THE UNIVERSITY OF CHICAGO

HELP FROM AN "OLD FRIEND": *BACILLUS SUBTILIS* EXOPOLYSACCHARIDE INTERFERES WITH TH2 PRIMING AND INHIBITS ALLERGIC INFLAMMATION

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This dissertation is dedicated to the memory of my grandfather, Henry James (Kimo) Hollinger (August 10, 1939 – October 31, 2023).

Table of Contents

List of Figures

List of Tables

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Abstract

Allergic asthma is an increasing burden in industrialized countries, where treatments are limited to treating symptoms rather than root causes. In recent years, the use of inhaled probiotic cocktails has shown promise in preventing allergic asthma. However, the mechanisms by which such probiotics prevent sensitization remain to be elucidated. In this study, we focused on exopolysaccharide (EPS) from *B. subtilis*, a ubiquitous soil bacterium. We found that intratracheal EPS treatment prior to sensitization with house dust mite (HDM) limited lung mucus hypersecretion and eosinophilia. Furthermore, EPS treatment inhibited accumulation of T_H2, but not T_H1 or T_H17 subsets. To determine whether EPS was affecting T_H2 cell priming by dendritic cells (DCs), we compared the composition and phenotype of DCs in the lung and lung-draining lymph node (LDLN) after EPS and HDM treatment. Surprisingly, pre-treatment with EPS induced DC activation, as measured by an increase in CD86, and a decrease in the ability to uptake HDM and migrate to the LDLN. Additionally, EPS-pretreated BMDCs were unable to induce antigen-specific T cell proliferation *in vitro*. Transcriptionally, EPS downregulated proteins associated with DC induction of T cell responses such as innate cytokines, costimulatory molecules, and chemokines. Together, our data suggest that EPS inhibits allergic sensitization by dysregulating DCs prior to HDM exposure, thereby leading to decreased T_H2 priming and allergic inflammation. Thus, we provide a potential mechanism by which inhalation of a single prebiotic molecule can prevent allergic sensitization.

Chapter 1: Introduction

Allergic asthma

Asthma is a chronic inflammatory condition that affects over 262 million people worldwide(2022). Rather than a single disease state, asthma covers a variety of disease etiologies, also known as endotypes, that result in similar symptoms. The prototypical endotype for asthma is allergic asthma, in which patients become sensitized to environmental antigens and, upon re-exposure, develop excess mucus in the airways and airway smooth muscle hyperreactivity. Upon exposure to allergen, these patients undergo acute exacerbations, more commonly known as "asthma attacks", in which airway constriction and development of airway mucus plugs can result in hypoxia and even death(2020).

Allergic asthma often manifests in young children during the course of the "atopic march", in which infants are diagnosed with atopic dermatitis and then suffer from comorbid food allergy, allergic asthma, and/or allergic rhinitis in later childhood(Hill and Spergel, 2018). Because there is no known cure for asthma, asthmatics diagnosed as children or teenagers must maintain control of their disease for life via corticosteroids or bronchodilators. Thus, there is an urgent need to understand how patients become sensitized to allergen if there is any hope of preventing or even reversing sensitization.

The allergic response to complex allergens such as house dust mite (HDM) begins at epithelial surfaces within the respiratory tract. Alarmins such as IL-33, TLSP, and IL-25 are stored within the endothelium at steady-state; however, in response to barrier damage or epithelial cell

death by allergenic proteases, these alarmins are released into the extracellular space(Gandhi et al., 2013; Lambrecht and Hammad, 2012). In response to IL-33 and IL-25, innate cells such as ILC2s expand and secrete cytokines IL-5 and IL-13(Kim et al., 2016). In turn, IL-5 induces differentiation of eosinophils and recruits eosinophil precursors from the bone marrow, while IL-13 induces smooth muscle hyperplasia, goblet cell metaplasia, and promotes mucus secretion from goblet cells(Lambrecht et al., 2019).

Simultaneously, dendritic cells (DCs) sample the lung epithelium(Thornton et al., 2012; Veres et al., 2011) and intercept antigen that has passed the compromised epithelial barrier(Hammad et al., 2009). At their basal state, DCs are considered immature, as they primarily phagocytose antigen, express low levels of immunostimulatory cytokines, and surveil a relatively small range of the local environment(Veres et al., 2011). In response to pathogen-associated molecular patterns (PAMPs) found in complex allergens, damage-associated molecular patterns (DAMPs) released by allergen protease activity, and epithelium-derived alarmins such as IL-33, these dendritic cells become activated(Schuijs et al., 2019). Activation is characterized by a shift from antigen uptake to antigen presentation on MHC molecules, upregulation of surface costimulatory molecules, enhanced pro-inflammatory cytokine secretion, and upregulation of CCR7, which enables migration of activated DCs to the lung-draining lymph node (LDLN)(Hammad et al., 2010). There, DCs “scan” naïve T cells through peptide-MHC interactions with TCRs. Upon recognition of an antigen-specific TCR, DC-derived costimulatory molecules and cytokines induce T cell proliferation and drive differentiation of the naïve T cell towards a Th2 phenotype. These differentiated Th2 cells then migrate back to the lung, where they secrete type 2 cytokines IL-4, IL-5, and IL-13(Schuijs et al., 2019).

While IL-5 and IL-13 from Th2 cells amplify the response initiated by ILC2s, IL-4

promotes B cell class switching to IgE. Antigen-specific IgE then crosslinks FcεRI on mast cells or basophils to induce degranulation and release of pro-inflammatory products such as histamine and prostaglandins(Platts-Mills, 2001). These products then induce bronchoconstriction, mucus secretion from airway goblet cells, vascular leakage, and further degranulation of basophils and

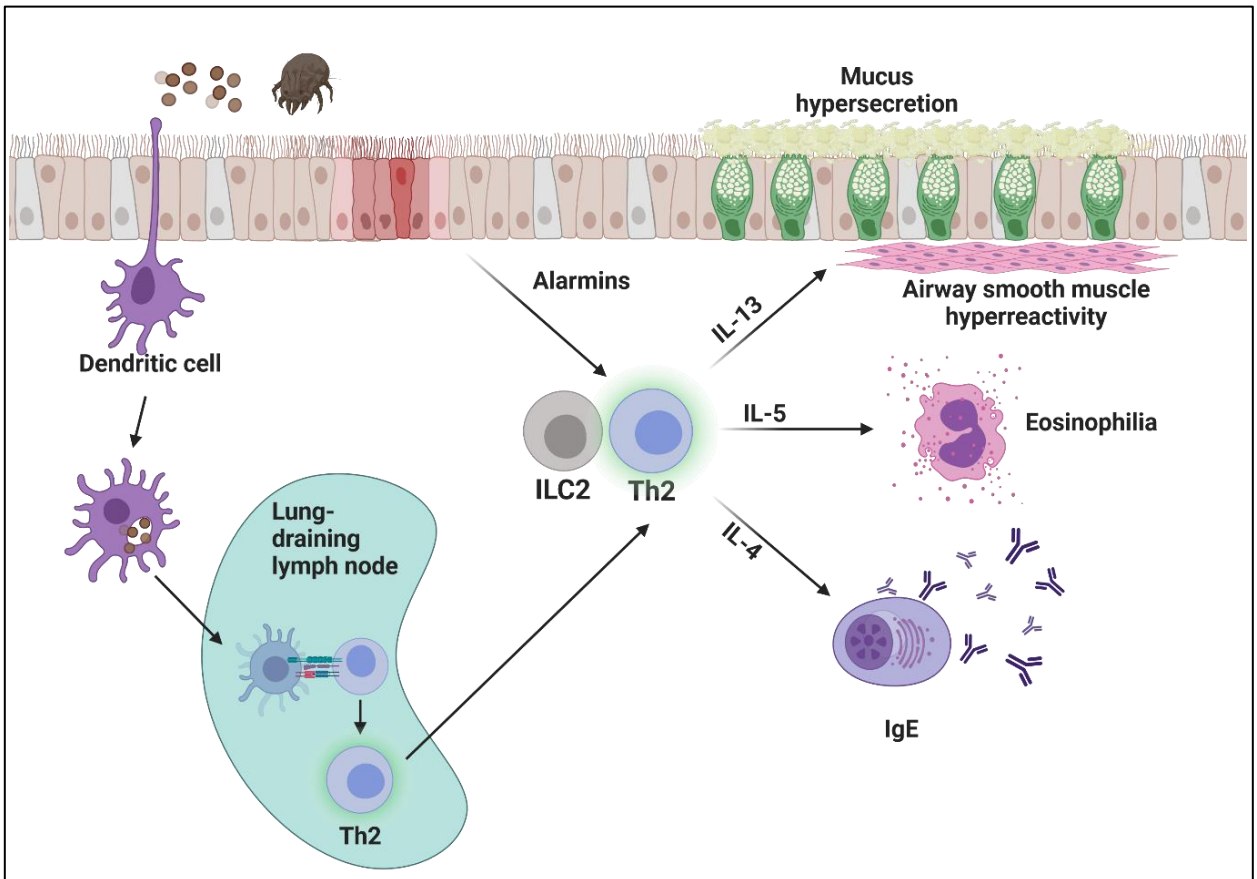

Figure 1-1: A simplified diagram of the cellular mediators of allergic asthma. Image generated in Biorender.

mast cells(Peebles, 2019; Yamauchi and Ogasawara, 2019). The cellular players involved in the pathogenesis of allergic asthma are summarized in Figure 1-1.

Dendritic cells: subsets and functions

As previously discussed, dendritic cells are crucial in translating microbial and damage-associated signals into a productive adaptive immune response. Dendritic cells are necessary for

sensitization to allergens such as house dust mite (HDM), as CD11c-DTR mice depleted of DCs upon receipt of diphtheria toxi, have greatly attenuated BAL eosinophilia and BAL levels of IL-5, IL-13, and IFN-γ. Allergen-pulsed DCs are also sufficient to induce allergic inflammation, as mice challenged with HDM after transfer of HDM-experienced $CD11c^{+}MHCII^{+}$ DCs experienced BAL eosinophilia and BAL secretion of IL-5 and IL-13(Hammad et al., 2010).

Pulmonary DCs comprise several subsets based on hematopoietic lineage, transcription factor usage, and relative contribution to T cell priming (summarized in Figure 1-2). Conventional DCs (cDCs) are derived from pre-DCs that migrate from the bone marrow to the lung (Shin et al., 2020). There, they differentiate into two main cDC subsets: cDC1s and cDC2s. Often characterized using the surface markers CD103 and/or XCR1, cDC1s rely upon the transcription factors BATF3 and IRF8 for their differentiation and are specialized in cross-presenting antigen to CD8 T cells. Thus, they are crucial for control of tumors and viral infection in peripheral tissues, including the lung(Zhang et al., 2023). cDC1s have also been implicated in restraining excess Th2 inflammation without triggering Th1 cell production during chronic HDM exposure through secretion of IL-12 in the mediastinal lymph node (Conejero et al., 2017).

The second group of conventional DCs upregulate the master transcription factor IRF4 and are identified in the lung by surface expression of CD11b and Sirpα(Shin et al., 2020). Unlike cDC1s, cDC2s are a heterogenous DC subset that are central in priming CD4 T cell responses, as deletion of IRF4 in myeloid cells prevents sensitization to HDM and abolishes protective Th17 responses in pulmonary *Aspergillus* infection (Schlitzer et al., 2013; Williams et al., 2013).

Identifying exactly which DC subtypes induce Th2 versus Th17 responses *in vivo* is challenging, as cDC2 surface markers are plastic upon challenge with an inflammatory

stimulus(Bosteels et al., 2020). Therefore, interrogation of transcription factors utilized by DCs has been an alternative and more fruitful approach. For example, myeloid-specific deletion of the transcription factor KLF4 resulted in fewer Sirpα(+)CD24(+) DCs in the lung. These mice also had reduced eosinophilia and peribronchial inflammation after HDM sensitization and challenge (Tussiwand et al., 2015).

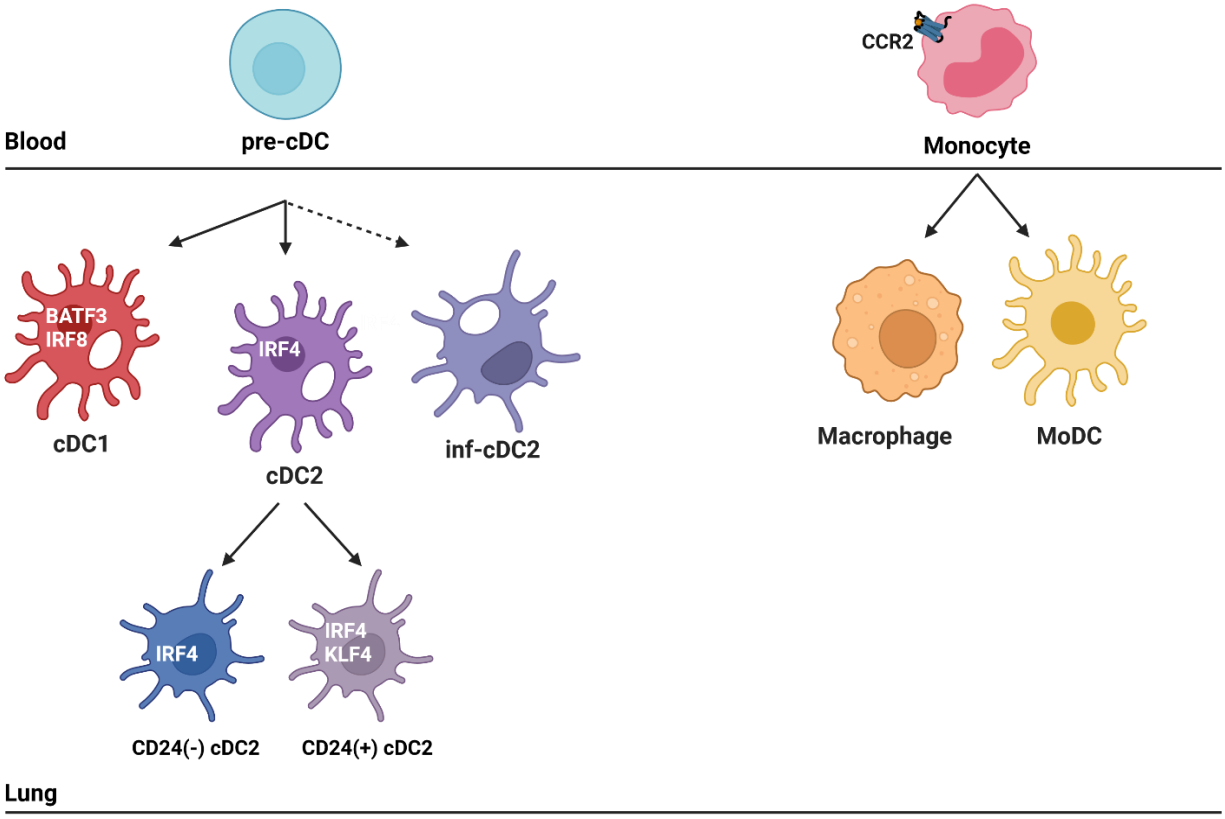

Figure 1-2: Myeloid cell subsets in the lung. Pre-cDCs from the bone marrow seed the lung parenchyma, where they differentiate into IRF8-dependent cDC1 or IRF4-dependent cDC2 at homeostasis. During inflammation, inflammatory cDC2s (inf-cDC2s) also differentiate from pre-cDCs in an IRF8-dependent manner and are capable of presenting antigen to both CD4 and CD8 T cells. Within the cDC2 lineage, KLF4-dependent DCs (identifiable by the surface marker CD24) are crucial in priming Th2 responses. Monocyte-derived cells primarily differentiate into macrophages; however, monocyte-derived dendritic cells (MoDCs) can behave similarly to cDCs by processing and presenting antigen, as well as migrating to lung-draining lymph nodes. Image generated in Biorender.

At homeostasis, the primary myeloid cells in the lung are alveolar macrophages, which phagocytose inhaled particulate matter, turn over surfactant in the alveoli, and enhance tissue repair in response to epithelial damage(Puttur et al., 2019). During an inflammatory event such as

sensitization to allergen, infection, or acute injury, CCR2-expressing monocytes migrate from the bone marrow to the lung and differentiate into interstitial macrophages(Gibbings et al., 2017). A subset of these monocyte-derived cells, however, upregulate MHCII molecules reminiscent of maturing DCs. These so-called monocyte-derived dendritic cells were shown to be sufficient for inducing Th2 responses, but only in response to high-dose allergen(Plantinga et al., 2013). Recently, MoDCs have also been identified migrating to the lymph node and priming T cells, further blurring the lines between conventional and monocyte-derived DCs(Rawat et al., 2023).

Impact of the environment on allergic asthma

A. The Hygiene Hypothesis and transformation into the "Old Friends" hypothesis

With the advent of antibiotics and widespread vaccination, deaths due to infectious disease have been reduced dramatically in the past 60 years in industrialized countries(Sakai and Morimoto, 2022). Concurrently, however, there has been an increase in the incidence of autoimmune and allergic diseases. In response to these observations, David Strachan coined the so-called "hygiene hypothesis": exposure to early-life infections, such as measles and rheumatic fever, prevented the development of allergic disease later in life(Strachan, 1989).

Though repeated respiratory infection in childhood has been shown to be non-protective(Bodner et al., 1998)—exposure to rhinovirus, for example, increases the risk of asthma diagnosis by school age(Jackson et al., 2008; Kusel et al., 2007)— other microbial exposures have been proposed to "train" the early immune system to be hyporesponsive to allergens. Specifically, the hygiene hypothesis has been revised into the "old friends" or "biodiversity" hypothesis, which specifies that not all bacterial and viral infections early in life protect against allergy. Rather, depletion of microorganisms that chronically infect humans, transiently pass through the GI tract

through ingestion, or reside in the mucosa results in lost protection from asthma (Figure 1-3; (Haahtela et al., 2013)).

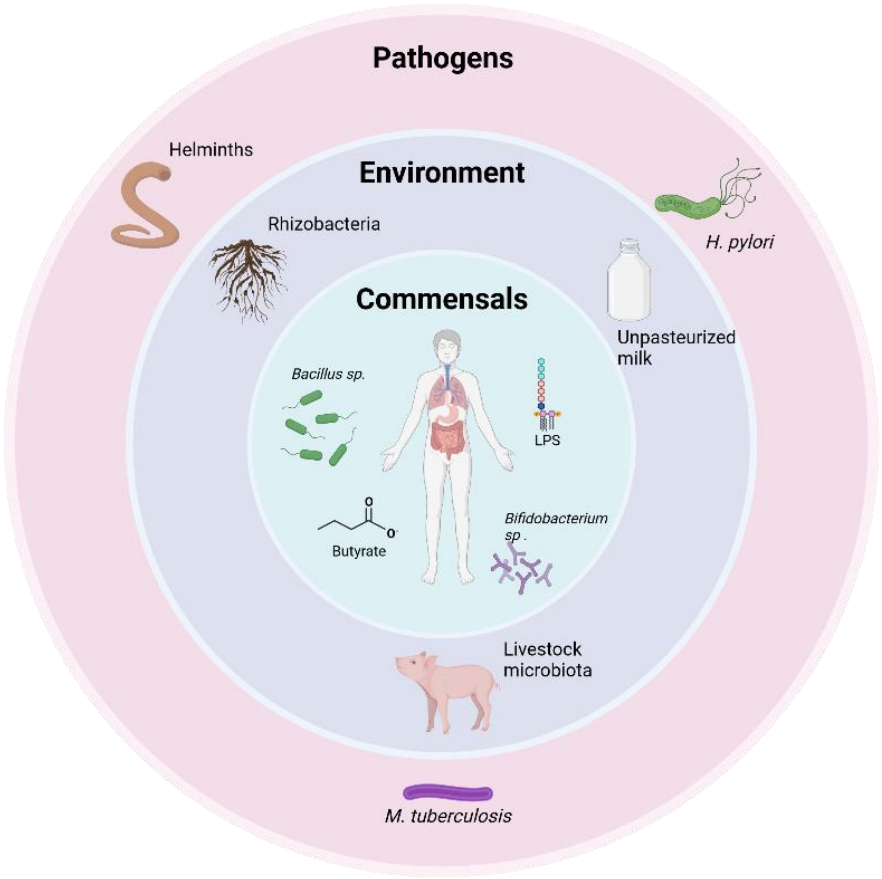

Figure 1-3: Protective exposures implicated in the "Old Friends" hypothesis. In pre-industrialized society, humans were exposed to greater numbers of low-dose pathogens (outside circle), as well as microbes from non-urban exposures such as soil, unpasteurized milk, or livestock (middle circle). Additionally, a more diverse microbiota resulted in low-level innate immune stimulation and circulation of immunomodulatory metabolites such as butyrate (inner circle). Combined, these exposures resulted in enhanced immunity at barrier surfaces, Treg generation, and tolerance to environmental antigens. In the absence of these exposures, humans lose enhanced barrier immunity and become sensitized to environmental and self-antigens. Image generated by Biorender, based on a review from (Rook, 2023).

