC II presentation to naïve T-cells [99, 106]. Similarly, CTLA-4 binds C80/86 on APCs with higher affinity than CD28, thereby blocking this important co-stimulatory signal for T-cells [99]. CTLA-4 ligation with CD80/86 may also induce tryptophan depletion in APCs, which leads to the production of pro-apoptotic factors [107].

Lastly, the simultaneous maturation of NK cells and decline in Treg potency over the course of development inspires investigation into a potential interplay between these cell types. In parallel to investigating the role of NK cells in our adult HDM model (see: **4.2 The Influence of Immune Maturation During Development on Allergen Responsiveness**), we will evaluate the influence that the more potent infant Tregs may have on NK cell maturation and/or activation. It has been demonstrated that Tregs can suppress NK cell responses to plasmid DNA vaccination and tumour growth via TGF-β or direct cytotoxic activity [108-110]. Interestingly, some *in vitro* studies highlight the ability of NK cells to suppress the expansion of $CD4^+CD25^+Foxp3^+$ Tregs [110, 111]. The direction in which the pendulum swings in the infant lung (i.e. whether Tregs suppress NK cell maturation, the reverse, or even if neither is the case) remains to be determined, and is certainly a point of future investigation.

4.8 Role of Immunosuppressive Alveolar Macrophages in Allergen Responsiveness

While we have demonstrated that Tregs play a vital role in maintaining immune homeostasis in the infant lung, we have also identified another potentially immunosuppressive cell that is prominent in the infant lung: the $CD101^+$ alveolar macrophage (AM). Our data show there is a rapid accumulation of AMs, identified as $F4/80^+CD11c^+CD11b^{low}Ly6C^-FITC^+$, within the first 2 – 3 days after birth. The speed at which they accumulate certainly speaks to the newly formed alveolar sacs and suggests a central homeostatic role in the face of the array of environmental exposures the newborn experiences. The functionality of these AMs, however, is yet to be elucidated, although much like the aforementioned Tregs, the number of $CD101^+$ AMs is particularly abundant after birth and dramatically decline to adult steady-state levels over the first two weeks of life. Interestingly, the pattern of MHC II expression follows a reverse trend, i.e. MHC II^+ AMs are sparse at day 1 and considerably increase over the first two weeks of life. This age-dependent inverse relationship in the proportion of AMs expressing CD101 and MHC II documents an immunological shift within the first two weeks of life, in that the alleged immunosuppressive environment of the neonatal lung is progressively replaced with the capacity to respond to ensuing challenges. Speculatively, as the newborn is rapidly confronted by multiple antigenic challenges, such as commensal colonization and the developing self, powerful immunosuppression in early life may confer a survival advantage by protecting from immunopathological responses. There is evidence to suggest that AMs in the adult lung have immunosuppressive as well as immune-enabling abilities [112-116]; however, we hypothesize that the immunosuppressive capacity is

more pronounced during infancy, and that CD101 expression, among other potential markers, mediate this enhanced immunosuppression.

The mechanisms through which $CD101^+$ myeloid cells mediate immunosuppression are unclear. Bouloc *et al.* (2000) noted that triggering CD101 on human cutaneous DCs inhibited T-cell proliferation thorough IL-10 production [83]. Moreover, recent evidence intimates that human macrophages derived from peripheral blood monocytes as well as lamina propria murine macrophages are important in the maintenance of the regulatory activity of Tregs, also through IL-10 [117, 118]. Given the appearance of $CD101^+$ AMs following birth and $CD101^+$ Tregs shortly thereafter, as well as the subsequent decline of both cell types into adulthood, it is reasonable to consider the possibility that $CD101^+$ AMs contribute, at least partly, to the pervasive immunosuppressive environment in the lung through maintenance of the Treg compartment. Consequently, in future experiments we plan to investigate the role of $CD101^+$ alveolar macrophages on allergen responsiveness through clodronate depletion, as well as study the impact of depletion on the content and/or immunosuppressive activity of Tregs. In addition, we will investigate classical immunomodulating mechanisms exerted by AMs, specifically the production of nitric oxide (NO), TGF-β, IL-10 and prostaglandin E2 (PGE2) in *in vitro* cultures of AMs or whole lung cell suspensions [119]. The latter two mediators, IL-10 and PGE2, have recently been shown to be upregulated by CD101 ligation of LPS-pulsed monocytes from human peripheral blood [82].

4.9 The Role of T-regulatory Cells and Alveolar Macrophages in Tolerance Induction

Taken together, our data show that remarkable allergen hyporesponsiveness in early life temporally coincides with a robust immunosuppressive environment in the lung that is mediated by Tregs and, possibly AMs. The significance of this proposition is that a large degree of immunological programming may take place during this interval, including the development of tolerance [27, 31]. In fact, we hypothesize that regulatory T-cells and AMs cooperate in a way to facilitate the imprinting of HDM tolerance.

The rapid accumulation of AMs immediately following birth brings up potential implications with respect to tolerance induction. FITC autofluorescence, low CD11b expression and lack of Ly6C (>90% are $Ly6C^-$) suggests that these cells are in fact AMs, not monocytes, though expression levels of another classical monocytic marker, CD115, remain to be elucidated [78, 120]. This, however, is not enough to confirm that they are not recruited from the periphery as it is entirely possible that they rapidly downregulate expression of Ly6C or are derived from $Ly6C^{low}$ monocytic precursors [79, 121]. Further analysis of chemokine markers, particularly the CCL2-CCR2 axis through which monocytes replenish AMs during inflammatory responses, as well as CX3CR1, which is highly expressed on $Ly6C^{low}$ monocytes, will allow for a better appraisal as to whether these AMs are recruited from the periphery or the result of differentiation of local precursor cells [122, 123]. Holt and colleagues have demonstrated that AMs recently recruited to the adult lung in the context of carbon-induced inflammation have superior antigen-presenting capacity insinuating a window of T-cell responsiveness immediately following recruitment [113, 124]. It is unlikely that neonatal AMs, if they are in fact recruited from the periphery, have a similar ability given their low expression of MHC II

and high expression of CD101. Nevertheless, it is important to confirm this with an evaluation of their antigen-presenting ability with a FITC-dextran test.

Overall, to study the potential tolerance 'window' in infancy it is imperative to determine the ideal immunological requirements to induce tolerance. We hypothesize that the two main variables are (I) timing of exposure and (II) allergen dose. With respect to timing, it is important to define the most powerful immunosuppressive environment in terms of Tregs and $CD101^+$ AMs among other suppressor cell types. Secondly, it is important to carefully consider the dose administered; over-exposure to HDM may induce a response, i.e. data seen by administering 125 μg HDM over 10 days, while under-exposure may promote ignorance as opposed to tolerance. To reconcile this problem, we speculate that sufficient HDM is required to initiate antigen presentation and subsequent generation of an adaptive response, so as to promote the expansion of Tregs, without actually inducing responsiveness. In this regard, Ostroukhova *et al.* (2004) have described that the expansion of $Foxp3^+TGF\text{-}\beta^+$ Tregs is important for the induction and maintenance of tolerance in adult mice, which was induced by repeated exposures of a low dose of aerosolized ovalbumin [71]. This *low* dose for tolerance induction has proven difficult to ascertain for HDM, but perhaps it can be identified in the context of the immunosuppressive environment in the infant setting. Thus, a more comprehensive dose-response relationship much be performed in infant mice using a spectrum of doses, where the expansion of Tregs is closely examined in order to better delineate the line between ignorance and potential tolerance induction. In this regard, we also plan to describe immune activity in the thoracic lymph nodes, which our laboratory and collaborators have

previously defined as the exclusive site of tolerance induction following OVA inhalation *in vivo* [125, 126]. From there, we can test whether tolerance is induced with subsequent exposure to a range of HDM doses once mice reach adulthood.

4.10 Summary

The following are conclusions drawn from this thesis in reference to our initial *Specific Objectives (1.5.2)* in Chapter 1 – Introduction:

I. There is a progressive development of immune-inflammatory responses to HDM as exposures, 25 μg of HDM for 10 consecutive days, are initiated later in development, as evidenced by total cell number and eosinophilia in the BAL and serum HDM-specific IgG_1 levels.

II. *NK Cells:* There is a progressive emergence of mature $CD3^-CD49b^+CD11b^+$ NK cells in the lung throughout development. The proportion of NK cells remains the same following HDM exposure, though there is a higher absolute level only in the HDM-exposed adults.

Myeloid Dendritic Cells: There is a small, resident population throughout development. Following HDM exposure, a subset of mDCs ($CD11c^{+/hi}CD11b^{hi}MHCII^{hi}$) expands in adult, but not infant, mice. The $CD11c^{hi}$ subset is thought to be a rich source of pro-inflammatory chemokines.

Alveolar Macrophages: There is a rapid accumulation of alveolar macrophages within the first 2 to 3 days of life that is maintained over the course of natural development. They

show differential expression of CD101 and MHC II over the first two weeks of life. Specifically, CD101 expression is greatest shortly after birth and progressively diminishes reaching steady state levels at two weeks. In contrast, MHCII expression follows a reverse trend; indeed, sparse MHCII expression was detected within the first 2 days after birth, was evident by week 1 and reached adult steady state levels by week 2. Moreover, HDM exposure does not elicit CD101 expression on alveolar macrophages regardless of age, though an increase in MHC II expression is only observed following HDM exposure in adult mice.

T-regulatory Cells: The number of $CD3^+CD4^+$ T cells is very low in the newborn, increases substantially at week 1 and remains constant until adulthood. The proportion $CD3^+CD4^+FoxP3^+$ T regulatory cells relative to total $CD4^+$ T-cells follows the same pattern. However, the expression level of FoxP3 on a per cell basis is significantly higher within the first 1-2 weeks of life. Additionally, CD101 expression on T regs is also greater during this period. Interestingly, this Treg population expands following HDM exposure in both adult and infant mice.

III. Infant mice depleted of Tregs, via α-CD25 treatment, mount a robust Th2-skewed response to HDM, comparable to that observed in adult mice, demonstrating a critical role of Tregs in mediating the hyporesponsiveness of infant mice to HDM.

In general, our studies have focused on understanding the mechanisms of allergen responsiveness in the respiratory tract during infancy, which is particularly important to

understanding the *origins* of allergic asthma since infancy is a period during which developmental processes play a key role in influencing the programming of the immune system to allergens [20]. This is especially relevant considering the wealth of epidemiological studies which purport that allergic sensitization predominantly occurs in the first few years of life [26, 27, 33]. We have supported this assertion by identifying a progressive acquisition in responsiveness to HDM as a BALB/c mouse emerges from the infancy period of murine development.

Furthermore, our data suggest that the hyporesponsiveness to HDM early in life may be explained by two connected events: a) the inherent immunosuppressive environment in the lung, i.e. regulatory T-cells and immunosuppressive alveolar macrophages and b) the immaturity of the machinery for effective immune responses, i.e. natural killer cells, dendritic cells and alveolar macrophages. This suggestion was brought on by investigations into specific components of the immune response from birth to adulthood with potential implications in allergen responsiveness and allergic sensitization. These studies have provided us with justification for the future study of the roles of previously unidentified cells in the context of allergy, such $CD101^+$ regulatory T-cells and $CD101^+$ alveolar macrophages, as well as the roles of cells that to date have not been extensively studied in models of allergic airway disease, i.e. NK cells.

Moreover, we have demonstrated that a disruption in the homeostatic immune balance in infancy, Treg depletion in our case, may lead to the imprinting of aberrant immune-inflammatory responses, like allergen sensitization. This is in line with epidemiological evidence that suggests Treg deficiency in infants is indicative of future

sensitization [64, 65]. Furthermore, our demonstration of the importance of the immunosuppressive environment during infancy in regulating HDM responsiveness has given us a platform from which to base future experimentation with respect to the induction of tolerance to HDM extract in a murine model, thus far not described in the literature. This is particularly enticing given that we will embark on experiments investigating tolerance induction in infant mice, a more physiologically relevant model compared with most of the evidence in the literature that studies tolerance to ovalbumin in adult murine models [71, 126].

Overall, from these studies, we have made advances in uncovering the conditions under which one becomes allergic and laid a foundation for future experimentation in this regard.

CHAPTER 5
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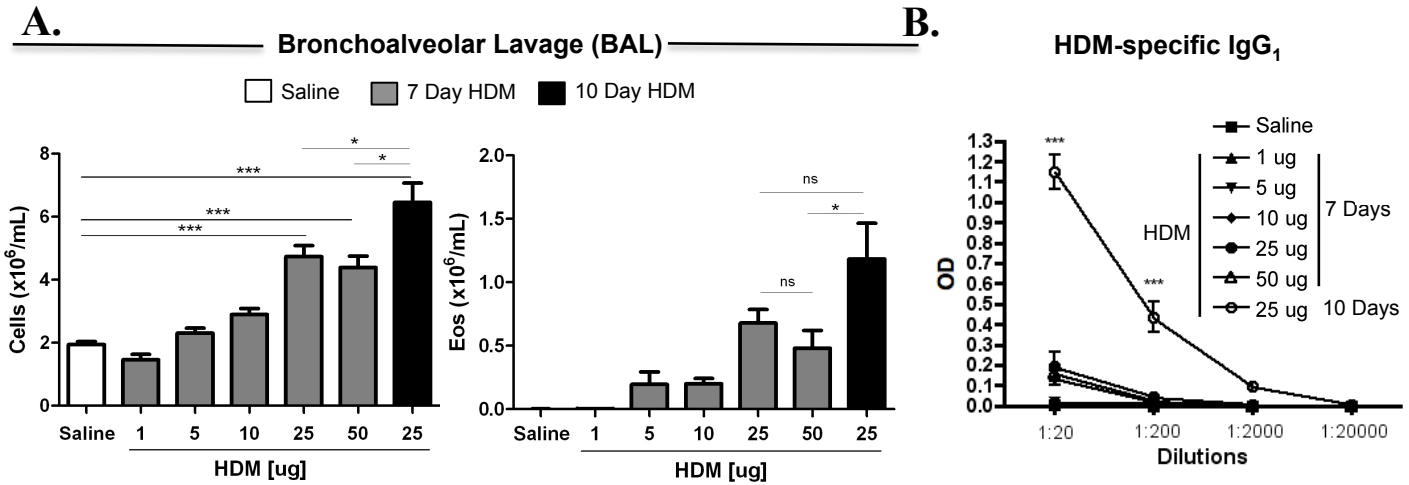
CHAPTER 6
FIGURES



C.

	25 ug HDM MHC II + expression	
APC	7 Day	10 Day
Alveolar Macrophages–$CD11c^{hi}CD11b^{-}F4/80^{+}$	✔ ✔	✔✔✔ ✔
B-Cells–$CD11c^{+}CD11b^{-}F4/80^{-}B220^{+}$		✔✔✔ ✔✔✔
DCs–$CD11c^{hi}CD11b^{hi}F4/80^{+}$	 ✔	✔ ✔✔
DCs–$CD11c^{+}CD11b^{-}F4/80^{-}B220^{-}$	✔✔ ✔	✔ ✔
DCs–$CD11c^{+}CD11b^{+}F4/80^{-}B220^{-}$	✔ ✔	✔✔ ✔✔
DCs–$CD11c^{+}CD11b^{hi}F4/80^{-}B220^{-}$	✔✔ ✔✔	✔
Lymphocyte		
Activated T-cells – $CD3^{+}CD4^{+}CD69^{+}$	✔	✔✔✔
Th2 cells – $CD3^{+}CD4^{+}T1ST2^{+}$		✔✔✔
Activated Th2 cells – $CD3^{+}CD4^{+}T1ST2^{+}CD69^{+}$		✔✔

Figure 1 – **Immune-inflammatory responses of adult mice following exposure to 1, 5, 10, 25 or 50 µg of HDM over 7 days.** Adult Balb/c mice were exposed to saline or 1, 5, 10, 25 or 50 µg HDM intranasally for 7 consecutive days and to 25 µg HDM for 10 consecutive days as a positive control. Mice were sacrificed ~24hrs after the last HDM exposure at which time serum and BAL fluid was collected. (A) Cellular profile in the BAL showing the number of total cells and eosinophils. (B) Serum levels of HDM-specific IgG_1. (C) Flow cytometric analysis of lung APC and lymphocyte populations; ✔denotes statistical significance over saline-exposed mice - ✔ $p < 0.05$ ✔✔ $p < 0.01$ ✔✔✔ $p < 0.001$. Data are representative of one of two independent experiments. n = 4-8/ group. TCN = Total Cell Number