B. The Farm Effect: an important corollary of the Old Friends hypothesis

In the last decade of the twentieth century, multiple groups observed that urban environments conferred a higher risk of atopic sensitization in children(L. Bråbäck, 1991; Popp et al., 1989).

Initially, the increased atopic sensitization in urban children was thought to be a result of increased air pollution. However, a landmark study in Switzerland by Braun-Fahrländer and colleagues found that, within the same rural population, farmers' children had significantly lower rates of hay fever, current wheeze, and IgEs against specific outdoor allergens than children of non-farmers(Braun-Fahrlander et al., 1999). In the next year, a cross-sectional survey of rural families in Austria, Germany, and Switzerland found that the prevalence of asthma was only 1% in children exposed to stables and farm milk during infancy, as opposed to 12% in children that were not exposed to either. Children with farm exposures were also less likely to have hay fever or atopic sensitization to common allergens(Riedler et al., 2001). From these findings, Dr. Erika von Mutius developed the "Farm Effect" hypothesis, in which farm-derived microbial exposures protect infants from the development of asthma and allergy at school age. These exposures are believed to result in "training" of the host immune system, such that the host remains hyporesponsive to environmental allergens upon re-exposure(Gerretsen and Schuijs, 2022).

What about the farm environment, then, prevents asthma in exposed individuals? While farm exposure consistently results in lower levels of atopic sensitization, hay fever, and wheeze(Braun-Fahrlander et al., 1999; Downs et al., 2001; Kloepfer et al., 2022), others have found no association between childhood farm exposure and asthma prevalence specifically (Brunekreef et al., 2012; Mazur et al., 2018). To clarify the role of the farm environment in protection from allergic asthma, Ege et al stratified specific farm exposures and correlated individual exposures with asthma incidence in rural families. In the PARSIFAL study group, children exposed to pig farming, farm milk, animal sheds, or haying had a lower odds ratio (OR) of ever acquiring asthma. Conversely, children exposed to hares or rabbits had a higher OR of acquiring asthma(Ege et al., 2007). These findings were consistent with other studies, in which exposure to non-pasteurized farm milk or

animal sheds(Debarry et al., 2007; Illi et al., 2012; Lluis et al., 2014) during pregnancy or infancy inversely correlated with asthma prevalence by age five. Consistent with this finding, exposures to farm dust have also been demonstrated to be protective against allergic sensitization in animal models(Peters et al., 2006; Stein et al., 2016). Even the presence of a "farm-like" microbiota signature in non-farming homes was found to be inversely correlated with asthma prevalence in children of those homes(Kirjavainen et al., 2019).

C. Endotoxin: A microbial agent implicated in the Farm Effect

"Endotoxin tolerance", or innate immune hyporesponsiveness to subsequent doses of endotoxin after initial exposure, was first described in 1946 when rabbits treated sequentially with typhoid vaccine had an attenuated fever response(Beeson, 1946). Successive studies revealed that mice injected with lethal doses of endotoxin survived if they were first pre-treated by endotoxin at a lower dose(Carey et al., 1958). *In vitro*, both mouse monocytes and human macrophages secrete less pro-inflammatory cytokines such as TNFα, IL-6, IL-12, and IL-1β upon exposure to a second dose of sublethal endotoxin. Endotoxin-tolerized myeloid cells instead secreted anti-inflammatory cytokines such as IL-10 and TGF-β(Biswas and Lopez-Collazo, 2009).

Concurrent with the development of the Farm Effect hypothesis, Dr. Erika von Mutius and colleagues found that indoor endotoxin levels were significantly higher in farm homes than homes of rural, non-farming families(von Mutius et al., 2000). Since the concept of endotoxin tolerance was already widely appreciated, multiple groups hypothesized that endotoxin in farm dust contributed to lower incidences of asthma in farm children. In other studies, endotoxin levels in household dust were and inversely correlated with the occurrence of atopic sensitization, hay fever, and allergic asthma in children(Braun-Fahrlander et al., 2002; Gehring et al., 2004; Karvonen et al., 2012; Stein et al., 2016). The anti-allergic effect of environmental endotoxin,

however, remains controversial; other groups have found no association between endotoxin exposure and prevalence of allergic asthma (Ege et al., 2007; Shekhar et al., 2017; Thorne et al., 2005), and endotoxin exposure in house dust from non-farming households appears to be positively associated with asthma (Kanchongkittiphon et al., 2015).

Mechanistic studies of endotoxin exposure and allergic asthma *in vivo* have also shown mixed results. In low doses (~100 ng), pulmonary endotoxin can be co-administered with ovalbumin (OVA) to induce Th2 responses upon subsequent challenges with OVA in mice. An increased dose of endotoxin (100 μg) upon sensitization, however, results in neutrophilia and Th1 immunity in the lung(Eisenbarth et al., 2002). In some contexts, low-dose LPS co-administered with OVA during sensitization results in an influx of Tregs after multiple OVA challenges(Whitehead et al., 2014). In the OVA/alum model of allergic sensitization, TLR4 deficiency augments eosinophilia, and challenge with endotoxin-contaminated OVA in TLR4-sufficient mice reduced serum IgE and eosinophilia(Hollingsworth et al., 2006), arguing for an anti-Th2 effect of endotoxin.

In the house dust mite extract (HDM) model of allergic asthma, endotoxin sensing by TLR4 on stromal cells was shown to be necessary and sufficient for DC activation and Th2 priming *in vivo*(Hammad et al., 2009). However, chronic low-dose endotoxin exposure two weeks prior to sensitization with HDM prevented eosinophilia, IgE production, and airway hyperresponsiveness. This anti-allergic effect was mediated through upregulation of A20, a negative regulator of TLR4 signaling, in epithelial cells(Schuijs et al., 2015). Graded dosing of endotoxin during HDM sensitization also revealed a dose-dependent effect on eosinophilia, in which higher endotoxin doses during sensitization suppressed eosinophilia and mucus secretion in airways, but also enhanced neutrophilia and disrupted alveolar architecture (Daan de Boer et al., 2013). While endotoxin—particularly endotoxin pre-exposure—could protect against eosinophilia and other

manifestations of type 2 inflammation, even a slight change in the dosage and/or timing of endotoxin exposure could exacerbate inflammation or even skew the lung towards a neutrophilic phenotype.

Harnessing the Farm Effect through probiotics

A. OM-85 BV

Originally developed as an immunomodulatory agent to prevent respiratory tract infections, OM-85 BV (also known by the brand name Broncho-Vaxom®) has also been studied in the context of preventing or treating allergic inflammation in mouse and rat models. OM-85, an endotoxin-low alkaline lysate initially produced from eight bacterial species, was demonstrated to suppress OVA-induced pulmonary allergic inflammation when given orally. At high doses, OM-85 was shown to induce conversion of $FoxP3^-$ Tconv cells into $FoxP3^+$ Tregs in a $CD103^+$ DC-dependent manner(Navarro et al., 2011). A subsequent study found that human-equivalent doses of OM-85 in a similar OVA-induced murine model of pulmonary allergic inflammation were unable to reduce lung eosinophilia or OVA-specific IgE, with only a modest improvement in pulmonary function (as measured by reduced airway resistance in the OM-85+OVA group)(Rodrigues et al., 2016).

Intranasal administration of OM-85 was also able to inhibit eosinophilia and improve pulmonary function in two different models of experimental asthma: OVA:alum and *Alternaria alternata* (Pivniouk et al., 2022). In both models, OM-85 induced $FoxP3^+$ Tregs downstream of an increase in $CD103^+$ cDC1 in the lung, indicating that OM-85 was acting through a similar mechanism as the 2011 study despite the change in administration site. In contrast to the 2011 study, use of intranasal OM-85 prior to *A. alternata* sensitization reduced BAL IL-33 levels and

lung ILC2s, both factors crucial for initiating allergic inflammation in mice and humans(Pivniouk et al., 2022).

B. Acinetobacter lwoffii

Multiple groups have attempted to isolate farm shed-associated microbes that have immunomodulatory properties *in vivo*. One Gram-negative bacterium, *Acinetobacter lwoffii*, stood out due to its relative abundance in cowshed environments, a protective exposure according to the 2007 Ege study. A cowshed isolate of *A. lwoffii*, when administered intranasally prior to sensitization with OVA/alum, prevented eosinophilia in the BAL and improved airway reactivity(Debarry et al., 2007). Consistent with the idea that prenatal exposures are also sufficient to protect against allergic inflammation, the pups of dams treated with *A. lwoffii* were protected against lung eosinophilia, goblet cell metaplasia, and overall heightened lung inflammation when challenged with OVA 6 weeks after birth(Conrad et al., 2009).

A recent study demonstrated that intranasal *A. lwoffii* administration triggers the release of TNF-α, IL-6, and IFN-β into the bronchoalveolar lavage fluid. Follow-up studies in cytokine-deficient mice demonstrated that IL-6 release by *A. lwoffii* treatment was necessary for protection from OVA-induced allergic inflammation(Alashkar Alhamwe et al., 2023). This low-level innate activation by *A. lwoffii* is reminiscent of the activated phenotype of PBMCs from Amish children, who are protected from asthma and have an elevated TNF-α signature relative to Hutterite children who have a higher prevalence of asthma(Stein et al., 2016). However, whether *A. lwoffii* is capable of protecting from sensitization to complex allergens such as HDM remains to be seen.

Bacillus subtilis as a candidate probiotic

While there has been much focus on microbial products from Gram-negative bacteria, the

potential anti-inflammatory effects of Gram-positive bacteria and their byproducts remain relatively unexplored. *Bacillus subtilis* is a Gram-positive, aerobic, spore-forming bacterium that is commonly found in soil as a plant-associated symbiont. It has been used for centuries as a fermentation agent in Africa and Asia, most notably in the production of the Japanese fermented soybean product natto(Gopikrishna et al., 2021). Because of its ubiquity and widespread use as a fermentation agent, *B. subtilis* is designated as GRAS, or "generally recognized as safe", by the Food and Drug Administration(Hutt, 2018). Since its first use as a probiotic in Europe and Southeast Asia in the 1980s(Green et al., 1999), *B. subtilis* has been demonstrated to modulate host immunity through a variety of processes. Namely, *B. subtilis*, when administered orally, can prevent growth of potentially pathogenic bacteria, improve intestinal barrier integrity, enhance IgA secretion(Lefevre et al., 2015), and increase levels of cecal short-chain fatty acids (SCFAs)(Freedman et al., 2021).

A. B. subtilis and exclusion of pathogenic bacteria

One of the most notable properties of *B. subtilis* is its ability to prevent the establishment of human pathogens within its host. A study of a rural Thai population, for example, found that the presence of *B. subtilis* in fecal samples precluded intestinal and nasal colonization by the human pathogen *Staphylococcus aureus*. Follow-up studies in mouse models demonstrated that *B. subtilis*-derived fengycin, a lipopeptide, prevented successful quorum sensing in *S. aureus*, thereby precluding *S. aureus* colonization(Piewngam et al., 2018). *B.* subtilis-mediated exclusion of *S. aureus* was also used therapeutically to treat *S. aureus*-induced mastitis in a mouse model(Qiu et al., 2022). This signalling interference effect was not specific to *S. aureus*, as oral gavage of *B. subtilis* spores also prevented dissemination of *Enterococcus faecalis* into the blood(Piewngam et al., 2021).

B. *B. subtilis* and mucosal integrity

As a ubiquitous plant and soil bacterium, *B. subtilis* spores are readily isolated from food products such as dairy milk(Qiu et al., 2022) and vegetables(Bartal et al., 2023; Koilybayeva et al., 2023). Because spores are resistant to the hostile conditions associated with the gastrointestinal tract, *B. subtilis* spores survive the acidic conditions of the stomach and germinate into vegetative cells in the ileum and jejunum(Elshaghabee et al., 2017). There, they can interact with and modulate intestinal epithelial cell function. In livestock such as pigs and broiler chickens, feed supplementation with *B. subtilis* significantly increases intestinal villus height and crypt depth while also inducing expression of tight junction proteins ZO-1, occludin, and claudin-1(Tong et al., 2023; Zhang et al., 2016). Within the ileum, *B. subtilis* induced differentiation of secretory cells, including Paneth, goblet, and enteroendocrine cells, when administered orally over a 21-day period. Furthermore, *B. subtilis* treatment increased expression of anti-microbial peptides (AMPs) in the ileum, which resulted in resistance to *S. typhimurium* infection(Hou et al., 2022). *B. subtilis* has also been demonstrated to prevent pro-inflammatory cytokine secretion and upregulate tight barrier junction proteins in intestinal epithelial cell culture(Azimirad et al., 2017; Rhayat et al., 2019; Zhang et al., 2022).

B. subtilis has also further been shown to increase serum and luminal IgA after supplemental feeding in broiler chicks(Min et al., 2016; Tong et al., 2023) and weanling pigs (Lewton et al., 2022). Increases in serum IgA were also associated with reduced severity of diarrhea in beagles challenged with enterotoxigenic *E. coli*(Yang et al., 2023).

C. Effects of *B. subtilis* on other components of the immune response

B. subtilis and its metabolites have also shown direct effects on hematopoietic cells.

Supernatants from natto-derived *B. subtilis* isolates induced IL-10 and IL-12 production in a human-derived THP-1 dendritic cell line. Subsequent fractionation of the supernatant from one isolate revealed that the chaperone protein GroEL was upregulated in *B. subtilis* supernatants, and purified GroEL induced transcription of IL-1, IL-10, TNF-α, and several CXCL ligands(Uesugi et al., 2023). In another study, RAW264.7 cells (a murine macrophage cell line) upregulated IL-10 but downregulated IL-12, IL-1β, TNF-α, and IL-6 when co-incubated with *B. subtilis* strain R0179(Zhao et al., 2021).

B. subtilis exopolysaccharide: A novel immunomodulatory agent

While *B. subtilis*'s immunomodulatory abilities have been described in the agricultural space, it is less clear which individual components of *B. subtilis* were responsible. An initial study by Dr. Katherine Knight's group found that *B. subtilis*, in combination with *B. licheniformis*, consistently induced gut-associated lymphoid tissue (GALT) and somatic diversification in GALT-associated B cells in rabbits(Rhee et al., 2004). In light of a previous study demonstrating that *B. subtilis* colonization at birth protects against *C. rodentium*-induced enteropathy(D'Arienzo et al., 2006), the Knight group hypothesized that a specific compound secreted by *B. subtilis* is capable of inhibiting enteric inflammation *in vivo*. Indeed, they screened *B. subtilis* mutants and found that mutants deficient in exopolysaccharide (EPS) production were not protected from *C. rodentium*-induced enteric inflammation(Jones and Knight, 2012).

A. Exopolysaccharides

Rather than growing as singular, vegetative cells, many microbes often exist together in complex communities known as biofilms. Biofilms are composed of DNA, polysaccharides, and proteins that act as a source of structural integrity and nutrients for the microbes residing within.

Exopolysaccharides (EPSs) are a class of high molecular-weight polysaccharides secreted by microbes within the biofilm. EPSs vary in structure depending on their microbe of origin, and a single microbe can secrete multiple EPSs(Amyx-Sherer and Reichhardt, 2023). However, the composition of EPSs within a single species remains remarkably consistent across carbon sources. As such, EPS production is easily scaled for use in food production, namely as thickening or gelling agents(Kornmann et al., 2003).

Bacillus subtilis alone produces a number of EPSs. The most well-known is levan, a fructose-based homopolysaccharide, which *B. subtilis* natto can produce up to 40-50 g per liter of culture(Netrusov et al., 2023). Due to levan's ability to form nanoparticles, large-scale manufacturing of *B. subtilis* levan is currently being optimized for use in cosmetic creams and serums(Domzal-Kedzia et al., 2023).

The anti-inflammatory strain of *B. subtilis* used in the Knight group's colitis studies (Jones and Knight, 2012; Jones et al., 2014), strain 3610, depends on the products of the *epsA-O* operon to secrete EPS (Arnaouteli et al., 2021). Deletion of the genes encoding the transcriptional repressor SinR and matrix protein TasA results in a *B. subtilis* mutant that hypersecretes EPS. Gas chromatography/mass spectrometry of EPS isolated from this strain revealed that the 300 kDa product was primarily mannose and glucose, making it distinct from levan(Jones et al., 2014).

B. EPS's anti-inflammatory effects in non-asthmatic disease models

A single dose of EPS isolated from the 3610-derived *sinRtasA B. subtilis* mutant improved diarrhea scores and reduced crypt height in a *C. rodentium* inflammation model. EPS's anti-inflammatory effects are dependent on TLR4, as TLR4-deficient mice treated with EPS were not

protected from diarrhea and intestinal crypt lengthening(Jones et al., 2014). Mechanistically, they found that EPS bound peritoneal macrophages and induced the expression of Arg-1, IL-4Rα, IL-4 and IL-13, all molecules associated with an anti-inflammatory, "M2"-like macrophage phenotype. These macrophages, when cocultured with anti-CD3-stimulated T cells, suppressed proliferation of both CD4 and CD8 T cells in a TGF-β-dependent manner(Paynich et al., 2017).

In light of the findings that EPS altered peritoneal macrophage phenotypes and prevented T cell proliferation in an antigen-independent manner, the Knight group hypothesized that EPS treatment could prevent pathology in disease states that involve macrophages and/or T cells. Indeed, EPS treatment prior to *S. aureus* infection improved survival, decreased weight loss, and reduced bacterial burden in the blood, spleen, and liver. At the cellular level, EPS induced a "hybrid" peritoneal macrophage subset that had enhanced ROS production and microbicidal activity, while also suppressing *S. aureus* SAg-dependent T cell proliferation. These macrophages upregulated both Arg-1 and iNOS, markers of M2-like and M1-like macrophages, respectively(Paik et al., 2019). EPS pretreatment in the context of murine graft-versus-host disease (GvHD) was also found to prolong recipient survival, suppress systemic IFN-γ, KC and MCP-1, and prevent caspase-1 activation in alloreactive donor cells(Kalinina et al., 2021).

C. Studies on *B. subtilis* and allergic disease

In the context of allergic disease, oral gavage of *B. subtilis* spores one day before and five days after sensitization to HDM prevented BAL eosinophilia after HDM challenge(Swartzendruber et al., 2019). This effect was robust, as mice sensitized up to three weeks after *B. subtilis* gavage were also protected from BAL eosinophilia(Rosalinda Monroy Del Toro, 2023). While the transfer of EPS-treated BMDCs also prevented allergic eosinophilia in the same HDM

model(Swartzendruber et al., 2019), it was not addressed whether this effect was dependent on T cell priming or whether changes in BAL reflected changes in lung histology or cell composition.