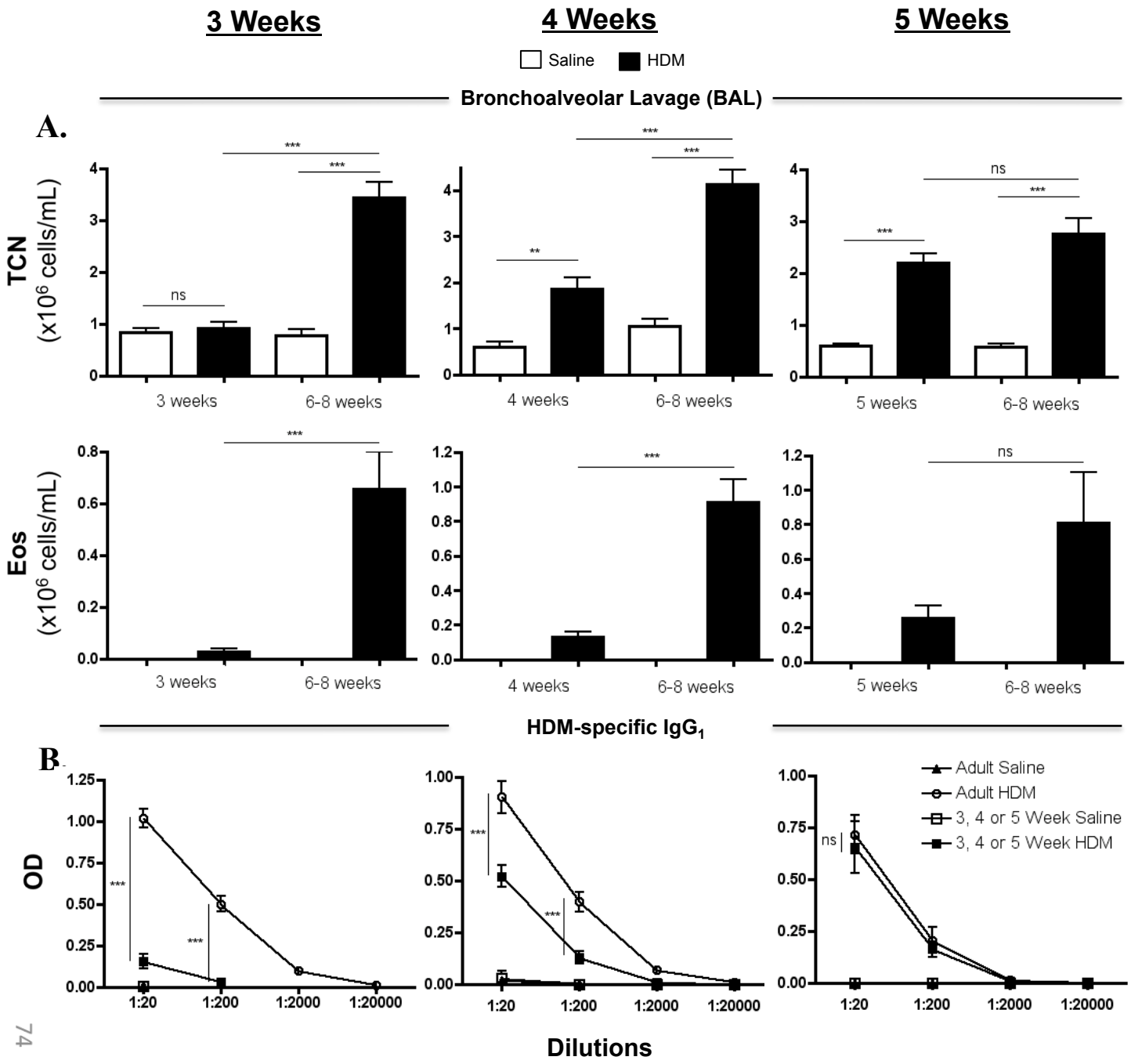
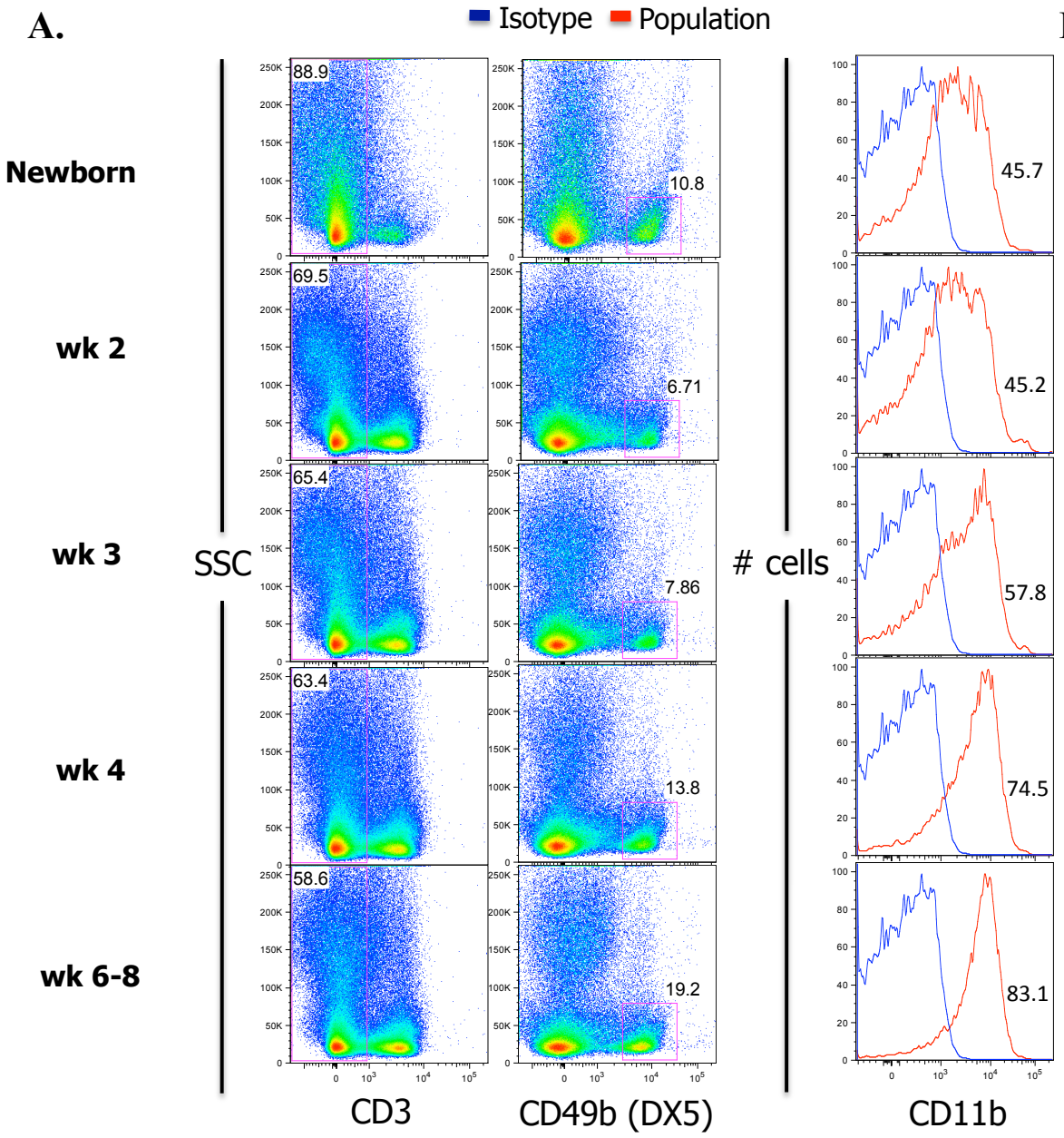


Figure 2 – **Immune-inflammatory responses of 3, 4, 5 and 6-8 week-old mice following HDM exposure.** Balb/c mice of different ages (3, 4, 5, 6-8 weeks-old) were exposed to saline or 25 µg HDM intranasally for 10 consecutive days and sacrificed ~24hrs after the last HDM exposure at which time serum and BAL fluid was collected. (A) Cellular profile in the BAL showing the number of total cells and eosinophils. (B) Serum levels of HDM-specific IgG_1. Data are representative of one of three independent experiments. n = 4-6/group. TCN = Total Cell Number



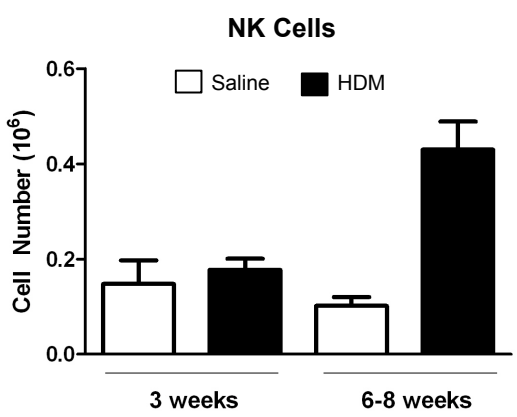
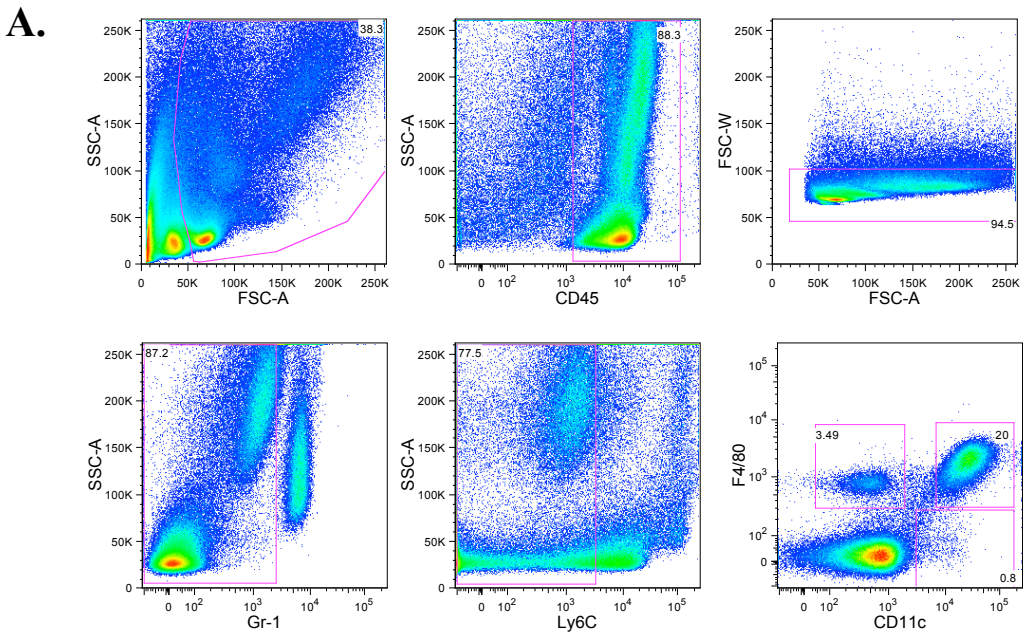
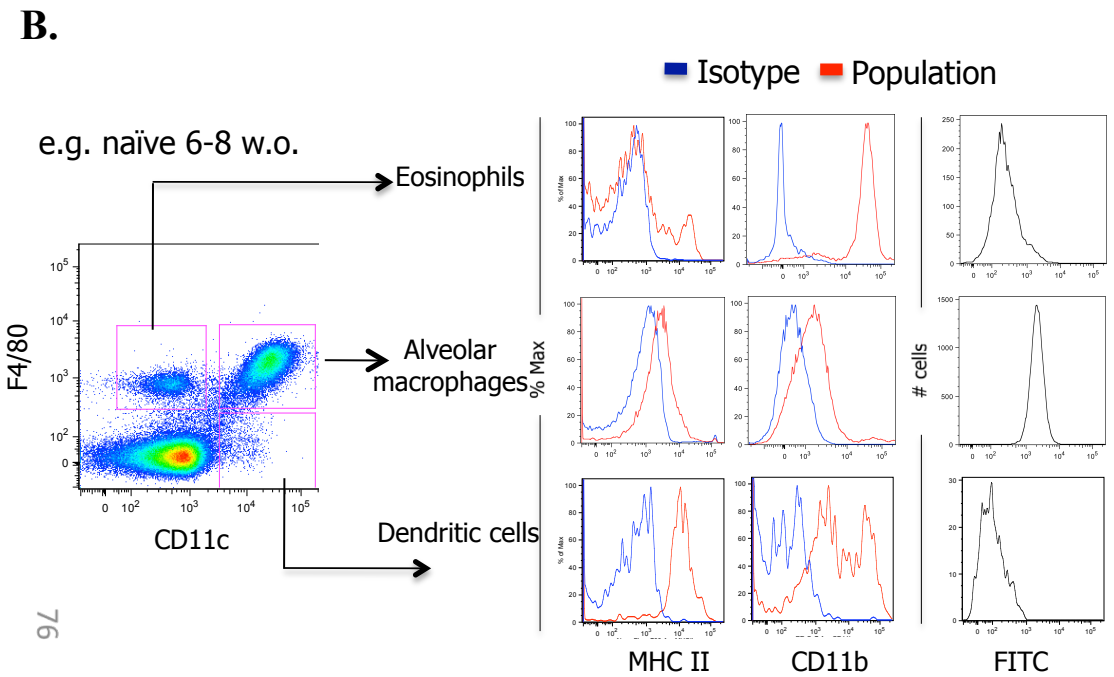


Figure 3 – **Maturation of natural killer (NK) cells over the course of development.** (A) Naïve Balb/c mice were sacrificed at the indicated points of development at which time lung tissue cells were isolated, stained with a combination of APC-Cy7-conjugated anti-CD45, PECy7-conjugated anti-CD11b, biotinylated CD49b (DX5), Pac Orange-conjugated anti-Gr1, AF700-conjugated anti-I-Ad, and PE-conjugated anti-CD101, followed by Qdot800-streptavidin. (A) First column shows FACS dot plots of a representative profile of CD3 after gating on live single CD45+ cells. Second column shows FACS dot plots of a representative profile of CD49b (DX5) after gating on $CD3^{-}Gr1^{-}$ cells. Third column includes histograms showing expression of CD11b on $CD3^{-}CD49b^{+}$ cells. Positive staining (red lines) relative to staining with the isotype control antibody (blue lines). Each time point analyzed includes pooled lung tissue cells from 3 to 4 littermates. (B) NK cell ($CD3^{-}CD49b^{+}$) absolute values from single cell lung suspension. Data are representative of one of 3 independent experiments.





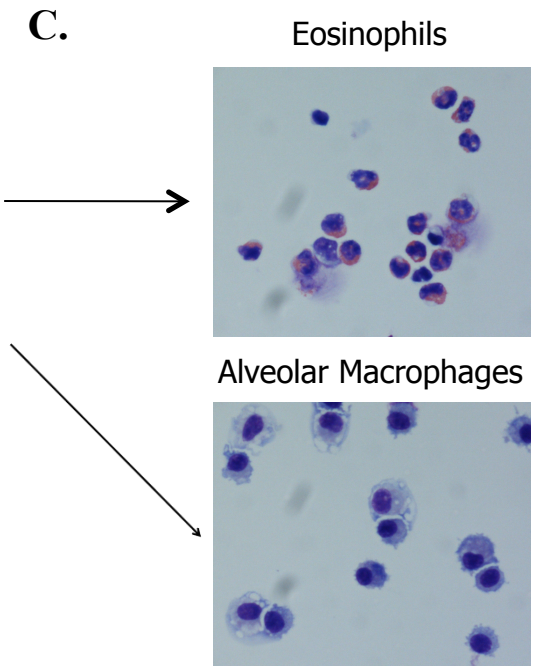


Figure 4 – **Phenotypic characterization of lung antigen-presenting cells (APCs) (A).** Gating Strategy for lung APCs. Lung tissue cells from a Balb/c mouse (6-8 w.o.) were isolated and stained with a combination of APC-Cy7-conjugated anti-CD45, APC-conjugated anti-CD11c, eF450-conjugated anti-F4/80, PECy7-conjugated anti-CD11b, AF700-conjugated anti-I-Ad, PerCpCy5.5-conjugated anti-Ly6C and Pac Orange-conjugated anti-Gr1 and analyzed by flow cytometry. **(B).** FACS dot plot shows a representative profile of CD11c versus F4/80 staining after gating on live CD45+ single Ly6C-Gr1-cells in a naïve mouse. Each of the APC subsets was further analyzed for the expression of MHC II, CD11b and FITC auto-fluorescence. Histogram plots show positive staining (red lines) relative to staining with the isotype control antibody (blue lines). **(C).** Live CD45+ single Ly6C-Gr1-F4/80+CD11c+ (alveolar macrophages) or CD11c-(eosinophils) were FACS sorted and cytospun for morphological analysis.