B. subtilis's ubiquitous distribution and EPS's broadly immunomodulatory effects led our group and others to hypothesize that EPS from *B. subtilis* would be capable of inhibiting allergic inflammation if administered *in vivo*. One group found that oral administration of high-dose EPS from *B. subtilis* strain xztubd1 prevented accumulation of eosinophils and lymphocytes in the BAL fluid, decreased lung oxidative stress biomarkers, and decreased lung expression of the Th2 cytokines IL-4 and IL-5. This group also found that large doses of oral EPS downregulated NF-κB p65, JAK1, and STAT6 expression in the lungs by qPCR, indicating that EPS was inhibiting canonical NF-κB signaling in lung cells(Zhang and Yi, 2022). These findings were consistent with a recent publication from the Knight group, where they found that ingested *B. subtilis* EPS from a different strain activated non-canonical NF-κB signaling to suppress IFN-γ, IL-2, and IL-6 secretion(Zamora-Pineda et al., 2023). However, whether local administration of *B. subtilis* EPS—reminiscent of inhaled exposure to farm dust thought to be responsible for the farm effect—can protect against allergic inflammation *in vivo* remains to be seen.

Summary

In this dissertation, I describe a novel anti-inflammatory role for *B. subtilis* EPS in preventing allergic inflammation *in vivo*. First, I will demonstrate that EPS administered directly to the respiratory mucosa prevents mucus secretion, eosinophilia, and accumulation of Th2 cells in a TLR4-dependent manner. This protective effect is most pronounced if EPS is given before sensitization to allergen, although I also show that EPS treatment prior to challenge prevents allergic inflammation *in vivo* to a weaker extent. I then trace this EPS-mediated defect in allergic

inflammation to the DC compartment, where EPS treatment alters the ability of antigen-positive DCs to uptake antigen in the lung and migrate to the lung-draining LN. These studies are followed by transcriptional analysis of EPS-treated DCs by RNA-sequencing, which reveals that EPS reduces expression of TNF-α, IL-1β, and IL-2 in sensitized DCs. As these cytokines are pro-survival factors for Th cell priming, we posit that EPS treatment reduces DC delivery of signal 3, preventing efficient T cell priming and downstream T cell differentiation. Thus, the studies described here unveil a mechanism by which probiotic-derived EPSs can inhibit allergic sensitization.

Chapter 2: Materials and Methods

Mice. *Chapter 3.* C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN) and bred in-house. Tlr4-lps-del mice were purchased from the Jackson Laboratory and maintained in-house. Animals were housed in a specific pathogen-free facility maintained by the University of Chicago Animal Resources Center. All studies conformed to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research. The experiments were approved by the University of Chicago Institutional Animal Care and Use Committee. *Chapter 4.* C57BL/6J mice were purchased from the Jackson Laboratory. Animals were housed in a specific pathogen-free facility maintained by the University of Virginia Center for Comparative Medicine. All studies conformed to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research. The experiments were approved by the University of Virginia Institutional Animal Care and Use Committee.

House dust mite (HDM) model of allergic inflammation. HDM extract (Stallergenes Greer, part #XPB82D3A25) was resuspended in sterile, endotoxin-free 1X PBS (Sigma-Aldrich, part TMS-012). For sensitization experiments, mice received 25 μg HDM intratracheally (i.t.) and were sacrificed 12-18 hours later. In experiments requiring fluorescent antigen tracking, HDM was labeled with Alexa Fluor 647 Protein Labeling Kit (Life Technologies #A20173) per manufacturer's instructions. For experiments requiring allergen challenge, sensitized mice received 10 μg HDM i.t. on days 7, 8, 9, and 10 after sensitization, and were sacrificed on day 13. At the time of sacrifice, mice received an intravenous injection of biotinylated anti-CD45 (clone 30-F11, BioLegend) to allow for identification of CD45iv(+) cells located in the vasculature. Five

minutes after anti-CD45 administration, cardiac puncture was performed to collect blood for serum IgE analysis. In some mice, bronchoalveolar lavage (BAL) was performed to analyze cells in the airspace by flow cytometry. Briefly, the tracheas of euthanized mice were cannulated with a 20G blunt-ended needle (Thomas Scientific, part 1176R94). After the blunt-ended needle was secured with surgical thread, the lungs were flushed with 0.8 ml 1x PBS and then collected into a 5 ml polypropylene tube. Each BAL consisted of four total washes with 0.8 ml PBS per wash. After BAL collection, lungs were perfused through the left ventricle of the heart using 10 ml of 1x non-sterile PBS(diluted from 10xPBS, Fisher BioReagents, catalog no. BP399-4). Then, lungs and mediastinal lymph nodes (medLN) were harvested for flow cytometric and histologic analyses.

Exopolysaccharide (EPS) generation and treatment. Exopolysaccharide (EPS) from *Bacillus subtilis* was generated by the Knight group at Loyola University Chicago. Briefly, supernatants from *B. subtilis* strain DS991 were ethanol precipitated, digested with DNase, RNAse, and proteinase K, and then purified by gel filtration on an S1000 column. After pooling EPS-positive fractions and a second ethanol precipitation, EPS was vacuum-dried and resuspended in sterile 1XPBS. EPS concentration was determined using a colorimetric phenol-sulfuric acid assay with xanthan gum standards.

Forty micrograms of EPS was administered i.t. to mice while under a plane of anesthesia. For sensitization experiments, mice received 40 μg EPS one day prior to i.t. with HDM. Half of mice undergoing the full HDM sensitization and challenge model were treated with 40 μg EPS i.t. at days -1, 2, and 5 relative to sensitization.

Periodic Acid-Schiff (PAS) staining and quantification. For histological analysis, left lung lobes were placed in a histology tissue cassette) and submerged in 10% PBS-buffered formalin (Azer Scientific, catalog no. PFNBF-360). After 24 hours at room temperature, cassettes were

rinsed twice with 70% ethanol and submerged in 70% ethanol. Lungs in 70% ethanol were embedded in paraffin, sectioned at 5 μm thickness, fixed onto a glass slide, and stained with Periodic Acid-Schiff (PAS) stain at either the Research Histology Core at the University of Virginia or the Biomedical Tissue Repository Facility at the University of Chicago. PAS-stained slides were scanned at 40x magnification at the Biomedical Tissue Repository Facility at the University of Virginia or the Integrated Light Microscopy Core at the University of Virginia. To score PAS staining, all airways from each lung section were given a score of 0 to 5, based on what percentage of the airway was positive for PAS stain. 0 = no staining; 1 = 1-25% staining; 2 = 26-50% staining; 3 = 51-75% staining; 4 = 76-99% staining; 5 = all cells positive for PAS and/or a score of 4 with a mucus plug present in the airway lumen. At least 20 small airways over each lung slide were averaged to give a final score per sample. To normalize scores across experiments, all samples within one experiment were divided by the mean PAS score of PBS/HDM-treated mice.

Tissue processing for flow cytometry. After collection, lung single-cell suspensions were generated by mincing with dissecting scissors for 90 seconds, followed by resuspension in complete Dulbecco's Modified Eagle Medium (Life Technologies, number 11-995-065) supplemented with 5% fetal bovine serum (X&Y Cell Culture, catalog no. FBS-500-HI), 1% penicillin and streptomycin solution (Life Technologies, catalog no. 15140122), 1x MEM non-essential amino acids (Life Technologies, catalog no. 11140050), and 1M HEPES (Life Technologies, catalog no. 15630130). Then, collagenase IV(Sigma-Aldrich, catalog no. C5138-500MG) and DNase I (Worthington Biochemical, DP grade) were added to the digested tissue mixture at a final concentration of 600 U/ml collagenase IV and 20 μg/ml DNase I, respectively. Tissue digests were incubated at 37°C for 1 hour with rocking, then cell suspensions were pipetted up and down with a p1000 pipette and filtered through a nylon mesh filter (Component Supply,

catalog no. U-CMN-120). After centrifugation for 5 minutes at 1350 rpm, red blood cells were lysed using Ammonium-Chloride-Potassium (ACK) lysis solution for one minute. ACK lysis was quenched by the addition of 9 ml of complete DMEM. After a second centrifugation for 5 minutes at 1350 rpm, cells were resuspended in 5 ml of complete DMEM and an aliquot of cells were taken for counting by hemacytometer or flow cytometry.

For BAL cell isolation, the volume of total BAL from each mouse was noted and then brought up to 3 ml using FACS buffer. After centrifugation, BAL pellets were resuspended in 1 ml of FACS buffer for counting.

To generate single-cell suspensions from mediastinal lymph nodes, isolated whole LNs were dissected by teasing apart with 20G needles in 1 ml of 5% complete DMEM, followed by incubation at 37°C for 1 hour with 600 U/ml collagenase IV and 20 μg/ml DNase I. After digestion, LNs were dissociated by pipetting up and down, then filtered through sterile nylon mesh into a 5 ml nonsterile round bottom polystyrene FACS tube (Corning Incorporated, 352052, Tewksbury, MA, USA). Wells containing the digested LNs were also washed with 1 ml complete DMEM following filtration and transfer. After centrifugation, LNs were resuspended in 1 ml complete DMEM and an aliquot was taken for counting.

Flow cytometry staining. After counting, $5x10^5$ or 10^6 BAL, lung, or LN cells were transferred to a 5 ml FACS tube and washed with 2 ml FACS. For some experiments, cells were washed again with 1 ml of PBS to remove any remaining protein for viability staining. Upon decanting the supernatant, PBS-washed cells were incubated with Zombie Aqua (Biolegend, 423102, San Diego, CA, USA) or Zombie NIR (Biolegend, catalog no. 423106, San Diego, CA, USA) viability dye for 10 minutes at room temperature. To quench viability staining, cells were washed with 2 ml FACS buffer and the supernatants decanted. Prior to surface staining, all samples were incubated

at RT for 5 minutes with 10 µl 2.4G2 hybridoma supernatant to prevent nonspecific antibody binding to Fc receptors. The staining conditions and dilution of stock antibodies for each panel are detailed in Table 2-1. Samples undergoing just surface staining were washed with FACS buffer, then resuspended in FACS buffer for acquisition. Samples were acquired on a BD Fortessa or Cytek Aurora.

Antigen	Fluorophore	Company	Catalog No.	Stock Ab/10^6 cells	Staining conditions
Inflammation panel (Chapter 3)					
CD45.2	FITC	Biolegend	109806	0.25 µl	30 minutes at 4C
CD11b	BV510	BD	562950	0.25 µl	30 minutes at 4C
SiglecF	PE	BD Horizon	552126	0.5 µl	30 minutes at 4C
CD11c	APC	Biolegend	117310	0.5 µl	30 minutes at 4C
I-A/I-E	BV785	Biolegend	107465	0.125 µl	30 minutes at 4C
CD19	BV650	Biolegend	115541	0.25 µl	30 minutes at 4C
CD3	PE/Cy7	Biolegend	100320	0.5 µl	30 minutes at 4C
CD4	BV605	Biolegend	100548	0.25 µl	30 minutes at 4C
CD8	PE/Dazzle594	Biolegend	100762	0.125 µl	30 minutes at 4C
T cell transcription factor panel (Chapter 3)					
ST2	PE	Biolegend	145304	0.5 µl	15 minutes at RT prior to surface staining
CD3	BUV395	BD	740268	1 µl	30 minutes at 4C
CD4	BV605	Biolegend	100548	0.25 µl	30 minutes at 4C
CD8	BV711	Biolegend	100759	0.25 µl	30 minutes at 4C
TCRγδ	APC/Fire750	Biolegend	118136	0.5 µl	30 minutes at 4C
CD45	biotin	Biolegend	103104	N/A	Injected iv 5 minutes prior to sacrifice
CD45.2	FITC	Biolegend	109806	0.25 µl	30 minutes at 4C
Streptavidin	BUV805	BD Horizon	564923	0.5 µl	30 minutes at 4C
CD44	Pacific Blue	Biolegend	103020	0.5 µl	30 minutes at 4C
CD62L	AlexaFluor700	Invitrogen	56-0621-82	0.5 µl	30 minutes at 4C
CD69	BUV737	BD	564684	0.5 µl	30 minutes at 4C

Table 2-1: Flow cytometry panels used to identify and phenotype lung cells, LDLN cells, and BMDCs.

Antigen	Fluorophore	Company	Catalog No.	Stock Ab/10^6 cells	Staining conditions
BMDC panel (Chapter 4)					
F4/80	PE	BioLegend	123110	0.25 µl	30 minutes at 4C
CD11c	FITC	BioLegend	117306	0.25 µl	30 minutes at 4C
I-A/I-E	PerCP/Cy5.5	BioLegend	107626	0.25 µl	30 minutes at 4C
CD103	BV421	BioLegend	121422	0.5 µl	30 minutes at 4C
CD11b	APC/Cy7	BioLegend	101226	0.125 µl	30 minutes at 4C
CD86	BV605	BioLegend	105037	0.5 µl	30 minutes at 4C
CD24	PE/Dazzle594	BioLegend	101838	0.25 µl	30 minutes at 4C
***In vivo* dendritic cell panel (Chapter 4)**					
CD45	BUV805	BD Horizon	612892	0.25 µl	30 minutes at 4C
SiglecF	PE-CF594	BD	B562757	0.125 µl	30 minutes at 4C
CD3	Biotin	BioLegend	100244	0.5 µl	30 minutes at 4C
CD19	Biotin	BioLegend	115504	0.5 µl	30 minutes at 4C
NK1.1	Biotin	BioLegend	108704	0.5 µl	30 minutes at 4C
Streptavidin	APCefluor780	BioLegend	405208	0.2 µl	15 minutes at room temperature
CD80	PE	Pharmingen	553768	0.125 µl	30 minutes at 4C
CD11c	FITC	BioLegend	117306	0.5 µl	30 minutes at 4C
I-A/I-E	PerCP/Cy5.5	BioLegend	107626	0.25 µl	30 minutes at 4C
CD11b	BV510	BD	562950	0.25 µl	30 minutes at 4C
CD103	BV421	BioLegend	121422	0.5 µl	30 minutes at 4C
CD24	BUV395	BD Horizons	744471	0.5 µl	30 minutes at 4C
CD88	PE/Cy7	Biolegend	135810	0.25 µl	30 minutes at 4C
CD86	BV605	BioLegend	105037	0.5 µl	30 minutes at 4C
PDL1	BV785	BioLegend	124431	0.5 µl	30 minutes at 4C

Table 2-1, continued: Flow cytometry panels used to identify and phenotype lung cells, LDLN cells, and BMDCs.

After surface staining, samples undergoing intracellular staining were washed with 1x PBS, then fixed overnight with the eBioscience FoxP3/Transcription factor Fixation/Permeabilization Kit (ThermoFisher Scientific, catalog no. 00-5521-00) according to the manufacturer's instructions. After fixation, cells were washed twice with permeabilization buffer and incubated with 10 µl FBS for 10 minutes at room temperature. Intracellular stains were performed at the dilutions listed in Table 2-1 at 4°C overnight. After overnight staining, cells were washed once in permeabilization buffer, then washed twice with FACS buffer and resuspended in FACS buffer

for acquisition. Samples that underwent intracellular staining were acquired on a Fortessa X20 or Cytek Aurora.

T cell transcription factor panel: intracellular stains					
Antigen	Fluorophore	Company	Catalog No.	Stock Ab/10^6 cells	Staining conditions
GATA3	PE-CF594	BD Horizon	563510	1 µl	Overnight at 4C
FoxP3	APC	Invitrogen	17-5773-82	1 µl	Overnight at 4C
RORγt	PerCP/efluor710	Invitrogen	46-6988-82	1 µl	Overnight at 4C
T-bet	PE/Cy7	BioLegend	644824	1 µl	Overnight at 4C

Table 2-2: Intracellular stains to identify T cell transcription factors.

BMDC generation and culture. To generate BMDCs, bone marrow was flushed through mouse tibias and fibulas using a 1 ml syringe equipped with a 25G needle. After flushing, bone marrow was mechanically disrupted by repeated pipetting with a P1000 micropipettor and filtered through sterile nylon mesh filter. After filtering, cells were pelleted at 1350 rpm for five minutes at room temperature and resuspended in 1 ml ACK buffer for red blood cell lysis. The lysis reaction was quenched with 9 ml of 10% complete DMEM, after which cells were pelleted and resuspended in 5 ml 10% complete DMEM for counting. A total of 10^7 cells per well were plated in 5 ml of complete medium containing 200 ng/ml Flt3L (Biolegend, catalog no. 550706) in a six-well plate (Fisher Scientific, catalog no. 08-772-49) and cultured for at 37°C with 10% CO_2. After eight days, BMDCs were collected and resuspended in complete DMEM containing 20 ng/ml GM-CSF at a concentration of $8x10^5$ cells/ml ($4x10^5$ cells/500 µl) in a 24-well plate. Some wells received 2 µg/ml EPS. After 24 hours in culture, some wells received 2 µg/ml OVA-AF647 (Invitrogen, catalog no. O34784) and 10 µg/ml HDM for three hours. After incubation with HDM and OVA, BMDCs were harvested with warm 10% complete DMEM, pelleted, and stained for flow cytometry as described above.

Dendritic cell sorting. For RNA-sequencing experiments, mice were treated intratracheally with 40 μg EPS, 5 μg LPS from *E. coli* O55:B5 (Sigma, catalog no. L6529), or 50 μl endotoxin-free PBS. For some experiments, mice were also treated with 25 μg HDM approximately 24 hours after EPS or PBS treatment. Eighteen hours after treatment with EPS, LPS, or HDM, lungs were harvested and processed into single-cell suspensions as described previously. Dendritic cells were enriched from each lung suspension using the Pan Dendritic Cell Isolation Kit according to the manufacturer's instructions (Miltenyi Biotec, catalog no. 130-100-875). Enriched cells were washed one time with MACS buffer, then incubated with 10 μl 2.4G2 for five minutes at room temperature. Then, cells were stained with the markers listed in Table 2-3 for 30 minutes at 4°C.

Antigen	Fluorophore/Conjugate	Company	Catalog No.	Stock Ab/10^6 cells
SiglecF	Biotin	BD Biosciences	567600	0.125 μl
Ly6G	Biotin	BioLegend	127604	0.125 μl
CD3	Biotin	BioLegend	100244	0.125 μl
CD19	Biotin	BioLegend	115504	0.125 μl
NK1.1	Biotin	BioLegend	108704	0.125 μl
CD11c	APC	BioLegend	117310	0.125 μl
I-A/I-E	PerCP/Cy5.5	BioLegend	107626	0.0625 μl
CD103	PE	BioLegend	121406	0.125 μl
CD11b	BV510	BD	562950	0.125 μl
CD88	PE/Cy7	Biolegend	135810	0.125 μl
CD45	FITC	BioLegend	103108	0.125 μl

Table 2-3: Primary staining panel for isolating MACS-purified DCs by fluorescence-assisted cell sorting (FACS).

After staining, cells were washed with 2 ml MACs buffer, resuspended in 1 ml MACS buffer, and stained with streptavidin-APC/efluor780 (eBioscience, catalog no. 47-4317-82) at a final concentration of 0.4 μg/ml. After staining for 15 minutes at room temperature, cells were washed with 4 ml MACS buffer, centrifuged, and resuspended in 1 ml MACS buffer for sorting. Immediately before sorting, samples were filtered through nylon mesh filter and spiked with 5 μl of DAPI to distinguish dead cells. Stained samples were sorted using a Sony MA900 cell sorter

into three main populations (see Figure 2-1 for sorting strategy) into TRI Reagent for cell lysis and RNA isolation (Zymo Research, catalog no. R20510-1-50, Irvine, CA, USA). Samples sorted into TRI Reagent were stored at -80°C until all samples were collected for RNA isolation.