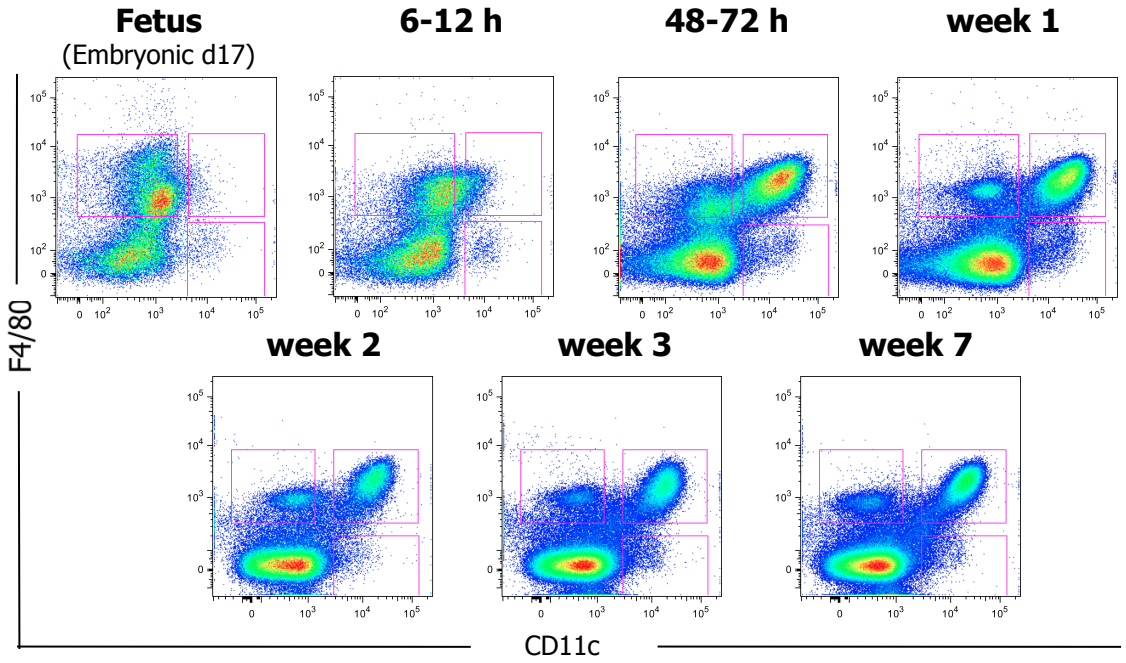


Figure 5 – **Lung APCs over the course of development.** Naïve Balb/c mice were sacrificed at the indicated points of development at which time lung tissue cells were isolated and prepared for flow cytometric analysis as described in Figure 4. FACS dot plots show a representative profile of CD11c versus F4/80 staining after gating on live CD45+ single cells cells at different points in development. Each time point analyzed included pooled lung tissue cells from 3 to 4 littermates. Data are representative of one of 3 independent experiments.

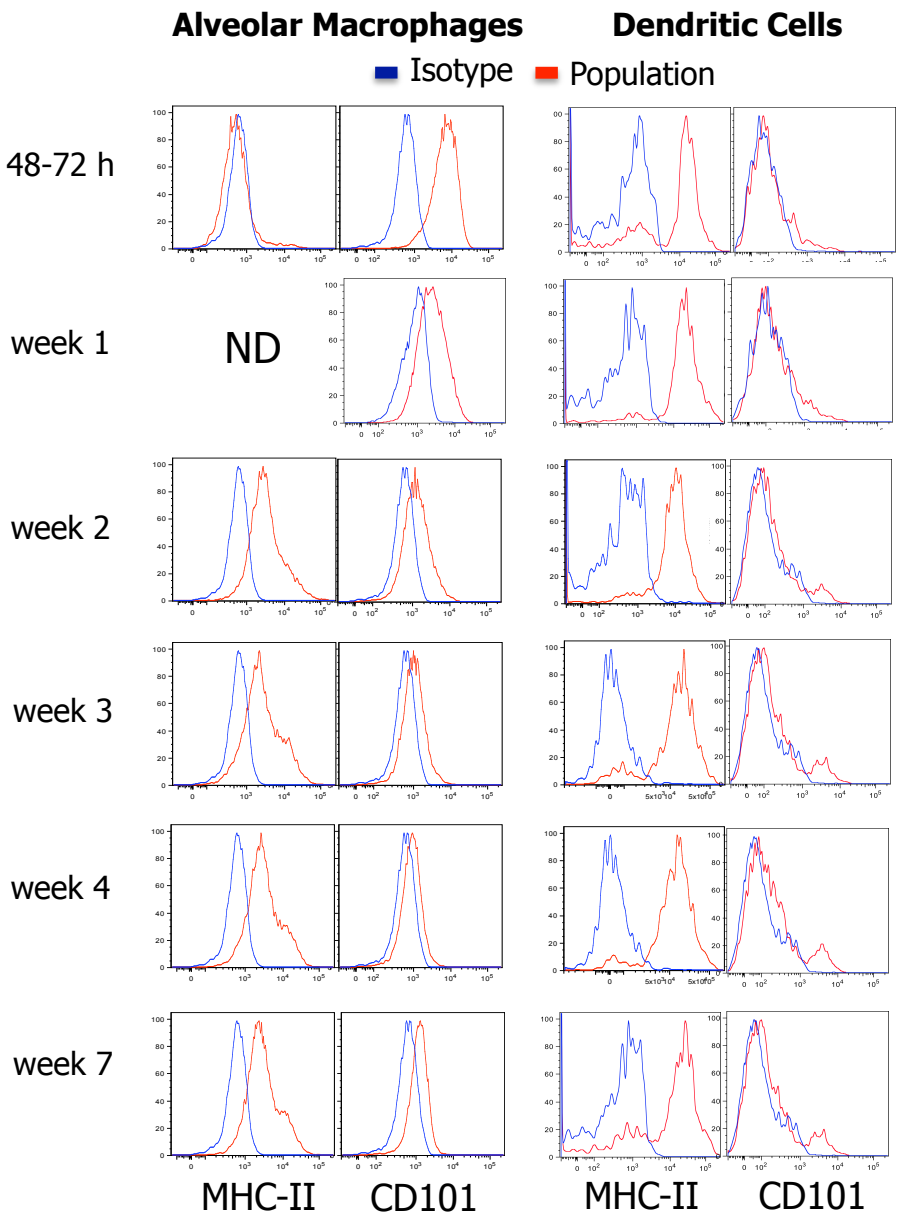


Figure 6 – **Expression of MHCII and CD101 on lung APCs over the course of development.** Naïve Balb/c mice were sacrificed at the indicated points of development at which time lung tissue cells were isolated, stained with a combination of APC-Cy7-conjugated anti-CD45, APC-conjugated anti-CD11c, eF450-conjugated anti-F4/80, PECy7-conjugated anti-CD11b, AF700-conjugated anti-I-Ad, PE-conjugated anti-CD101, PerCpCy5.5-conjugated anti-Ly6C and Pac Orange-conjugated anti-Gr1 and analyzed by flow cytometry. Each of the APC subsets indicated in Figure 4 was further analyzed for the expression of MHC II and CD101. Histogram plots show positive staining (red lines) relative to staining with the isotype control antibody (blue lines). Each time point analyzed included pooled lung tissue cells from 3 to 4 littermates. Data are representative of one of 3 independent experiments.
ND= No data