RNA isolation for RNA-seq. DCs sorted into TRI Reagent were brought to room temperature after storage in -80°C, after which total RNA was isolated using the QIAGEN RNeasy Micro kit (Catalog no. 74004, Germantown, MD, USA) according to the manufacturer's instructions under

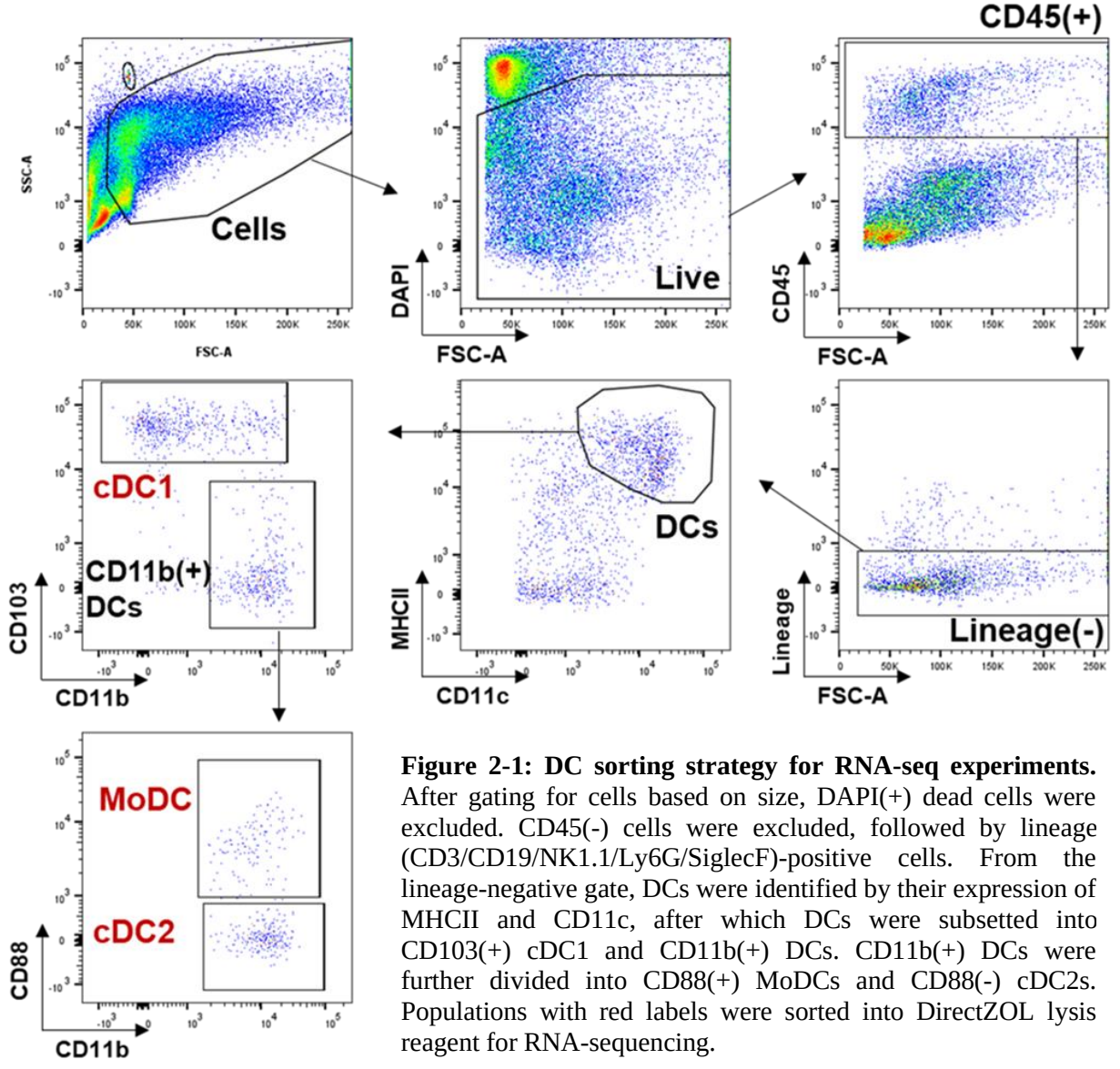

Figure 2-1: DC sorting strategy for RNA-seq experiments. After gating for cells based on size, DAPI(+) dead cells were excluded. CD45(-) cells were excluded, followed by lineage (CD3/CD19/NK1.1/Ly6G/SiglecF)-positive cells. From the lineage-negative gate, DCs were identified by their expression of MHCII and CD11c, after which DCs were subsetted into CD103(+) cDC1 and CD11b(+) DCs. CD11b(+) DCs were further divided into CD88(+) MoDCs and CD88(-) cDC2s. Populations with red labels were sorted into DirectZOL lysis reagent for RNA-sequencing.

"Appendix C: RNA Cleanup after Lysis and Homogenization with QIAzol Lysis Reagent". After elution, RNA was immediately stored at -80°C.

RNA library preparation and quality control were performed at Novogene (Sacramento, CA, USA). Briefly, whole RNA was purified using magnetic enrichment of poly-adenylated mRNA, followed by mRNA fragmentation and cDNA synthesis. Non-directional libraries were generated after end repair, A-tailing, adapter ligation, size selection, and PCR amplification, then quantified by Qubit and real-time PCR. Libraries that passed QC were pooled and sequenced on Illumina platforms.

RNA-seq filtering, normalization, and differential gene expression analysis. Data were cleaned and aligned at Novogene. Briefly, raw data were processed to remove adapter sequences, sequences in which poly-N reads were greater than 10% and reads where low-quality scores constituted more than 50% of the sequence read. Reads were aligned with mouse genome assembly GRCm39 (mm39) using HISAT2.

Subsequent normalization and differential gene expression analysis were performed in R using a pipeline generously provided by Alexandra Cassano (https://github.com/amcassano/AnalysisWorkflowTemplate). Total read counts were pre-filtered to remove genes that had fewer than 800 total counts per sample, to minimize noise and remove outliers. Filtered counts were normalized and log2 transformed using DESeq2 (version 1.42.0). Differential gene analysis and annotation were also performed in DESeq2. Differentially expressed genes were defined as a log2 fold change of at least 1.5 from DCs of PBS-treated cells, with an adjusted *p* value of less than 0.05. Heatmaps were generated using the heatmapFromCSV function within the analyzeRNA package in R.

Statistical analysis. *Chapter 3*. Data were plotted and analyzed in GraphPad Prism version 10.1.2. A p value of less than 0.05 was considered statistically significant (ns: not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Data are plotted as truncated violin plots, with solid lines indicating the mean and dotted lines indicating the SEM. In experiments with three or more groups, statistical significance was determined via one-way ANOVA with Sidak's multiple comparisons test. For plots displaying only two groups, statistical significance was determined via unpaired two-tailed t test. *Chapter 4*. Multiple unpaired t tests with a Holm-Sidak post-test were used to compare the MFI of CD86 in antigen-positive and antigen-negative DCs. Statistical significance was set at $p < 0.05$.

Chapter 3: Exopolysaccharide from *Bacillus subtilis* protects against allergic inflammation *in vivo*.

Introduction

As the incidence of infectious disease drops due to more effective antibiotics and vaccination, there has been a concurrent rise in the incidence of allergic and autoimmune disorders. This phenomenon has been proposed to be due to reduced microbial exposures, resulting in an immune system hyperresponsive to environmental stimuli. In agreement with this ”old friends hypothesis”, numerous studies have found that children exposed to a farm environment and the microbe(s) associated with these farms have a reduced prevalence of asthma, a phenomenon known as the farm effect(Braun-Fahrlander et al., 1999; Ege et al., 2007; Illi et al., 2014; Karvonen et al., 2012; Mazur et al., 2018; Muller-Rompa et al., 2018; Riedler et al., 2001; Stein et al., 2016). Understanding which farm-derived microbial exposures are protective from the development of allergic asthma provides a new avenue for training (or possible re-training) the immune system to become hyporesponsive to common allergens.

Mechanistically, other work has shown that farm dust-associated microbial products, such as endotoxin(Braun-Fahrlander et al., 2002; Delayre-Orthez et al., 2005; Ding et al., 2018; Karvonen et al., 2012; Natarajan et al., 2012; Schuijs et al., 2015), render the immune system refractory to allergic stimuli. However, the therapeutic utility of agents such as endotoxin are limited by potential off-target inflammation and morbidity. For example, endotoxin also induces acute lung injury in sufficient doses, and is used to model acute respiratory distress syndrome (ARDS) in mice(D'Alessio, 2018). Therefore, there is a need to isolate farm-associated microbial

products that inhibit allergic inflammation while not inducing other, deleterious inflammatory events.

Exopolysaccharides (EPSs) are a class of carbohydrate polymers secreted by bacteria and fungi into the extracellular space and are a major component of most biofilms. These polymers protect their host from harsh external conditions and are hypothesized to shield microbes within the biofilm from immunosurveillance(Arnaouteli et al., 2021). The common hay bacterium *Bacillus subtilis*, a Gram-positive, soil-resident bacterium, produces massive amounts of EPS, is readily isolatable from animal sheds(Andersson et al., 1999), improves intestinal barrier integrity in poultry and piglets(Abd El-Hack et al., 2020; Bai et al., 2017; Deng et al., 2020; Tsukahara et al., 2020), and prevents pulmonary allergic inflammation when provided as an intestinal probiotic(Swartzendruber et al., 2019). EPS derived from *B. subtilis* is alsoimmunomodulatory on its own, as it can limit inflammation in several mouse models such as T cell-mediated colitis(Jones et al., 2014), systemic *S. aureus* infection(Paik et al., 2019), and graft-versus-host disease(Kalinina et al., 2024; Kalinina et al., 2021). Therefore, *B. subtilis* EPS is an attractive farm-associated microbial product that may also act as an anti-inflammatory agent in allergic asthma.

In this study, we sought to determine whether EPS could also prevent inflammation in a murine model of house dust mite (HDM)-induced pulmonary allergy. Three intratracheal (i.t.) treatments around the time of HDM sensitization was sufficient to reduce mucus hypersecretion, eosinophilia, and Th2 cell accumulation in the lung. Consistent with previous reports(Zamora-Pineda et al., 2023), we found that TLR4 was necessary for EPS-mediated protection from allergic inflammation Excitingly, EPS given after the sensitization window was also able to increase alveolar macrophage (AM) numbers relative to eosinophils. These findings implicate EPS as an anti-allergic agent in both prophylactic and therapeutic contexts.

Results

EPS administration prevents eosinophilic inflammation in a murine model of allergic asthma.

Allergic inflammation characteristic of asthma can be modeled in mice by intratracheal (i.t.) sensitization and challenge to HDM extract. To determine whether exopolysaccharide (EPS) derived from the common hay bacterium *Bacillus subtilis* could inhibit allergic inflammation *in vivo*, mice were treated i.t. with 40 μg EPS three times around HDM sensitization (Figure 3-1A). After four challenges with HDM a week after sensitization, mice were euthanized at day 13 to quantify the extent of allergic inflammation via PAS staining for airway mucus, serum IgE, and flow cytometry analysis of lung hematopoietic cells. HDM treatment alone induced mucus secretion in small airways (Figure 3-1B, bottom left) which, when scored, was significantly greater in the lungs from HDM-treated mice than mice treated with saline or EPS alone (Figure 3-1C). Excitingly, EPS treatment around the time of sensitization significantly reduced mucus secretion into the airways, as measured by mean PAS score (Figure 3-1C). Additionally, IgE produced in response to HDM sensitization was downregulated by EPS treatment (Figure 3-1D).

We hypothesized that the reduced PAS scores and IgE in EPS-treated mice corresponded with a reduction in type 2 inflammatory mediators, such as eosinophils and CD4 T cells. Flow cytometric analysis of digested lungs revealed that EPS treatment, significantly reduced total lung cellularity and numbers of lung eosinophils, with a corresponding increase in alveolar macrophages (Figure 3-2A-B). To account for variation in cell yields and digestion efficiency across mice, the ratio of eosinophils to alveolar macrophages was also plotted in each treatment group. In HDM-treated mice, eosinophils outnumbered alveolar macrophages at an average ratio of 30.75 to one. With co-administration of EPS, this ratio was reduced to an average of 6.15 to

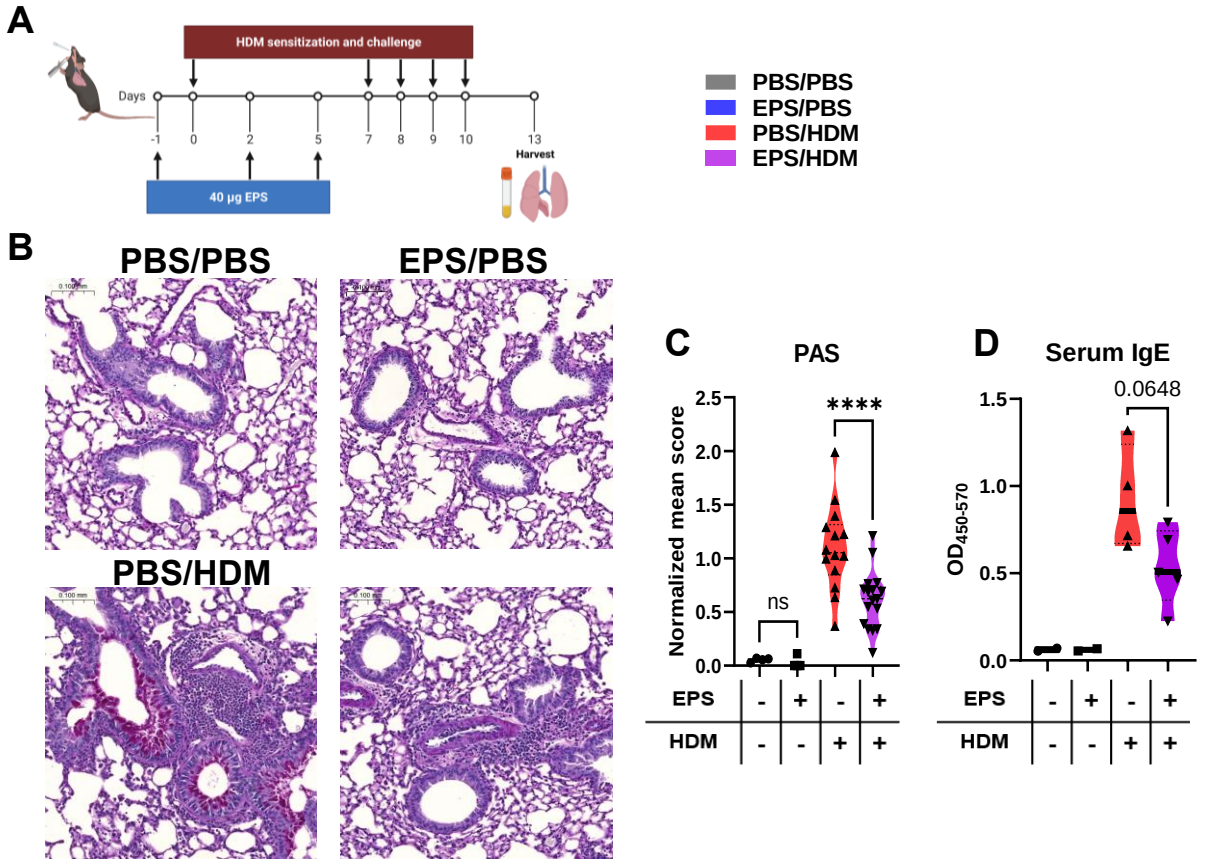

Figure 3-1: B. subtilis-derived EPS inhibits mucus secretion and circulating IgE during HDM-induced allergic inflammation. (A) Experimental schematic of EPS treatment during HDM sensitization and challenge. All distillations were performed intratracheally. At day 13, serum, BAL, lung, and mediastinal LNs were harvested for analysis. (B) Representative PAS staining from the lungs of mice treated as in (A). Magenta staining within the airways indicates mucus. Scale bar in the upper right of each image is 0.100 mm. (C) Mean PAS scores of airways in each stained lung section. All scores were normalized to the mean PAS of PBS/HDM-treated mice within each experiment. (D) Serum IgE measured by ELISA at day 13. ****, $p < 0.0001$ by one-way ANOVA with Sidak's multiple comparisons test. Diagram in (A) was generated in Biorender.

one, indicating that EPS is suppressing the eosinophilic response to HDM (Figure 3-2C). Interestingly, fewer CD4 T cells and DCs were recovered from the lungs of EPS/HDM-treated mice (Figure 3-2D, E).

Previous work in the HDM model has demonstrated that agents such as *A. lwoffii* inhibit allergic inflammation through inducing IL-6-dependent low-level, chronic inflammation (Alashkar Alhamwe et al., 2023). EPS does not appear to be inducing inflammation either on its

own or synergistically with HDM (Figure 3-2). If EPS induced an IL-6 dependent response, we would anticipate an increase in neutrophils in mice treated with EPS alone or EPS+HDM, as seen with *A. lwoffii*. To this end, we quantified the total number of neutrophils in the lungs of EPS+HDM-treated mice. EPS did not increase the number of neutrophils alone or in the context of HDM (Figure 3-2F). As such, we concluded that EPS is not suppressing allergic inflammation

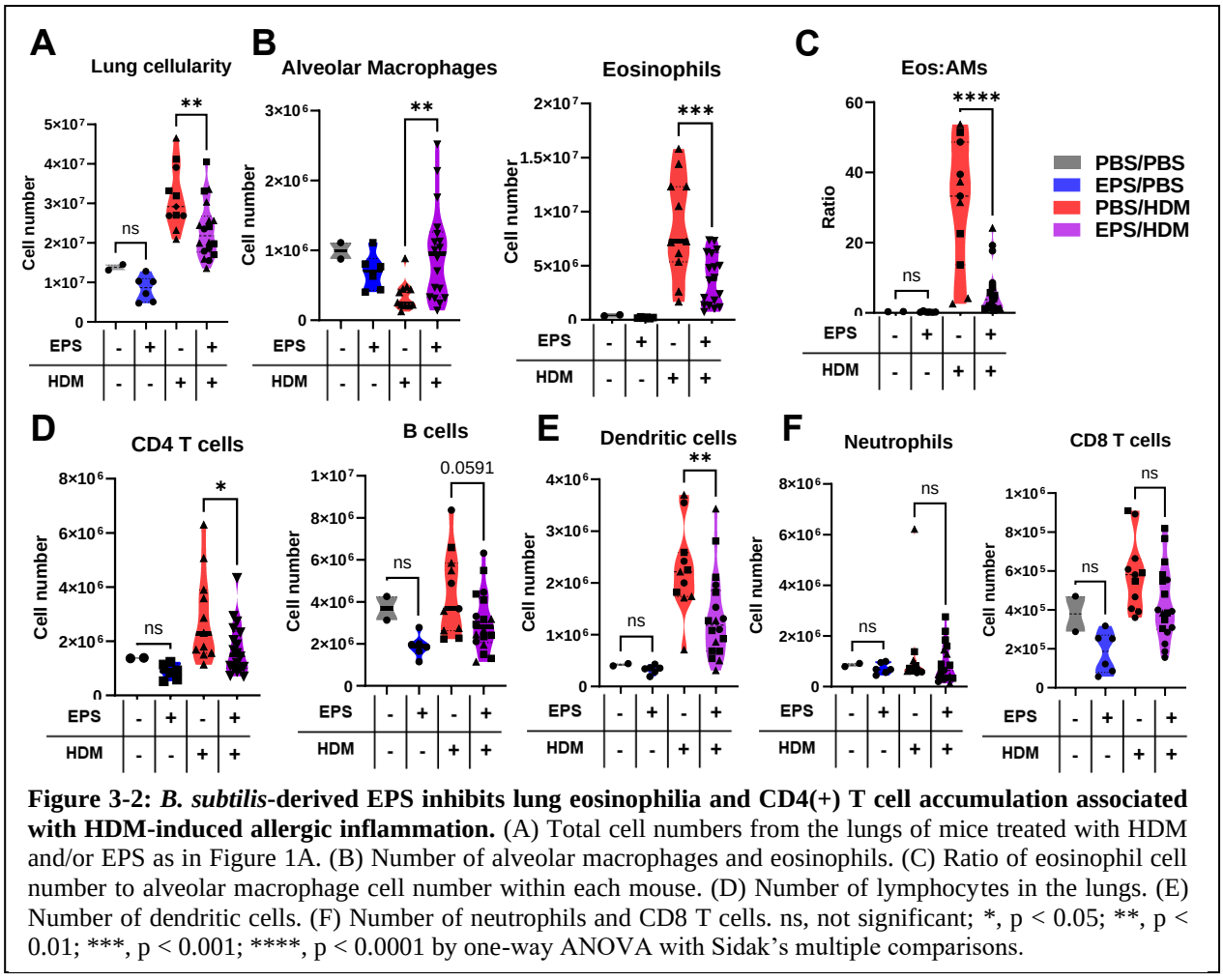

Figure 3-2: *B. subtilis*-derived EPS inhibits lung eosinophilia and CD4(+) T cell accumulation associated with HDM-induced allergic inflammation. (A) Total cell numbers from the lungs of mice treated with HDM and/or EPS as in Figure 1A. (B) Number of alveolar macrophages and eosinophils. (C) Ratio of eosinophil cell number to alveolar macrophage cell number within each mouse. (D) Number of lymphocytes in the lungs. (E) Number of dendritic cells. (F) Number of neutrophils and CD8 T cells. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ by one-way ANOVA with Sidak's multiple comparisons.

by skewing the lung towards a neutrophilic environment.