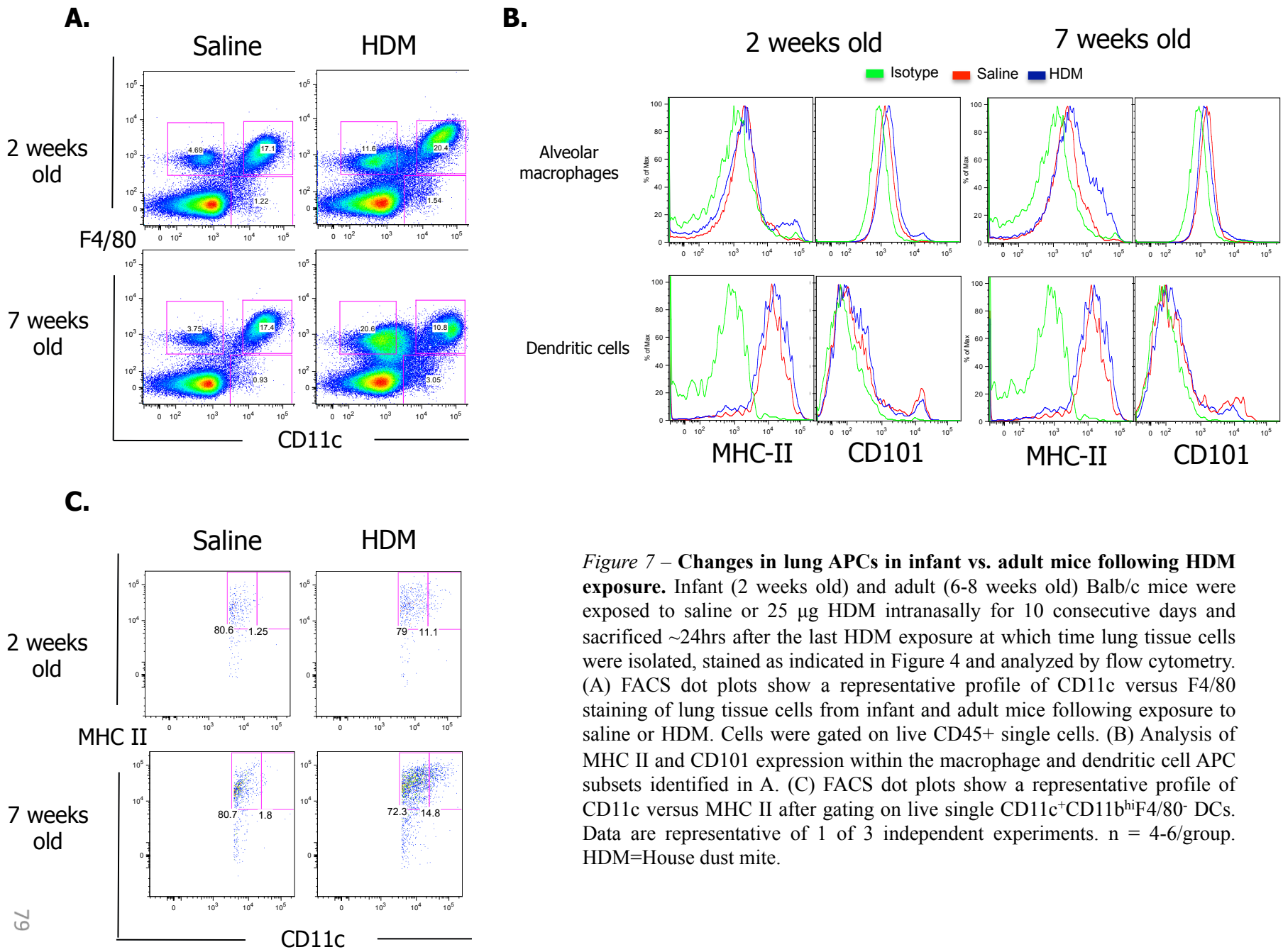
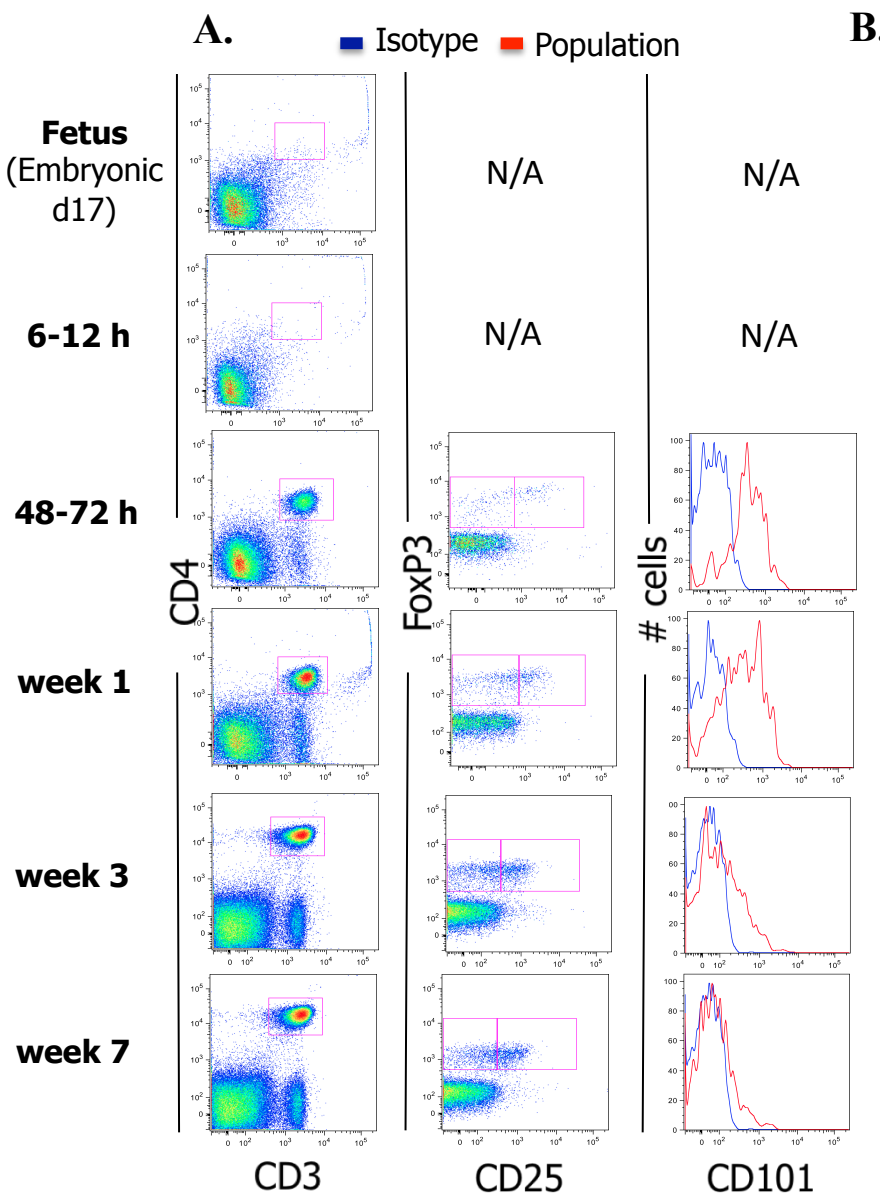


Figure 7 – **Changes in lung APCs in infant vs. adult mice following HDM exposure.** Infant (2 weeks old) and adult (6-8 weeks old) Balb/c mice were exposed to saline or 25 µg HDM intranasally for 10 consecutive days and sacrificed ~24hrs after the last HDM exposure at which time lung tissue cells were isolated, stained as indicated in Figure 4 and analyzed by flow cytometry. (A) FACS dot plots show a representative profile of CD11c versus F4/80 staining of lung tissue cells from infant and adult mice following exposure to saline or HDM. Cells were gated on live CD45+ single cells. (B) Analysis of MHC II and CD101 expression within the macrophage and dendritic cell APC subsets identified in A. (C) FACS dot plots show a representative profile of CD11c versus MHC II after gating on live single $CD11c^{+}CD11b^{hi}F4/80^{-}$ DCs. Data are representative of 1 of 3 independent experiments. n = 4-6/group. HDM=House dust mite.



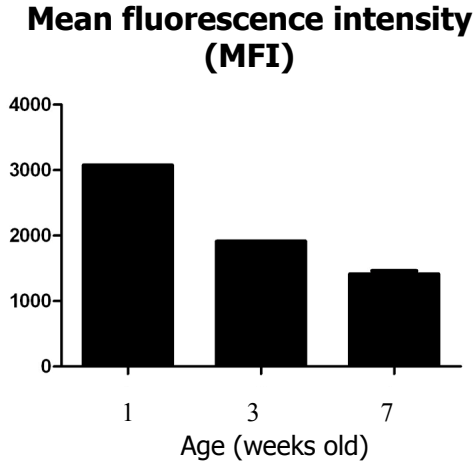


Figure 8 – **T regulatory cells ($CD4^+CD25^+FoxP3^+CD101^+$) in the lung over the course of development. (A).** Naïve Balb/c mice were sacrificed at the indicated points in development at which time lung tissue cells were isolated and stained with a combination of APC-Cy7-conjugated anti-CD45, AF-conjugated anti-CD3, APC-conjugated anti-CD4, biotinylated CD25 and PE-conjugated CD101, followed by Qdot800-streptavidin. FoxP3 protein expression was examined by ICS performed according to the manufacturer's protocol (eBioscience). (A) First column shows FACS dot plots of a representative profile of CD3 versus CD4 staining after gating on live single cells. Second column shows FACS dot plots of a representative profile of CD25 versus FoxP3 staining after gating on $CD3^+CD4^+$ cells. Third column includes histograms showing expression of CD101 on $CD3^+CD4^+CD25^+FoxP3^+$ cells. Positive staining (red lines) relative to staining with the isotype control antibody (blue lines). Each time point analyzed includes pooled lung tissue cells from 3 to 4 littermates **(B).** Mean Fluorescence Intensity of FoxP3 on $CD4^+CD25^+Foxp3^+$ at the indicated points of development. Naïve Balb/c mice were sacrificed at week 1, 3 and 7 of life at which time lungs were prepared for flow cytometric analysis as indicated above. Each time point analyzed included pooled lung tissue cells from 3 to 4 littermates. Data are representative of 1 of 3 independent experiments. N/A= Not analyzed.

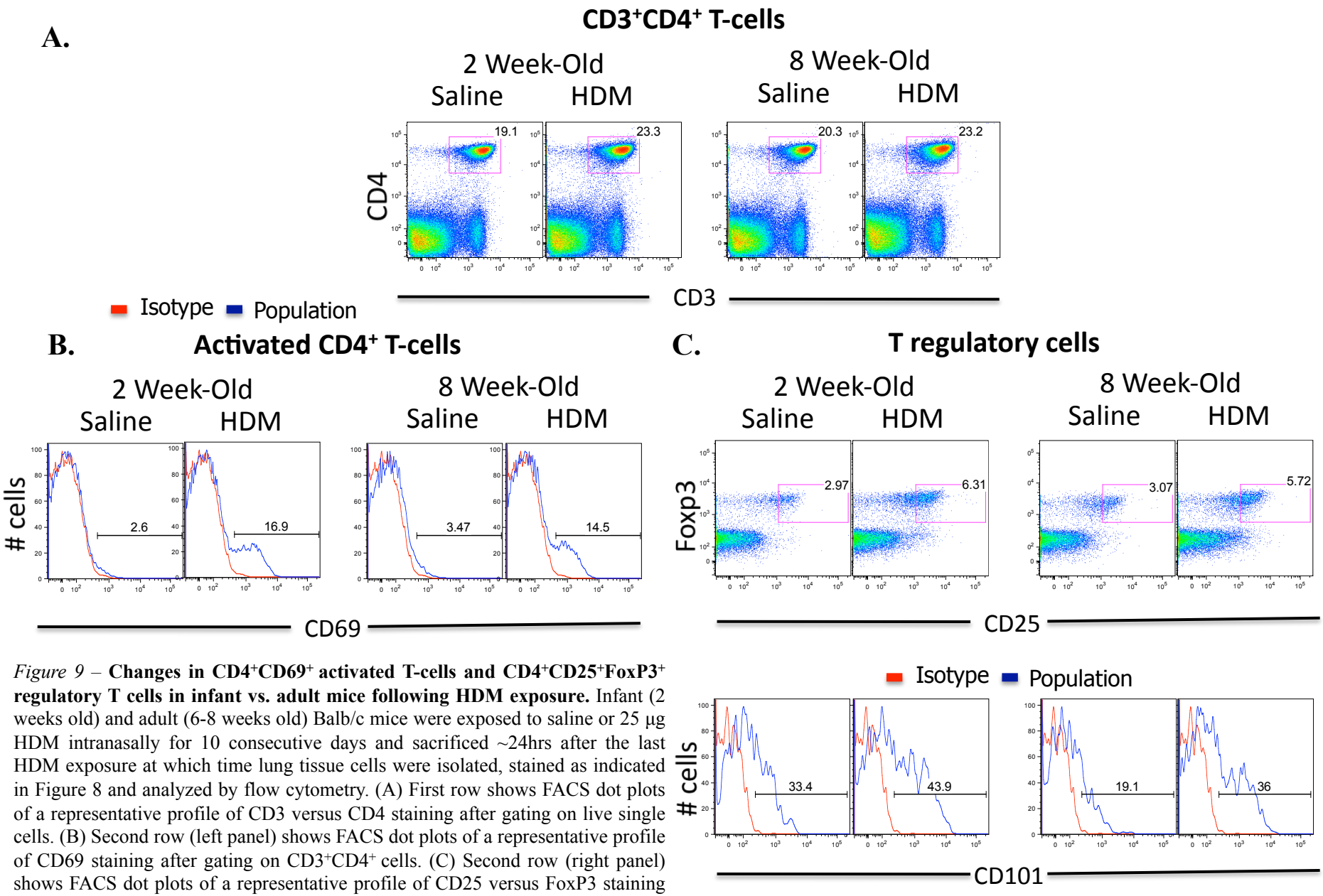


Figure 9 – **Changes in CD4⁺CD69⁺ activated T-cells and CD4⁺CD25⁺FoxP3⁺ regulatory T cells in infant vs. adult mice following HDM exposure.** Infant (2 weeks old) and adult (6-8 weeks old) Balb/c mice were exposed to saline or 25 µg HDM intranasally for 10 consecutive days and sacrificed ~24hrs after the last HDM exposure at which time lung tissue cells were isolated, stained as indicated in Figure 8 and analyzed by flow cytometry. (A) First row shows FACS dot plots of a representative profile of CD3 versus CD4 staining after gating on live single cells. (B) Second row (left panel) shows FACS dot plots of a representative profile of CD69 staining after gating on CD3⁺CD4⁺ cells. (C) Second row (right panel) shows FACS dot plots of a representative profile of CD25 versus FoxP3 staining after gating on CD3⁺CD4⁺ cells. Third row includes histograms showing expression of CD101 on CD3⁺CD4⁺FoxP3⁺ cells. Positive staining (blue lines) relative to staining with the isotype control antibody (red lines). Data are representative of 1 of 3 independent experiments. n = 4-6/group.