Because pathology in the HDM-driven model of allergic inflammation is localized to the airways as well as the lung parenchyma, we also quantified total cells in the bronchoalveolar lavage (BAL) of mice treated with EPS+HDM. EPS treatment around HDM sensitization also reduced

the total number of BAL cells (Figure 3-3A), BAL eosinophils (Figure 3-3B), BAL eosinophil to alveolar macrophage ratio(Figure 3-3C), and BAL CD4 T cells and B cells (Figure 3-3D), reflecting EPS-mediated dampening of the type 2 response in the lung parenchyma (Figure 3-2). Unlike the lung, EPS treatment reduced the accumulation of neutrophils and CD8 T cells (Figure 3-3G). Taken together with the histologic and flow cytometric analysis of the lung, we find that

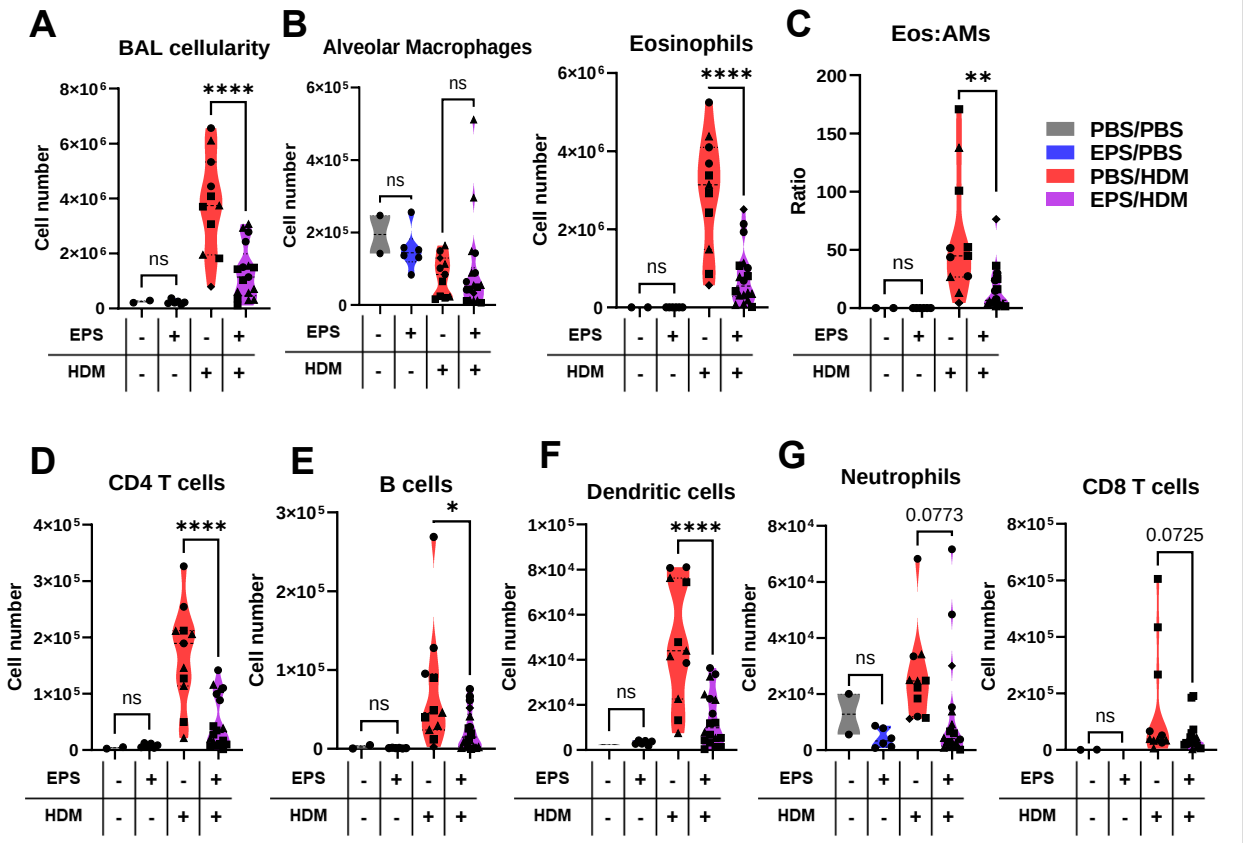

Figure 3-3: *B. subtilis*-derived EPS inhibits BAL eosinophilia and CD4(+) T cell accumulation associated with HDM-induced allergic inflammation. (A) Total cell numbers from the bronchoalveolar lavage (BAL) of mice treated with HDM and/or EPS as in Figure 1A. (B) Number of alveolar macrophages and eosinophils. (C) Ratio of eosinophil cell number to alveolar macrophage cell number within each mouse. (D) Number of lymphocytes in the BAL. (E) Number of dendritic cells. (F) Number of neutrophils and CD8 T cells. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$ by one-way ANOVA with Sidak's multiple comparisons.

EPS inhibits HDM-mediated allergic inflammation without skewing pulmonary immunity towards a neutrophilic phenotype.

Attenuated allergic inflammation by EPS exposure corresponds with fewer Th2 cells. *B. subtilis*-derived EPS has been demonstrated to inhibit CD4 T cells in other inflammatory contexts (Jones

et al., 2014; Paynich et al., 2017); as such, we hypothesized that the dramatic reduction in HDM-induced mucus secretion and eosinophilia was due to a defect in Th2 generation. To this end, we

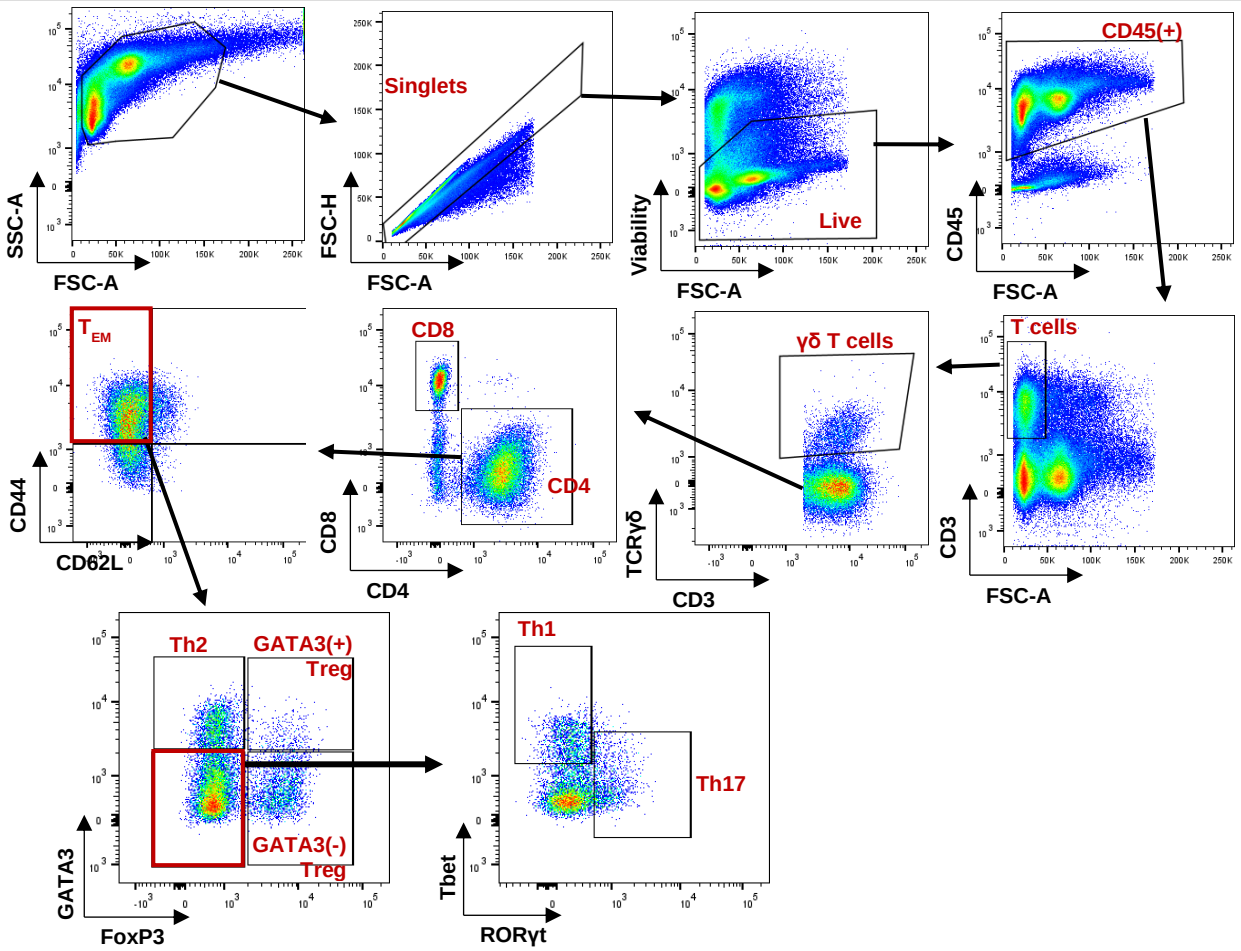

Figure 3-4: Gating strategy to identify different Th populations in the lung and lung-draining lymph node. After excluding doublets and dead cells, T cells were identified as CD45(+) and CD3(+). γδ T cells were excluded from downstream analysis by staining for the γδ T cell receptor. CD4 T cells were then subsetted by CD44 and CD62L. CD44(+)CD62L(-) T effector memory cells were then split into GATA3(+) Th2 cells, FoxP3(+)GATA3(+) Tregs, and FoxP3(+)GATA3(-) Tregs. Cells negative for GATA3 and FoxP3 were then subsetted into Tbet(+) Th1 cells and RORγt(+) Th17 cells.

quantified Th cells in the lungs of EPS+HDM-treated mice at day 13 of our model (gating strategy is described in Figure 3-4).

After HDM sensitization and challenge alone, GATA3(+) Th2 cells expanded dramatically in the lungs, as expected (compare the top left and bottom left panels of Figure 3-5A). However,

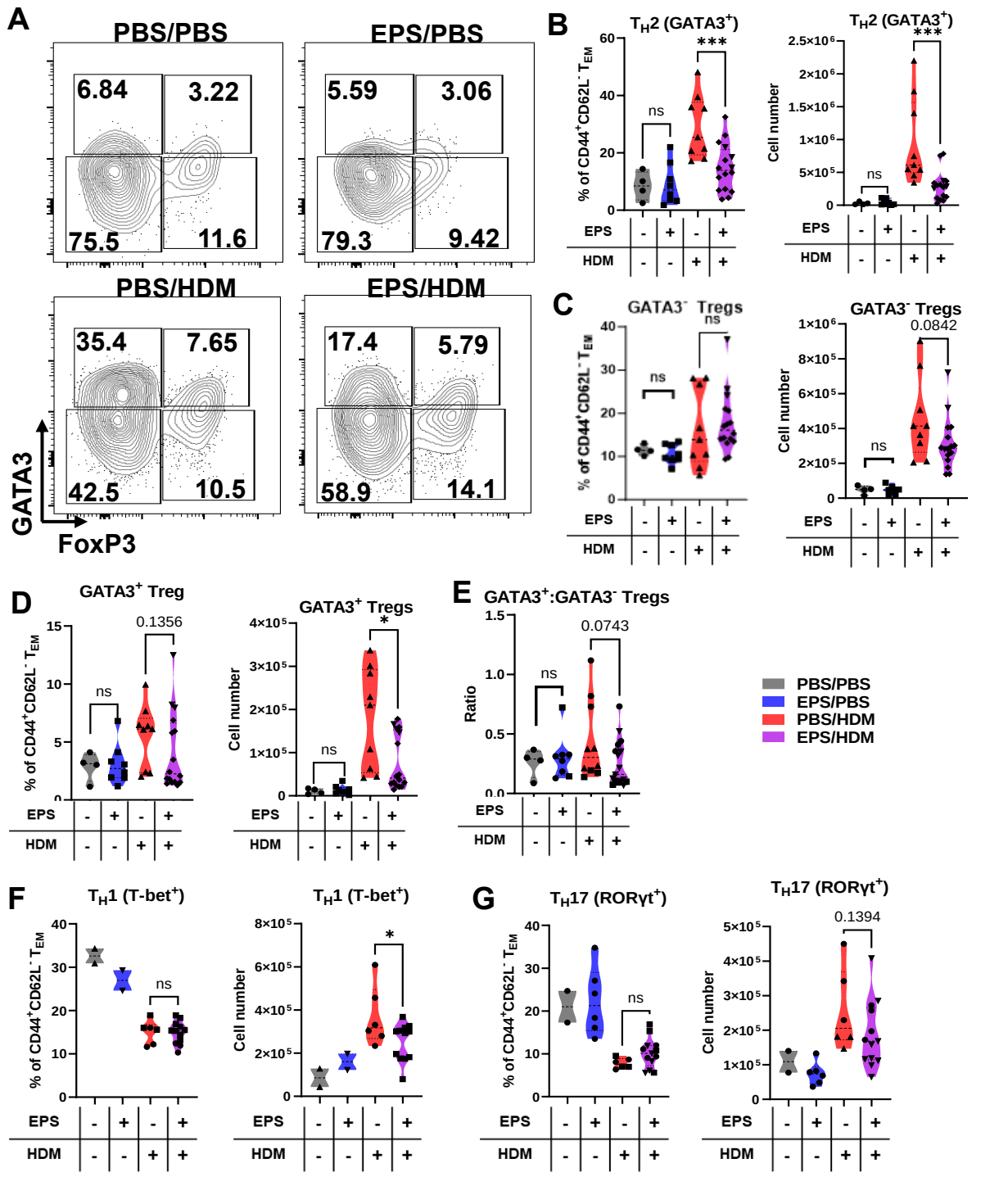

Figure 3-5: EPS inhibits GATA3-dependent T cell responses in the lung. (A) Representative flow plots of GATA3 and FoxP3 staining on lung CD4 T cells. Cells were pregated on live, CD45(+), CD3(+), TCRγδ(-), CD44(+)CD62L(-) populations. Numbers in boxes represent percentages of parent, i.e. CD44(+)CD62L(-) T effector memory (Tem) cells. (B) Quantification of GATA3 single-positive Th2 cells from the lungs of mice treated as in Figure 3-1A, by percentage of Tem cells (left) and backcalculated total cell numbers (right). (C) Quantification of GATA3(-) FoxP3(+) regulatory T cells as in (B). (D) Quantification of FoxP3(+)GATA3(+) cells as in (B). (E) Ratio of GATA3(+) to GATA3(-) regulatory T cells.

Figure 3-5, continued
(F) Quantification of T-bet(+)RORγt(-) cells as in (B). Tbet+ cells were subsetted from FoxP3-GATA3- cells. (G) Quantification of Rorgt+ cells as in (B). Rorgt(+) cells were subsetted from FoxP3-GATA3- cells. Plots (B)-(E) are three separate experiments with n ≥ 2 mice per group; plots (F)-(G) are two separate experiments with n ≥ 2 mice per group. Ns, not significant; *, p < 0.05; ***, p < 0.001; ****, p < 0.0001 by one-way ANOVA with Sidak's multiple comparisons.

addition of EPS to the HDM model inhibited accumulation of lung Th2 cells, both by percentage of effector memory T cells (Tem) and total cell numbers (Figures 3-5B). The reduction in Th2 cells was not due to Treg expansion, as FoxP3(+) Tregs were not significantly increased in EPS+HDM-treated mice as compared to HDM-only controls (Figure 3-5C). In analyzing these data, we observed that Tregs in HDM-treated mice could be divided into those that expressed the master Th2 transcription factor GATA3, and those that did not (Figure 3-5A). Little is known about GATA3(+) Tregs in the lung, but a recent study found that Tregs activated by IL-33 upregulated GATA3 and secreted type 2 effector cytokines IL-5 and IL-13(Chen et al., 2017), hinting at a potential pathogenic role for GATA3(+) Tregs in allergic inflammation. In line with these findings, EPS decreased the absolute number of these GATA3(+) Tregs (Figure 3-5D), and the ratio of GATA3(+) to GATA3(-) Tregs were reduced in EPS+HDM-treated mice (Figure 3-5E). Whether the reduction of GATA3(+) Tregs by EPS represents a specific mechanism by which EPS inhibits pathogenic Tregs remains a subject of future study.

Paralleling our findings that EPS does not induce neutrophilia or cytotoxic T cell expansion in the lung (Figure 3-3), EPS did not increase T-bet(+) Th1 (Figure 3-5F) or RORγt(+) Th17 (Figure 3-5G) cells within the CD4 T cell compartment. If anything, EPS slightly reduced Th1 and Th17 cell numbers, indicating that EPS was suppressing Th priming in general.

EPS dampens T cell priming in the lung-draining lymph node. After EPS treatment, we observe a reduction of total lung CD4 T cells and, more specifically, loss of GATA3(+) Th2 cells that are key effector cells in allergic responses. These data are consistent with previous work from the

Knight group, who have demonstrated that EPS suppress T cell proliferation *in vitro*(Paynich et al., 2017) and in the lamina propria of mice challenged with *Citrobacter rodentium* (Zamora-Pineda et al., 2023). Prior to homing to the site of inflammation, lung CD4 T cells are primed by DCs in the lung-draining lymph nodes (LDLNs). It is possible that, rather than negatively impacting Th2 generation in the HDM model, EPS treatment "traps" CD4 T cells in the lymph node, resulting in fewer CD4 T cells homing back into the lung parenchyma. Therefore, we analyzed Th cell frequencies in the LDLN at day 13 of the model depicted in Figure 3-1A.

In HDM-treated mice, approximately five percent of the CD4 Tem cells were GATA3-positive, reflecting that the majority of Th2 cells primed in the LN had already migrated to the lung by day 13. In mice treated with both EPS and HDM, however, the percentage of GATA3-positive CD4 Tem had dropped to 1-2%, which was comparable to controls that did not receive HDM (Figure 3-6A, left). This change in the relative proportion of Th2 cells in the LDLN was also reflected in fewer total GATA3(+) CD4 Tem (Figure 3-6A, right). The proportion and total number of GATA3(+)FoxP3(+) cells trended downward in mice given EPS and HDM relative to HDM-only controls (Figure 3-6B), reflecting the reduction in GATA3(+) Tregs in the lung. The percentage and number of GATA3(-) Tregs did not change in EPS+HDM-treated mice relative to HDM controls (Figure 3-6C); as such, the ratio of GATA3(+) to GATA3(-) Tregs was reduced in the LN, again consistent with the lower ratio of GATA3(+) Tregs to GATA3(-) Tregs in the lung (Figure 3-6D). Neither T-bet(+) Th1 cells (Figure 3-6E) nor RORγt(+) Th17 cells (Figure 3-6F) were reduced in EPS+HDM-treated mice, indicating that EPS is specifically dampening Th2 responses in the lung and LDLN during the peak of allergic inflammation.

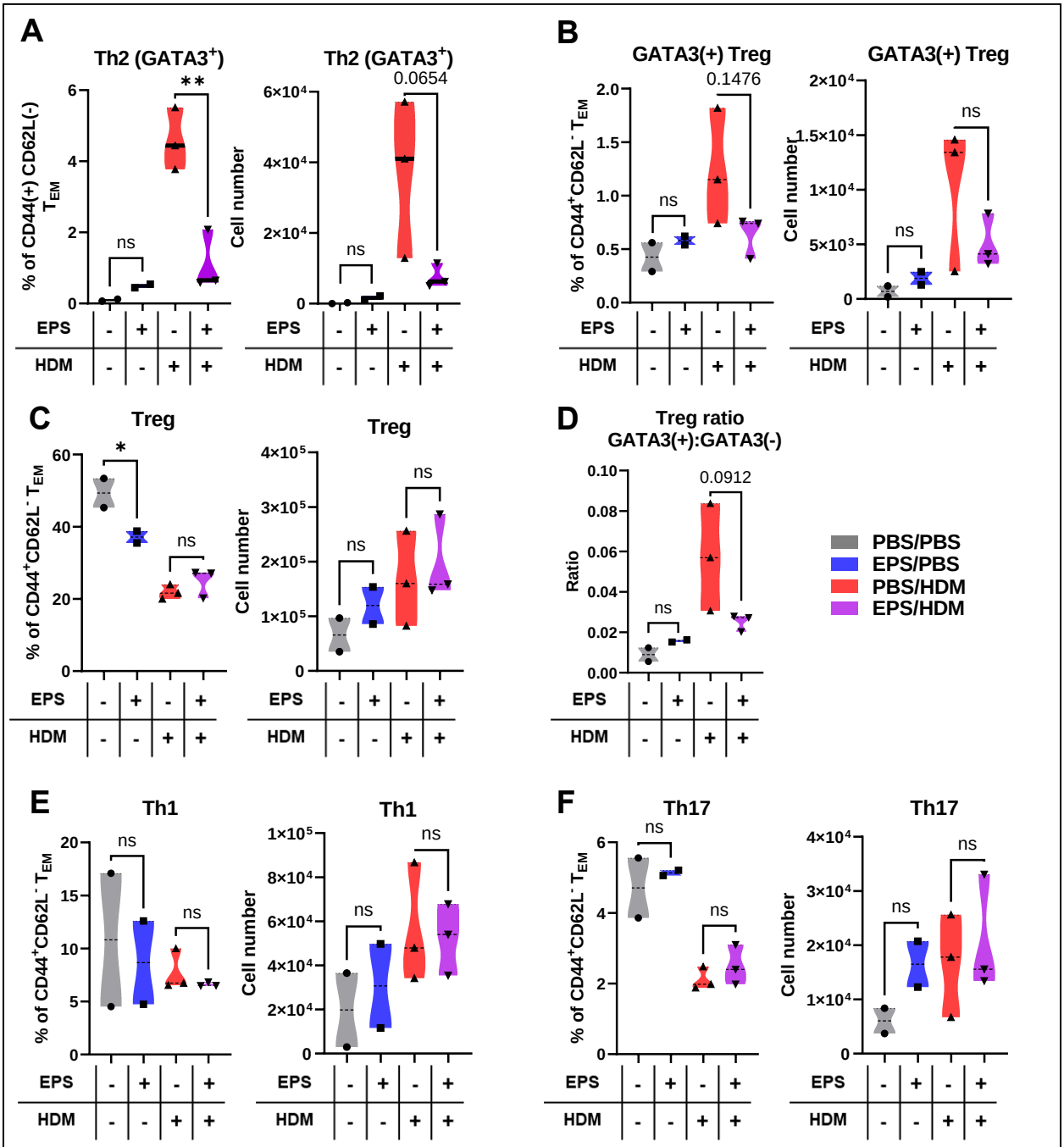

Figure 3-6: EPS also reduces GATA3-positive Th2 cells in the lung-draining lymph node. (A) Quantification of GATA3 single-positive Th2 cells from the lungs of mice treated as in Figure 3-1A, by percentage of Tem cells (left) and backcalculated total cell numbers (right). (B) Quantification of FoxP3+GATA3+ cells as in (A). (C) Quantification of GATA3- FoxP3+ regulatory T cells as in (A). (D) Ratio of GATA3(+) Tregs to GATA3(-) Tregs in the LDLN. (E) Quantification of GATA3(-)FoxP3(-)T-bet(+) Th1 cells as in (A). (F) Quantification of GATA3(-)FoxP3(-)RORγt(+) Th17 cells as in (A). ns, not significant; *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA with Sidak's multiple comparison test.

TLR4 is required for EPS's anti-inflammatory effects. The Knight group has previously demonstrated that EPS treatment does not reduce disease scores and crypt height in TLR4-deficient *C. rodentium*-infected mice(Jones et al., 2014). Furthermore, EPS-treated peritoneal myeloid cells are incapable of inhibiting T cell proliferation if derived from a TLR4-deficient animal(Paynich et al., 2017). From these data, they concluded that EPS prevents colitis pathology through engaging TLR4 on myeloid cells. To test whether TLR4 was also necessary for EPS to inhibit allergic inflammation *in vivo*, we subjected TLR4-lps-del mice, in which the entire *Tlr4* coding sequence is removed(Poltorak et al., 1998), to HDM sensitization and challenge as in Figure 3-1A. We expected that TLR4-deficient mice would not respond to HDM, as previous work has demonstrated that TLR4 on structural cells is necessary and sufficient for allergic responses to HDM(Hammad et al., 2009). Surprisingly, we observed that eosinophils comprised 15-30% of live CD45(+) cells in the lungs of TLR4-deficient mice, which was not significantly different from the proportion of eosinophils present in TLR4 heterozygous mice and significantly increased compared to HDM-naïve controls (Figure 3-7A). While unexpected, the finding that TLR4 deficiency doesn't completely abolish allergic inflammation allowed us to further investigate whether EPS-mediated protection from allergic inflammation depended on TLR4. After normalizing eosinophil counts across experiments, we found that EPS did not significantly affect eosinophil counts in TLR4-deficient mice (Figure 3-7B). Additionally, EPS treatment in TLR4-deficient mice did not significantly increase lung AMs (Figure 3-7C), unlike in B6 mice where EPS treatment increased lung AMs (Figure 3-1B). Taken together, these data indicate that EPS's dampening of HDM-mediated inflammation is TLR4-dependent.

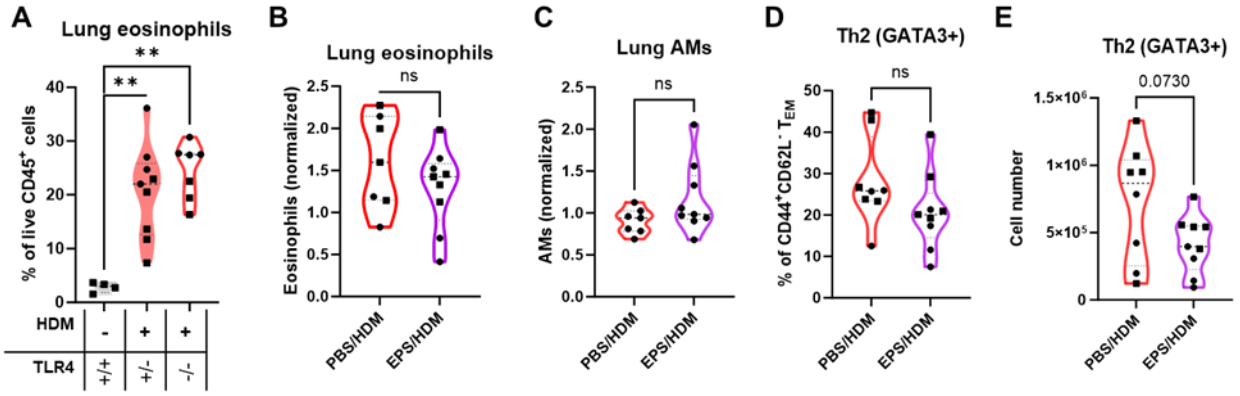

Figure 3-7: EPS-mediated protection from allergic inflammation is TLR4-dependent. (A) Lung eosinophilia as a percentage of lung hematopoietic cells after HDM sensitization and challenge. (B) Total number of eosinophils from the lungs of TLR4-deficient mice mice treated with PBS/HDM or EPS/HDM as in Figure 3-1A. Cell numbers were normalized to the mean number of eosinophils in PBS/HDM-treated mice. (C) Total numbers of AMs in the lung of TLR4-deficient mice treated with PBS/HDM or EPS/HDM, normalized as in (B). (D) GATA3(+) Th2 cells in the lungs of PBS/HDM- or EPS/HDM-treated TLR4-deficient mice, quantified as the percentage of Tem cells. (E) Total numbers of lung Th2 cells in PBS/HDM- or EPS/HDM-treated mice, normalized as in (B). (A): **, $p < 0.01$ by one-way ANOVA with Sidak's multiple comparisons test. (B)-(E): ns, not significant by unpaired two-tailed t test.

To determine if TLR4 deficiency also affects Th2 cell generation in the HDM model, we stained for Th transcription factors in the lungs of TLR4-lps-del/del mice treated with EPS and/or HDM. Curiously, the percentage of GATA3(+) Th2 cells was not significantly altered by loss of TLR4 (compare PBS/HDM groups in Figure 3-5B and Figure 3-7D). This finding was unexpected, as LPS sensing through TLR4 was previously thought necessary for dendritic cell priming of Th2 responses (Hammad et al., 2009). Regardless, in TLR4-deficient mice, Th2 cells as a percentage of Tems were not significantly affected by the addition of EPS (Figure 3-7D). The total number of Th2 cells in TLR4-lps-del/del mice treated with EPS and HDM was slightly reduced, although this reduction did not reach significance due to variation in cell counts in the HDM-treated group (Figure 3-7E). Taken together with the eosinophil and AM data, we concluded that EPS inhibits allergic inflammation in the HDM model by signaling through TLR4.

Continuous administration of EPS during the HDM model optimally inhibits allergic inflammation. Because EPS given around the time of sensitization reduced Th2 cell numbers in the lung and LDLN, we hypothesized that our current treatment schedule prevented allergic inflammation by interfering with T cell priming, which reaches its peak at approximately day 5 in our model. However, it is possible that EPS given around the time of HDM challenge would prevent re-activation of Th2 cells and blunt the response to HDM. To test this hypothesis, we modified the EPS treatment schedule to occur at days 5, 7, and 9, i.e. centered around the first challenge with HDM. We also included a group in which EPS was given at both early and late timepoints relative to HDM sensitization (Figure 3-8A).

We then quantified eosinophilia and lung Th2 cells at day 13 relative to sensitization. As expected, early treatment with EPS prevented eosinophil accumulation in the lung. In mice treated with EPS throughout the model, eosinophils trended downward relative to PBS/HDM controls ($p = 0.2247$ by one-way ANOVA with Sidak's multiple comparisons test; Figure 3-8B, orange versus red groups). If EPS was only given during the challenge phase, there was a minor decrease in eosinophils that failed to reach statistical significance ($p = 0.399$; Figure 3-8B, orange versus blue groups). Analysis of AMs within the lung tissue revealed that EPS treatment increased AMs somewhat in the early treatment group, almost significantly in the late treatment group, and very significantly in the continuous treatment group (Figure 3-8C). When data were normalized by taking the ratio of eosinophils to macrophages within lungs, EPS treatment—whether early, late, or continuous—significantly reduced the relative quantity of eosinophils (Figure 3-8D). These trends were also found in the BAL: eosinophilia was not significantly reduced in mice treated with EPS+HDM around challenge (Figure 3-8E) and AM numbers were increased, albeit not significantly (Figure 3-8F); when normalized by taking the ratio of eosinophils to AMs, however,

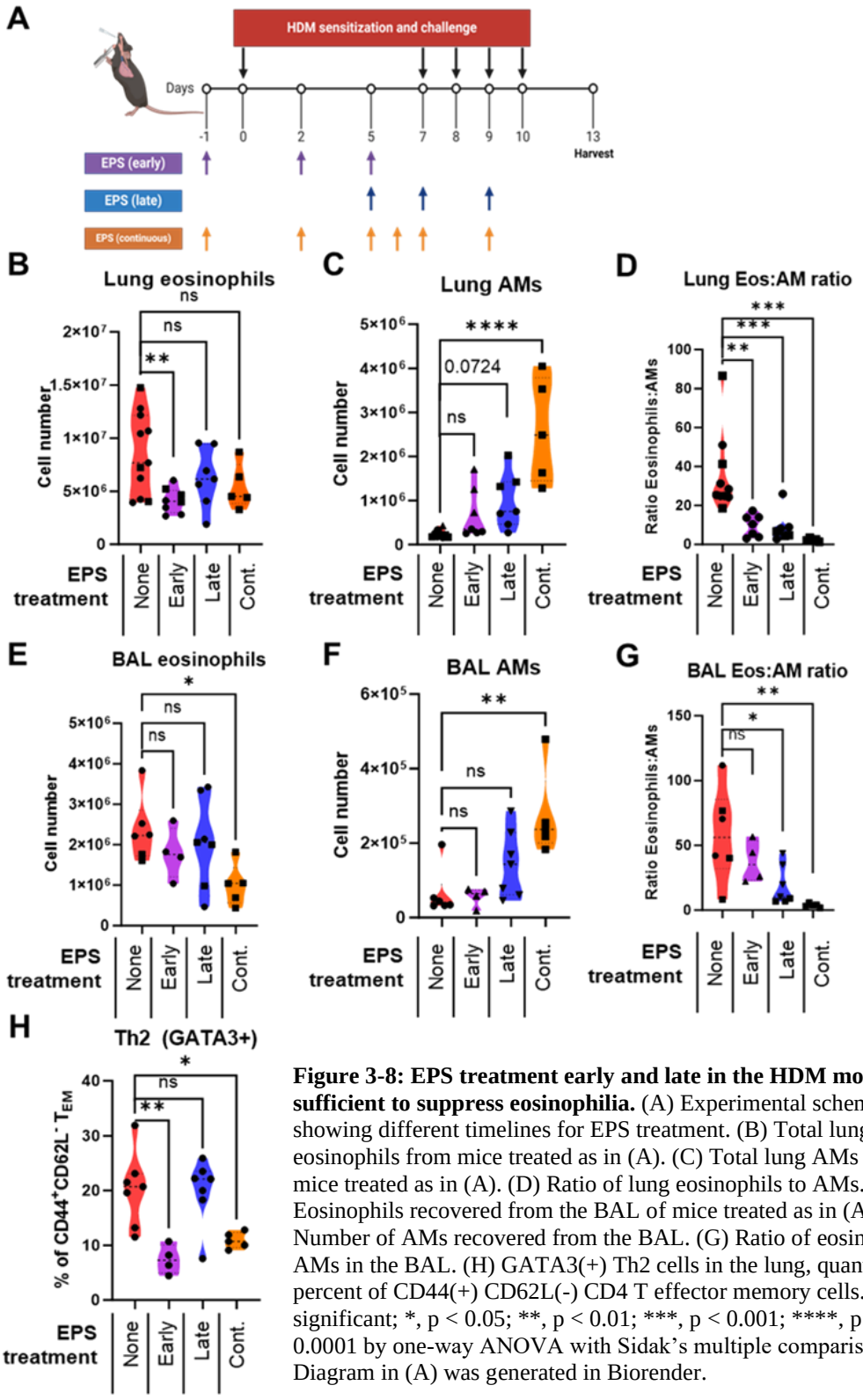

Figure 3-8: EPS treatment early and late in the HDM model is sufficient to suppress eosinophilia. (A) Experimental schematic showing different timelines for EPS treatment. (B) Total lung eosinophils from mice treated as in (A). (C) Total lung AMs from mice treated as in (A). (D) Ratio of lung eosinophils to AMs. (E) Eosinophils recovered from the BAL of mice treated as in (A). (F) Number of AMs recovered from the BAL. (G) Ratio of eosinophils to AMs in the BAL. (H) GATA3(+) Th2 cells in the lung, quantified as percent of CD44(+) CD62L(-) CD4 T effector memory cells. Ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ by one-way ANOVA with Sidak's multiple comparisons test. Diagram in (A) was generated in Biorender.

EPS treatment late or throughout the HDM model suppressed eosinophil enrichment in the BAL (Figure 3-8G). These data suggest that EPS treatment during challenge also plays a role in increasing AMs in the lung. Whether this increase results in improved lung function or reduced pathology requires further studies.

We next wanted to determine whether EPS treatment late in the model interfered with Th2 cell accumulation at day 13. To this end, we quantified Th2 cells in the lungs of mice that underwent early, late, or continuous EPS treatment. After continuous (orange) or early (purple) EPS treatment, the proportion of Th2 cells out of total T effector memory cells was significantly reduced relative to HDM-treated controls (Figure 3-8E). In mice given EPS after challenge, however, there was no significant difference in the percentage of Th2 cells within the Tem compartment as compared to HDM-treated controls (Figure 3-8E, blue group). Thus, the increase in AMs relative to eosinophils when EPS was given around challenge may occur independently of suppressing Th2 responses.

Discussion

In this study, we identified a novel role for the *B. subtilis* by-product EPS in preventing allergic inflammation *in vivo*. When administered intratracheally, three doses of EPS around the time of sensitization was sufficient to prevent a number of parameters associated with HDM-induced pulmonary inflammation, such as mucus hypersecretion as measured by PAS staining, serum IgE, lung and BAL eosinophilia, and lung CD4 T cell expansion (see Figure 3-9 for a summary of key results). This suppressive effect of EPS on allergic inflammation was dependent on TLR4, as TLR4-deficient mice treated with EPS were not protected from allergic eosinophilia (Figure 3-7). Finally, EPS rescued the eosinophil:AM ratio if given around challenge without

affecting Th2 cell numbers, arguing that, depending on the timing of treatment, EPS can interfere with different arms of the allergic response (Figure 3-8).

Although late EPS treatment does not significantly affect the number of lung and BAL eosinophils on its own, the increase in AMs relative to eosinophils suggests that EPS signaling through TLR4 affects the lung cell milieu in a T cell-independent number. AMs increase in the lung after HDM challenge, as the lung returns to homeostasis. Furthermore, AM depletion during the resolution phase of the HDM model delays clearance of neutrophils, Th2 cells, and interstitial macrophages (Mathie et al., 2015), suggesting that AMs play a central role in resolving lung inflammation. Therefore, the increase in AMs observed when EPS is given around HDM challenge could be a pro-resolving response induced by EPS on AMs directly. Whether EPS also accelerates resolution of the allergenic response to HDM, and whether EPS can positively impact lung function after the establishment of memory to HDM, would be an exciting future direction for this work. If EPS is accelerating resolution of inflammation after HDM exposure, it could have further utility as a therapeutic agent during asthma flares.

Other bacteria and bacterial products have been used to prevent allergic inflammation *in vivo*, namely endotoxin, *Acinetobacter lwoffii*, and bacterial lysate OM-85. These agents work through different mechanisms to inhibit allergic inflammation: bacterial lysates such as OM-85 induce regulatory T cells through activating CD103(+) dendritic cells(Pivniouk et al., 2022); *A. lwoffii* shifts the lung environment to a neutrophilic environment at the expense of eosinophilia(Debarry et al., 2007); and endotoxin is proposed to suppress epithelial release of the pro-Th2 cytokines IL-33, CCL20, and GM-CSF through inducing the ubiquitin-modifying enzyme A20(Schuijs et al., 2015). In contrast to *A. lwoffii* and OM-85, EPS does not increase the number of neutrophils in the lung (Figure 3-2F) or BAL (Figure 3-3F) or the number of regulatory T cells

in the lung (Figure 3-5C). Rather, EPS given around the time of sensitization specifically appears to blunt the generation of Th2 cells. As Th2 cells are an important source of crucial mediator cytokines IL-4, IL-5, and IL-13 (Lambrecht et al., 2019), it is likely that EPS, by reducing total Th2 cells in the lung, also reduces total lung IL-4/IL-5/IL-13, thereby preventing maximal IgE production, eosinophilia, and mucus hypersecretion, respectively.