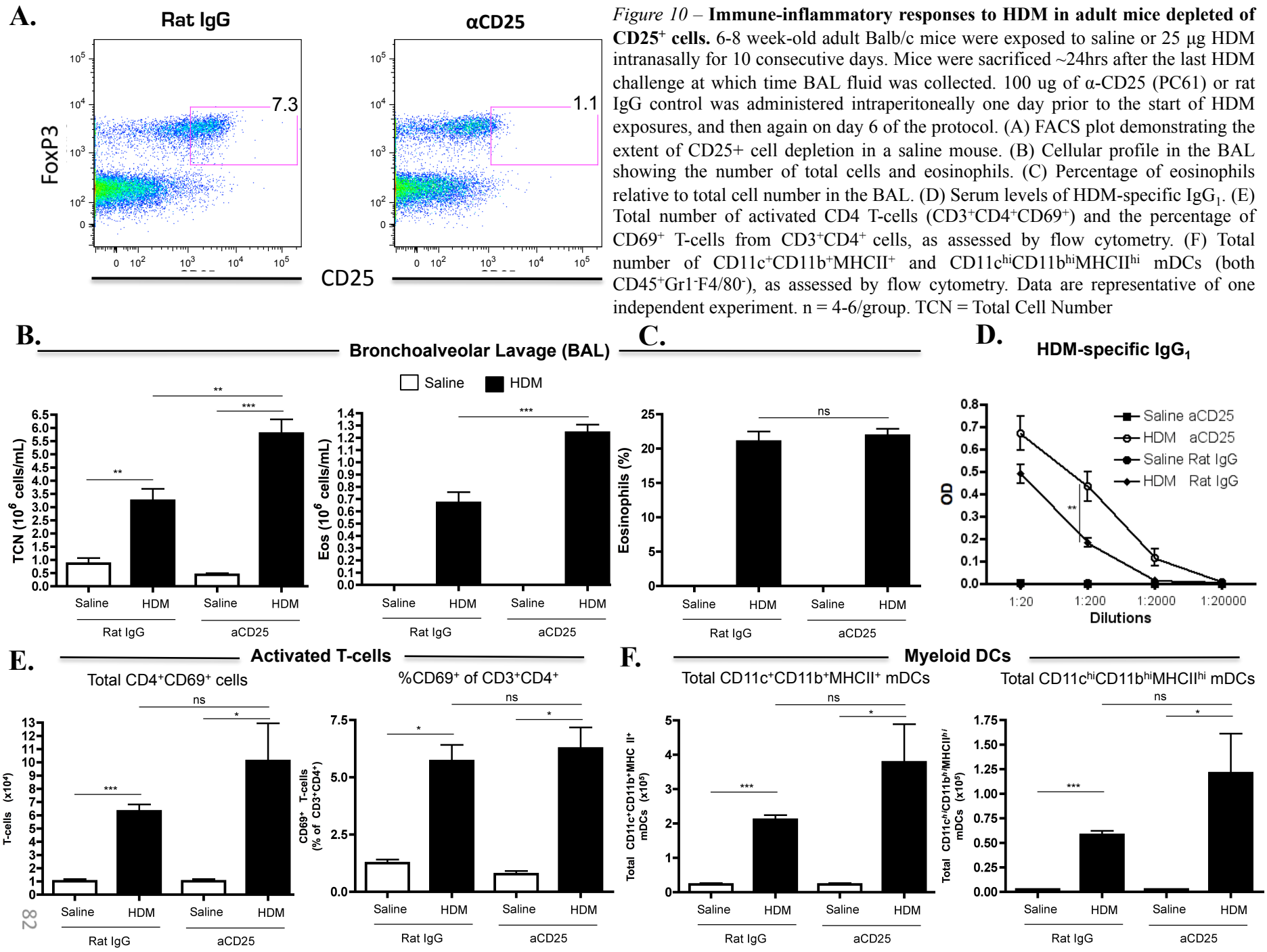


Figure 10 – **Immune-inflammatory responses to HDM in adult mice depleted of CD25$^+$ cells.** 6-8 week-old adult Balb/c mice were exposed to saline or 25 µg HDM intranasally for 10 consecutive days. Mice were sacrificed ~24hrs after the last HDM challenge at which time BAL fluid was collected. 100 ug of α-CD25 (PC61) or rat IgG control was administered intraperitoneally one day prior to the start of HDM exposures, and then again on day 6 of the protocol. (A) FACS plot demonstrating the extent of CD25+ cell depletion in a saline mouse. (B) Cellular profile in the BAL showing the number of total cells and eosinophils. (C) Percentage of eosinophils relative to total cell number in the BAL. (D) Serum levels of HDM-specific IgG_1. (E) Total number of activated CD4 T-cells ($CD3^+CD4^+CD69^+$) and the percentage of $CD69^+$ T-cells from $CD3^+CD4^+$ cells, as assessed by flow cytometry. (F) Total number of $CD11c^+CD11b^+MHCII^+$ and $CD11c^{hi}CD11b^{hi}MHCII^{hi}$ mDCs (both $CD45^+Gr1^-F4/80^-$), as assessed by flow cytometry. Data are representative of one independent experiment. n = 4-6/group. TCN = Total Cell Number

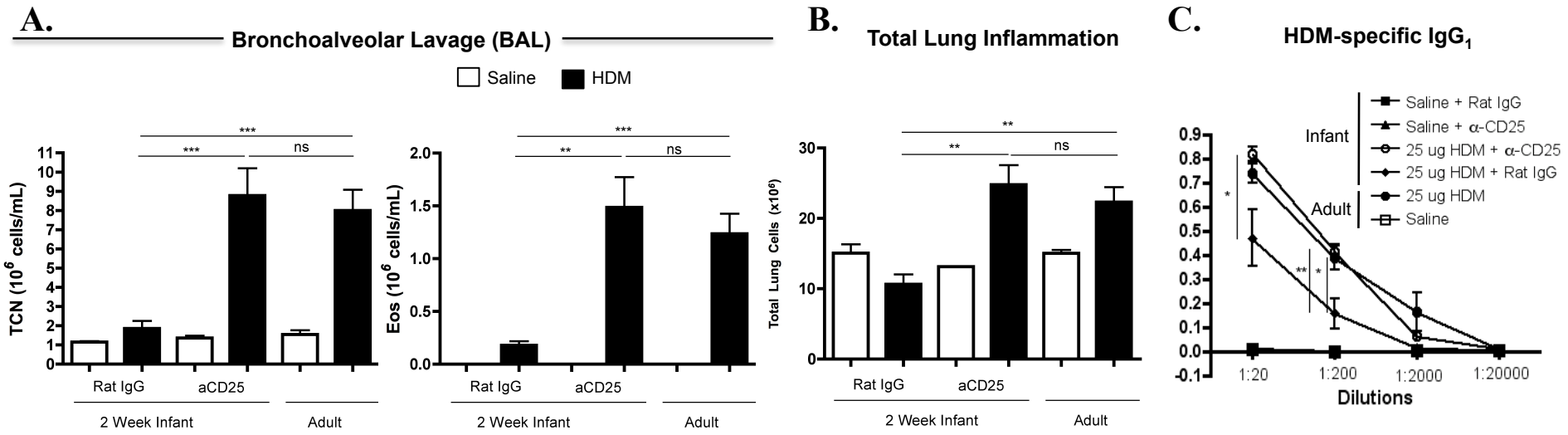


Figure 11 – **Immune-inflammatory responses to HDM in infant mice depleted of CD25$^+$ cells.** 2 week-old infant mice were exposed to saline or 25 µg HDM intranasally for 10 consecutive days. Mice were sacrificed ~24hrs after the last HDM challenge at which time BAL fluid was collected. 100 ug of α-CD25 (PC61) or rat IgG control was administered intraperitoneally one day prior to the start of HDM exposures, and then again on day 6 of the protocol. Untreated 6-8 week-old adult mice were used as saline and HDM controls. (A) Cellular profile in the BAL showing the number of total cells and eosinophils. (B) Total lung cell counts following single-cell isolation (C) Serum levels of HDM-specific IgG_1. Data are representative of one independent experiment. n = 4-6/group. TCN = Total Cell Number

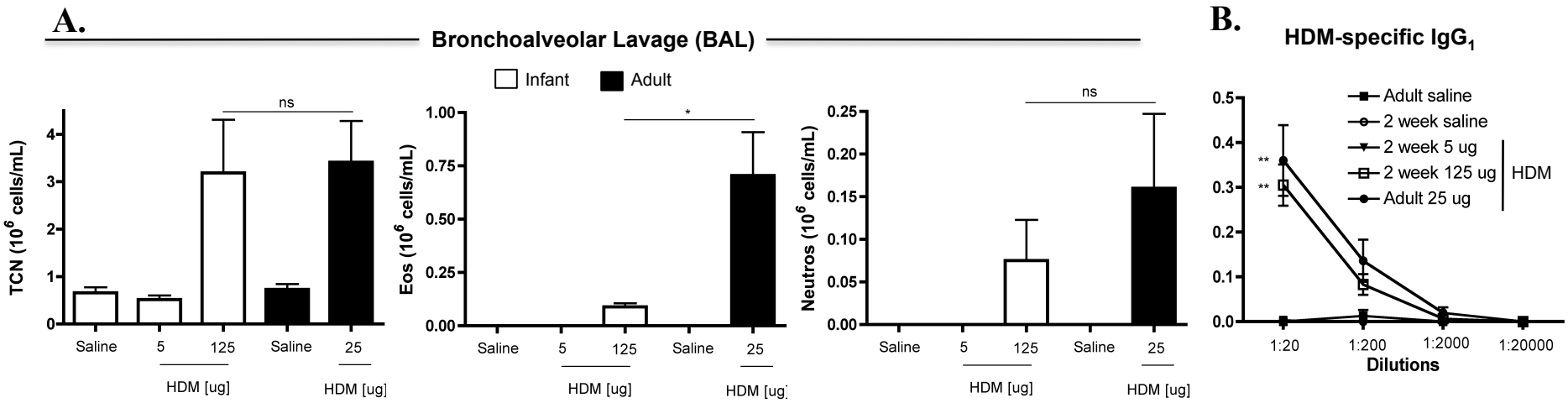


Figure 12 – **Immune-inflammatory responses of infant mice following exposure to 5 or 125 µg of HDM.** 2 week-old infant mice were exposed to saline, 5 or 125 µg HDM intranasally for 10 consecutive days. Adult mice exposed to 25 ug HDM over 10 days served as the positive adult control. Mice were sacrificed ~24hrs after the last HDM challenge at which time BAL fluid was collected. (A) Cellular profile in the BAL showing the number of total cells, eosinophils and neutrophils. (B) Serum levels of HDM-specific IgG_1. Data are representative of one independent experiment. n = 4-6/ group. TCN = Total Cell Number