The observation that EPS treatment around the time of challenge also rescues mice from eosinophilia without reducing lung Th2 cells(Figure 3-8) may suggest that EPS treatment in an already-sensitized animal can still protect mice from allergic inflammation. Notably, there are more AMs in the BAL of mice treated during the challenge phase rather than the sensitization phase (Figure 3-8F, $p = 0.0921$ by unpaired t test). AMs are primarily responsible for surveying the alveolar space and capturing inhaled particulates; as such, they are crucial for maintaining lung homeostasis (Aegerter et al., 2022). AMs also secrete anti-inflammatory cytokines such as TGF-β that are crucial for suppressing accumulation of inflammatory monocytes in the alveoli after allergen exposure (Branchett et al., 2021). Therefore, it is possible that TLR4 engagement by EPS can "turn on" the anti-inflammatory functions of AMs in an attempt maintain homeostasis, even in the presence of allergen. Alternatively, the increase in AMs in the late model could be a marker, rather than a causal player, in preventing allergic inflammation.

Alveolar macrophages express many pattern recognition receptors, including TLRs such as TLR4 (Schuijs et al., 2019). In the context of systemic *S. aureus* infection, EPS treatment generates peritoneal macrophages with increased intracellular ROS that are superior at bacterial clearance yet suppress T cell activation in response to *S. aureus* (Paik et al., 2019). One could imagine such "dual functioning" macrophages also at work in an HDM-sensitized lung, where they may perform similar anti-inflammatory functions. Whether AMs are functionally altered in

the presence of EPS during HDM treatment would shed insight on how EPS affects the innate immune milieu other than dendritic cells, which are discussed in the next chapter. In addition, studies in which EPS is given after establishment of an allergen-specific T cell population would shed exciting new light on whether EPS could suppress pre-existing allergy and open up the possibility of EPS as a treatment, rather than a prophylactic, for allergic asthma.

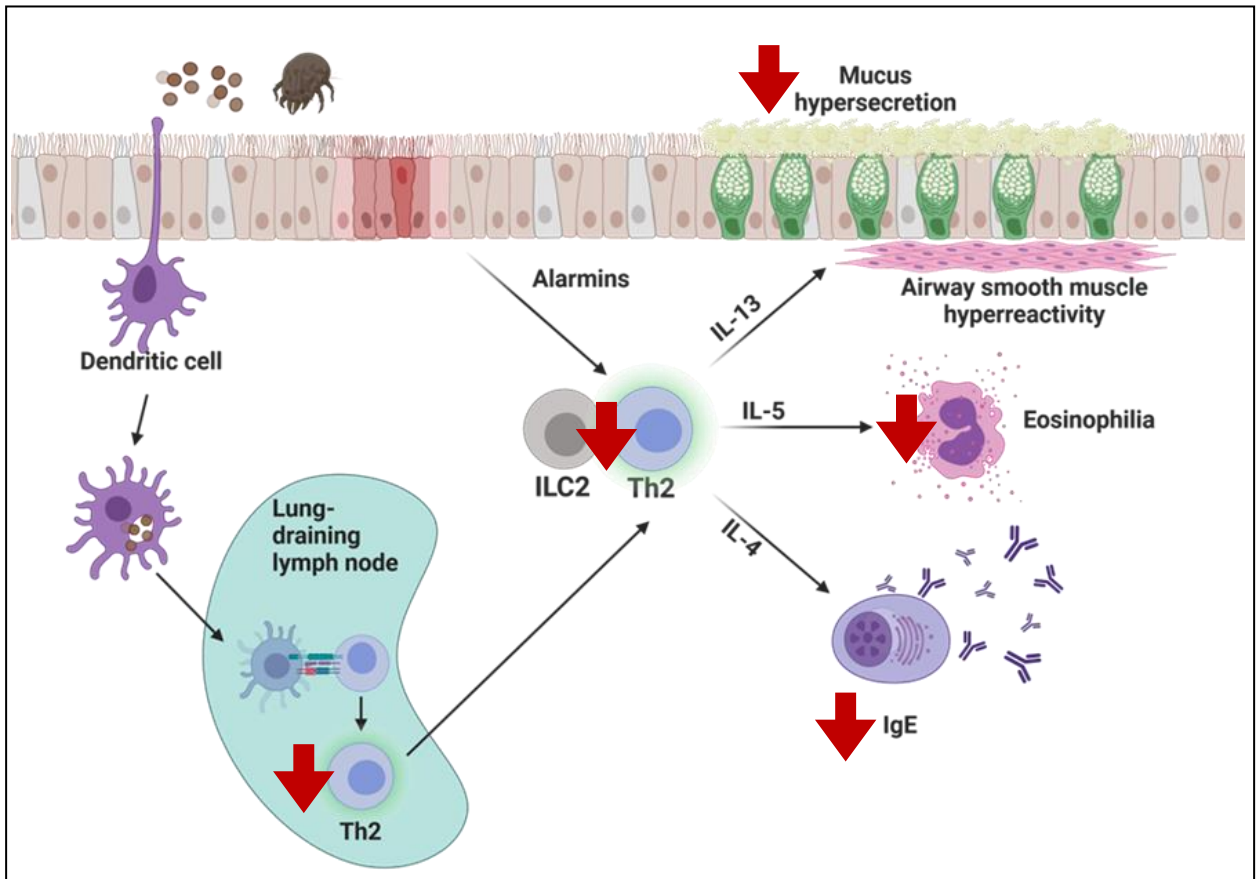

Figure 3-9: Inhaled EPS inhibits HDM-induced inflammation *in vivo*. Diagram depicting the downstream effectors of HDM-mediated allergic inflammation, with red arrows indicating which effector(s) are suppressed by EPS treatment. Image generated in Biorender.

It is intriguing to consider that endotoxin and EPS, which share a receptor, may act through the same molecular pathways to prevent sensitization to allergen. However, an important difference between EPS and LPS is the effects of each agent given alone. Throughout our studies, EPS did not significantly affect mucus staining or serum IgE (Figure 3-1) or accumulation of any

hematopoietic cell subset in the lung or BAL (Figures 3-2 and 3-3). One way to interpret these findings is that EPS may be inert in the lung without another immune stimulus. LPS, however, can induce type 2 immunity, type 1 immunity, or tolerance, depending on the dose and frequency of administration (Delayre-Orthez et al., 2005; Eisenbarth et al., 2002; Hammad et al., 2009; Hollingsworth et al., 2006). Therefore, LPS is not a viable prophylactic candidate for preventing allergic asthma. The ability of EPS to prevent allergic sequelae without affecting the pulmonary hematopoietic compartment is yet another piece of evidence to support that EPS may be superior to endotoxin as an anti-allergenic treatment.

In the context of endotoxin tolerance during HDM responses, loss of epithelial-derived CCL20 and GM-CSF results in the recruitment of fewer DCs to the lung and LDLN within 24 hours of HDM sensitization. In turn, fewer Th2 cells were generated, thereby preventing amplification of the type 2 response(Schuijs et al., 2015). Early EPS treatment (i.e. around sensitization) may also compromise the ability of DCs to induce Th2 cell priming and differentiation, as there are fewer Th2 cells in the lung (Figure 3-5B) and LDLN (Figure 3-6A) at day 13 post-sensitization. This possibility will be explored further in Chapter 4.

Chapter 4: EPS modulates cDC function to suppress Th2 priming.

Introduction

The primary function of DCs is to integrate innate signals such as LPS and extracellular ATP from the immediate environment and transform these environmental cues into “instructions” for Th cell priming and differentiation. There are three types of DC signals necessary to successfully prime a naïve T cell. First, peptide:MHC complexes on the surface of the DC engage with the T cell receptor, ensuring that the activated T cell is antigen-specific. Second, costimulatory molecules on the surface of the DC engage with their cognate receptors on the T cell to enhance TCR signaling, T cell survival, and proliferation. Third, cytokines secreted by the DC into the extracellular space are sensed by nearby T cells and, for most CD4 T cells, drive differentiation into a specific Th subset(Bakdash et al., 2013).

As previously discussed, multiple microbes and their by-products have been used to dampen allergic responses *in vivo*. In the context of OVA/alum-induced allergic inflammation, addition of moderate levels of LPS (70 EU/mg) to OVA reduced total lung eosinophils, an effect that was not seen in TLR4-deficient mice. During subsequent challenge with OVA, TLR4-deficient mice had elevated numbers of CD11c(+)CD11b(+) parenchymal DCs relative to wild-type mice, indicating that LPS exposure may be preventing the accumulation of cDC2s in this model (Hollingsworth et al., 2006). A subsequent study in Science found an increase in CD11b(+) cDCs after sensitization to HDM, which was attenuated in LPS-pretreated mice. This lack of DC infiltration was dependent on LPS sensing by epithelial cells, after which epithelial cells reduced

expression of DC chemoattractants CCL20 and GM-CSF(Schuijs et al., 2015). Hence, endotoxin tolerance in the context of type 2 inflammation was due to TLR4 sensing by epithelial cells, rather than direct sensing by DCs.

Studies of the bacterial lysate OM-85 in OVA/alum-induced allergic inflammation has found that OM85-treated animals had attenuated airway hyperreactivity, BAL eosinophilia, and airway goblet cell hyperplasia. Similar to the Schuijs study, Pivniouk and colleagues found that treatment with OM-85 decreased total CD11b(+) cDCs in the lung tissue. OM-85 pretreated animals, however, had expanded CD103(+) cDC1 in the lung and LDLN, which correlated with expanded lung Tregs. In another departure from the mechanisms by which endotoxin prevent allergic inflammation, transfer of OM-85-treated, OVA-pulsed BMDCs to hosts prior to challenge with OVA resulted in increased lung Tregs, decreased airway hyperreactivity, and reduced lung eosinophils. These findings indicate that OM-85, unlike LPS, can act directly on myeloid cells to prevent generation of a Th2 response. Specifically, OM-85 treatment expands CD103(+) cDC1s and, in turn, regulatory T cells. The molecular agent(s) within OM-85 responsible for this expansion remain to be elucidated, though the signaling pathway(s) necessary for the response to OM-85 require Trif/MyD88(Pivniouk et al., 2022).

The bacterium *A. lwoffii* also induces an anti-asthmatic phenotype *in vivo* through multiple parallel mechanisms, including reprogramming of lung macrophages (Kang et al., 2022) and induction of low-level, chronic IL-6-dependent inflammation in the lung tissue and serum. Direct stimulation of BMDCs with *A. lwoffii* results in enhanced secretion of IL-6, IL-1β, and TNF-α to an even greater degree than treatment with LPS(Alashkar Alhamwe et al., 2023). Because *A. lwoffii* is a Gram-negative bacterium, it is possible that its anti-allergenic effects are primarily mediated through an endotoxin tolerance-like mechanism. Current published studies of *A. lwoffii*

do not comment on the necessity of *A. lwoffii* recognition by DCs to exert its allergy-protective effects.

B. subtilis EPS is known to blunt inflammatory responses through TLR4 signaling on myeloid cells, as previous studies by the Knight group have demonstrated that TLR4-deficient BMDCs treated with EPS are unable to suppress CD4 T cell proliferation *in vitro*(Kalinina et al., 2021). Subsequent work by Zamora-Pineda et al demonstrated that fluorescent EPS binds directly to CD11c(+) and F4/80(+) cells in the colonic lamina propria after i.p. injection with EPS, highlighting that EPS interacts directly with myeloid cells *in vivo* (Zamora-Pineda et al., 2023). Consistent with the idea that EPS acts through myeloid cell engagement, previous work by our colleagues has found that the adoptive transfer of EPS-treated bone marrow-derived dendritic cells (BMDCs) was sufficient to protect mice from HDM-mediated allergic inflammation(Swartzendruber et al., 2019). However, the manner in which EPS affects DC function *in vitro* and *in vivo,* and whether these effects impact the capacity of these DCs to prime T cell responses, is not currently known. In this Chapter, I will dissect the mechanism(s) by which EPS impairs T cell priming by DCs.

Results.

EPS-treated BMDCs are inferior at inducing antigen-specific T cell proliferation.

EPS reduces CD4 T cells, particularly GATA3(+) Th2 cells, in the lung (Figure 3-5) and LDLN (Figure 3-6). As such, we hypothesized that EPS exposure results in suboptimal T cell priming and/or differentiation. To test this hypothesis, we first employed a bone marrow-derived dendritic cell (BMDC):T cell coculture system. In this system, we generated bone marrow-derived dendritic cells (BMDCs) using Flt3L, which biases differentiation of common myeloid progenitors

towards DCs and away from monocytes and macrophages(Lutz et al., 1999; Naik et al., 2005). After eight days of differentiation, cells were washed and resuspended in fresh media containing GM-CSF and EPS for overnight maturation. The next day, cells were stimulated for 2.5 hours with HDM and OVA, then co-cultured with purified OTII T cells labeled with Cell Trace Violet (CTV). After four days, T cell proliferation was quantified by CTV dilution. A schematic summarizing our experimental approach is found in Figure 4-1A.

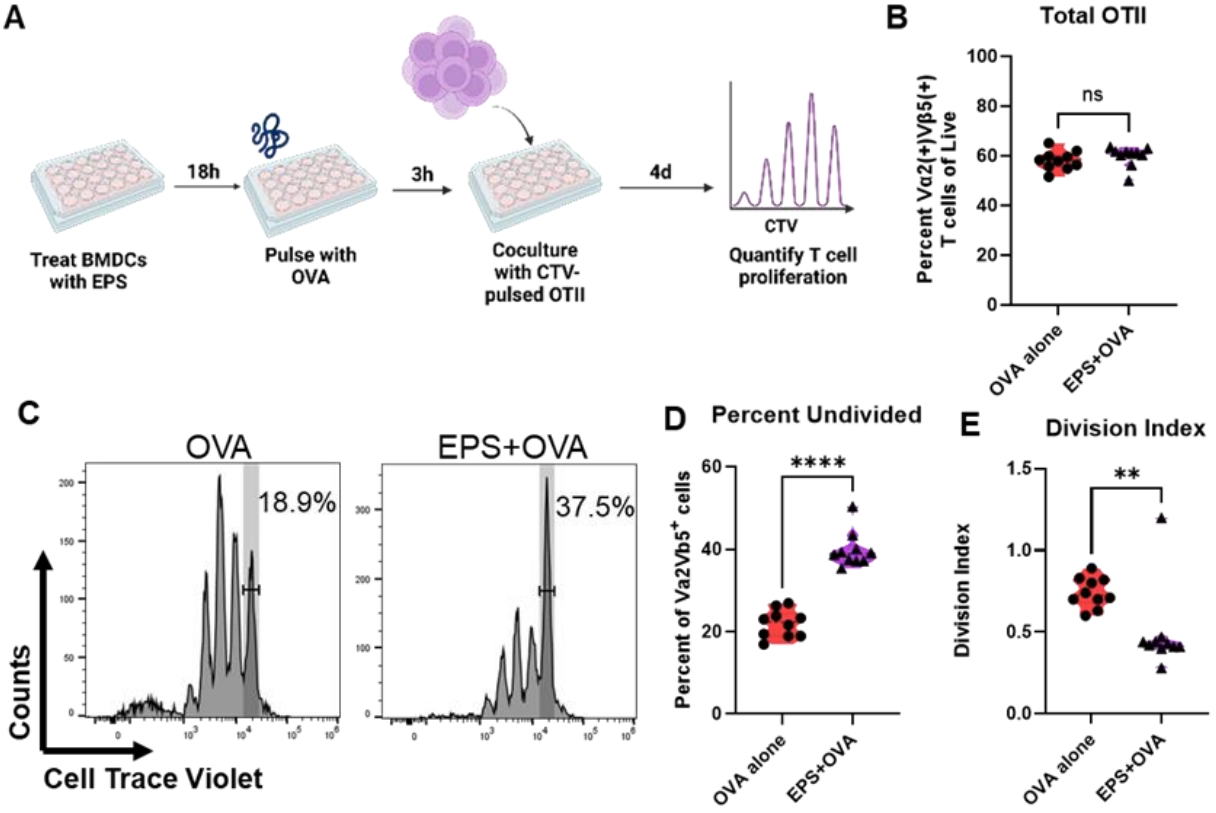

Figure 4-1: EPS-treated BMDCs are inferior at inducing antigen-specific T cell proliferation. (A) Schematic depicting experimental design. (B) Total OTII cells, quantified as the percentage of Vα2(+)Vβ5(+) of live cells. (C) Representative CTV dilution histograms of OTII cells cocultured with OVA-pulsed BMDCs or EPS-pretreated, OVA-pulsed BMDCs. The gray bar represents the peak of undivided cells. (D) Percentage of undivided cells. (E) Division index of OTII cells from FlowJo proliferation modeling. Ns, not significant; **, $p < 0.01$; ****, $p < 0.0001$ by unpaired t test. Diagram in (A) generated in Biorender.

In vitro, EPS exposure did not result in fewer OTII cells four days after culture (Figure 4-1B). In response to OVA exposure, the majority (>80%) of OTII cells proliferated at least once,

with only ~20% of OTII cells remaining undivided (Figure 4-1C, left panel). If OTII cells were co-cultured with EPS-treated BMDCs, however, the fraction of OTII cells that did not proliferate doubled to 40% (Figure 4-1C,D). Furthermore, OTII cells cocultured with EPS-treated BMDCs underwent fewer divisions on average, as measured by the division index (Figure 4-1E). From these data, we concluded that EPS treatment *in vitro* blunted antigen-specific T cell proliferation.

EPS directly induces a maturation-like phenotype on BMDCs.

The ability of EPS to negatively impact T cell proliferation *in vitro* indicated that EPS was preventing BMDCs from providing Signal 1 and/or Signal 2 to antigen-specific T cells. As such, we investigated the phenotype of BMDCs treated overnight with EPS. In this assay, we tracked the ability of BMDCs to phagocytose antigen using fluorescent OVA-AF647, as well as surface costimulatory molecule expression by flow cytometry (Figure 4-2A).

Antigen uptake in BMDCs varied based on their expression of CD103—25-30% of CD103(+) BMDCs were positive for OVA-AF647 (Figure 4-2B,C), as compared to only ~18% of CD103(-) CD11b(+) BMDCs (Figure 4-2B,D). Regardless of BMDC subset, overnight treatment with EPS dramatically reduced the frequency of OVA-AF647(+) cells (Figure 4-2C,D). We also quantified CD86 MFI on the surface of treated BMDCs and found that BMDCs exposed to EPS dramatically upregulated CD86 relative to untreated or HDM+OVA-647 only controls (Figure 4-2E). BMDCs treated only with EPS also expressed more CD86, indicating that EPS is impacting costimulation in an antigen-independent manner. The activation of these BMDCs in the absence of antigen may have resulted in an APC capable of providing Signal 2, but not Signal 1, to naïve T cells, thereby hindering their proliferative capacity. Intriguingly, this the first time in our studies that we have observed samples treated with EPS alone acting differently than PBS-treated control samples.

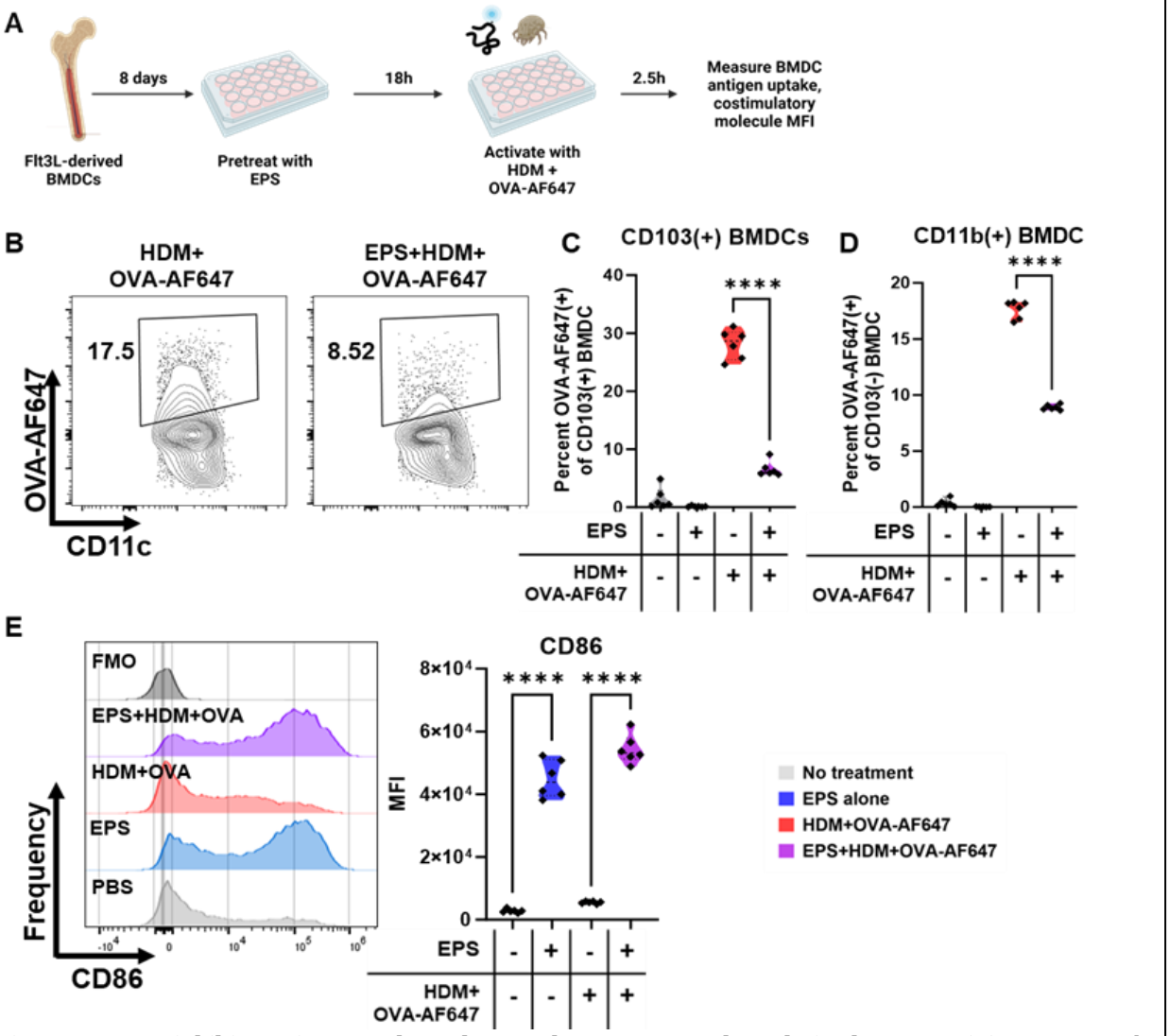

Figure 4-2: EPS inhibits antigen uptake and upregulates CD86 on Flt3L-derived BMDCs. (A) Experimental schematic. (B) Representative flow plots of CD11b(+) BMDCs, represented by antigen uptake (OVA-AF647, y axis) and CD11c (x axis). (C) Antigen uptake in CD103(+) BMDCs, measured by the percentage of CD103(+) BMDCs positive for OVA-AF647. (D) Antigen uptake in CD103(-) CD11b(+) BMDCs, quantified as in (C). (E) Representative histograms of CD86 fluorescence intensity (left) and quantification of CD86 median fluorescence intensity (right). ****, $p < 0.0001$ by one-way ANOVA with multiple comparisons. Diagram in (A) generated in Biorender.

EPS prevents accumulation of antigen-positive cDCs in the lung-draining lymph node.

We and others have previously demonstrated that cDC2s, particularly IRF4-expressing cDC2s, are necessary and sufficient for Th2 generation and inflammation in response to HDM(Lambrecht et al., 2000; Williams et al., 2013). As EPS was negatively impacting the ability

of BMDCs to uptake antigen *in vitro*, we hypothesized that EPS treatment *in vivo* also prevented antigen uptake in cDCs. To test this hypothesis, we treated mice with EPS i.t. one day prior to sensitization with HDM conjugated to AlexaFluor647 (hereafter HDM-AF647). Eighteen hours after sensitization, the number of antigen-positive DCs were enumerated in both the lung and LDLN (Figure 4-4A). Within the migratory cDC compartment of the LDLN, we quantified CD103(+) cDC1s, CD24(-) cDC2s, and CD24(+) cDC2s, with particular focus on CD24(+) cDC2s since they are necessary for priming Th2 cells in response to HDM (Tussiwand et al., 2015). The gating strategy used to identify these migratory DC subsets is depicted in Figure 4-3.

When HDM-sensitized mice were pre-treated with a single dose of EPS, significantly fewer antigen-positive migratory CD24(+) cDC2s were present in the LDLN (Figure 4-4B,C), consistent with the antigen uptake defect observed in EPS-treated BMDCs. This phenomenon may be due to EPS preventing migration of antigen-positive DCs from the lung to the LDLN; however, the proportion of antigen-positive CD24(+) cDC2s in the lung was also reduced in EPS-treated mice (Figure 4-4D). Fewer LDLN CD103(+) cDC1s in EPS-treated mice were positive for HDM-AF647, indicating that EPS prevented accumulation of LDLN antigen-positive cDCs in both subsets (Figure 4-4E,F). Interestingly, lung cDC1s from EPS-treated mice were not defective in HDM-AF647 uptake compared to controls (Figure 4-4G). Taken together, these data show that EPS prevents accumulation of antigen positive migratory DCs in the LDLN, likely due to preventing antigen uptake by cDCs in the lung.

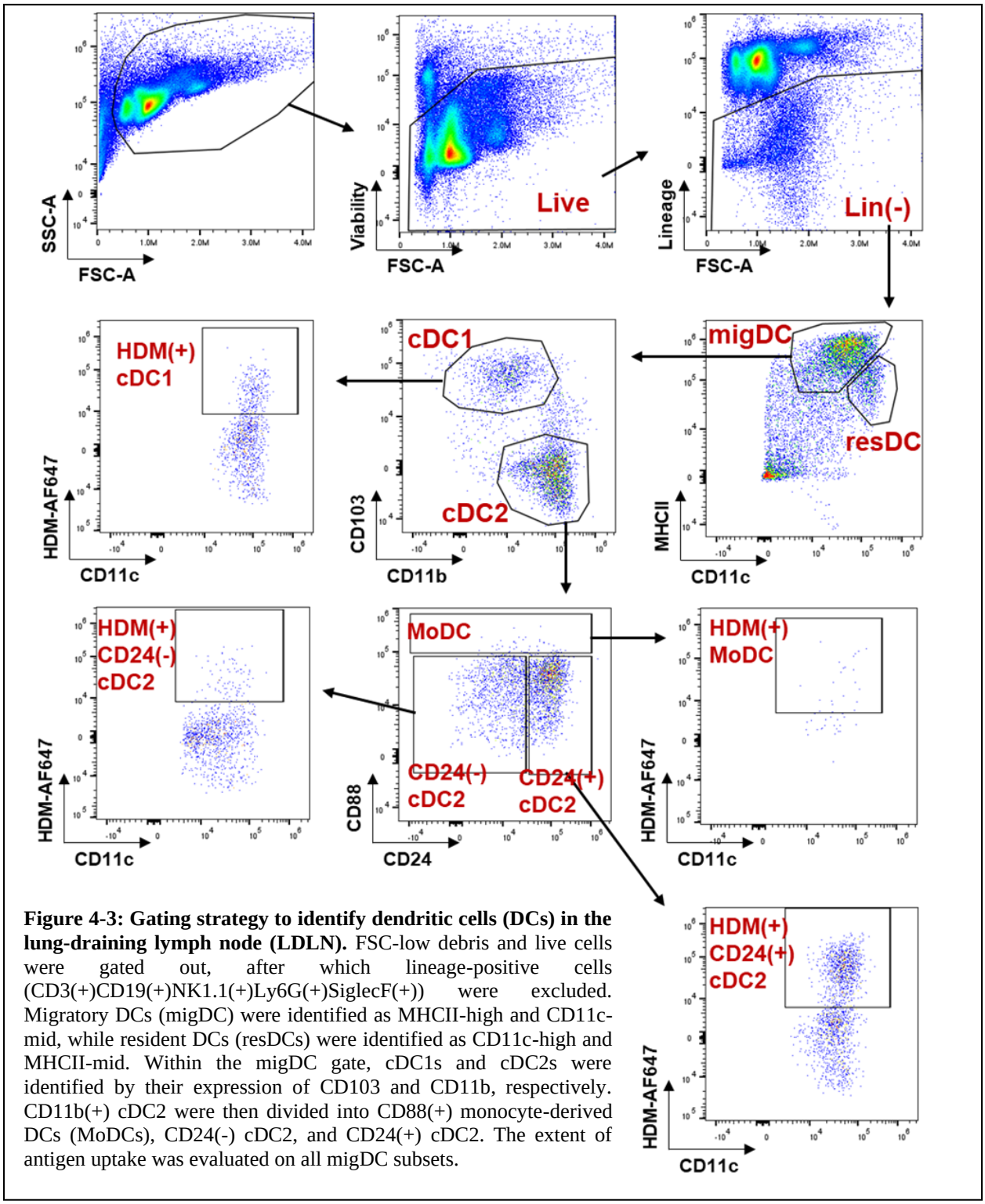

Figure 4-3: Gating strategy to identify dendritic cells (DCs) in the lung-draining lymph node (LDLN). FSC-low debris and live cells were gated out, after which lineage-positive cells (CD3(+)CD19(+)NK1.1(+)Ly6G(+)SiglecF(+)) were excluded. Migratory DCs (migDC) were identified as MHCII-high and CD11c-mid, while resident DCs (resDCs) were identified as CD11c-high and MHCII-mid. Within the migDC gate, cDC1s and cDC2s were identified by their expression of CD103 and CD11b, respectively. CD11b(+) cDC2 were then divided into CD88(+) monocyte-derived DCs (MoDCs), CD24(-) cDC2, and CD24(+) cDC2. The extent of antigen uptake was evaluated on all migDC subsets.

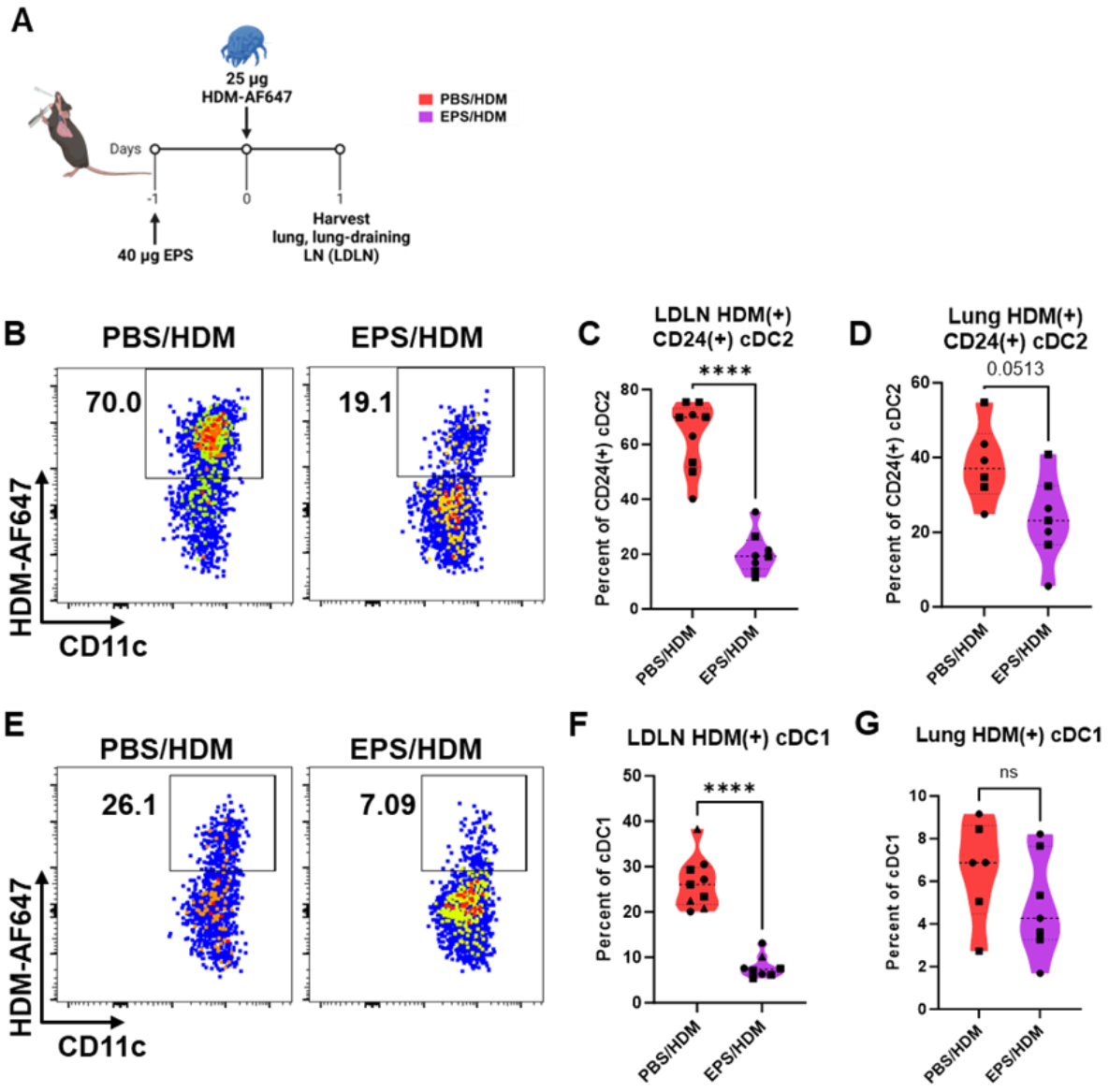

Figure 4-4: EPS treatment one day prior to HDM sensitization prevents accumulation of antigen-positive DCs in the LDLN. (A) Experimental schematic. (B) Representative flow plots of CD24(+) cDC2s by fluorescent antigen (y axis) and CD11c (x axis). (C) Quantification of lung-draining lymph node (LDLN) antigen-positive CD24(+) cDC2s, as percent of total cells falling within the gate in (B). (D) Quantification of lung antigen positive CD24(+) cDC2s as in (C). (E) Representative flow plots of CD103(+) cDC1s by fluorescent antigen (y axis) and CD11c (x axis). (F) Quantification of LDLN antigen-positive cDC1. (G) Quantification of lung antigen-positive cDC1. Ns, not significant; ****, $p < 0.0001$ by unpaired t test. Diagram in (A) generated in Biorender.

EPS does not induce excessive cDC migration from the lung to LDLN.

It is possible that antigen-negative, EPS-treated cells have infiltrated the LDLN before HDM treatment, driving up the percentage of antigen-negative migratory cDCs. If that was the case, we would see an enrichment of migratory cDCs in the LDLN relative to PBS/HDM controls. However, quantification of total LDLN migDCs revealed only a modest increase in total migratory cDC1s (Figure 4-5A), while total migratory CD24(+) cDC2s did not change (Figure 4-5B).

Next, we tested whether EPS was preventing migration of a particular DCs subset from the lung to the LDLN by comparing total numbers of CD24(+) cDC2 and CD103(+) cDC1 within the lung tissue. If EPS prevented migration of either of these subsets, then we would expect an enrichment of this subset in the lung relative to mice not given EPS. However, we found that EPS-pretreated mice do not contain more lung CD24(+) cDC2s (Figure 4-5C) or cDC1s (Figure 4-5D) than mice given just HDM-AF647.

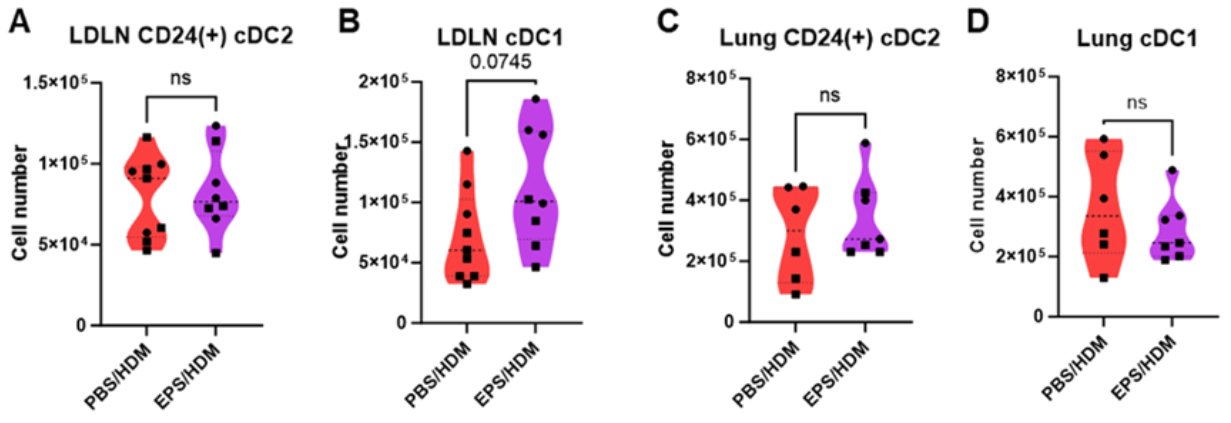

Figure 4-5: EPS does not affect cDC numbers in the lung or lung-draining lymph node (LDLN). (A) Total number of CD24(+) cDC2s in the lung-draining lymph node (LDLN). (B) Total number of cDC1s in the LDLN. (C) Total number of CD24(+) cDC2s in the lung tissue. (D) Total number of cDC1s in the lung tissue. Ns, not significant by unpaired t test.

EPS upregulates CD86 on antigen-negative migDCs in the LDLN, but not the lung.

Because dendritic cells lose antigen uptake capacity upon activation(Liu et al., 2021), we

hypothesized that EPS was inducing DC activation. As such, we assessed the surface expression of CD86, a costimulatory molecule upregulated upon DC activation (Lambrecht and Hammad, 2014). When migratory DCs were assessed for expression of costimulatory molecule CD86, we initially saw no difference in CD86 MFI between migratory DCs from PBS/HDM- and EPS/HDM-treated mice. When cDC1s and CD24(+) cDC2s were divided by antigen positivity, however, differences emerged. In those DCs that were positive for HDM, CD86 was upregulated, indicating that the DC had phagocytosed antigen and was maturing into an antigen-presenting cell (Figure 4-6A,B; PBS/HDM groups). In the context of EPS treatment, however, both HDM-negative and HDM-positive DCs had a similar CD86 MFI, indicating that even antigen-negative DCs were upregulating costimulation (Figure 4-6A,BI, EPS/HDM groups). This phenomenon was also observed in LDLN cDC1s from EPS/HDM-treated mice (Figure 4-6C,D). From these data, we concluded that EPS induced maturation in migratory DCs such that they were less capable of phagocytosing HDM.

Next, we measured CD86 on the surface of lung cDCs from EPS-treated mice. CD24(+) cDC2s that were positive for antigen had increased CD86 expression, as was seen in the LDLN (Figure 4-6E,F; PBS/HDM groups). Unlike the LDLN, however, antigen-negative CD24(+) cDC2s from EPS/HDM-treated mice had surface CD86 levels comparable to that of antigen-negative CD24(+) cDC2s from PBS/HDM mice (Figure 4-6E,F). The low CD86 MFI in EPS/HDM, antigen-negative CD24(+) cDC2s was also observed in lung cDC1s (Figure 4-6G,H). Taken together, these data indicate that EPS does not induce a maturation-like phenotype (suppressed antigen uptake and high CD86 expression) in cDCs in the lungs two days after administration. It is likely that EPS exposure induces maturation of cDC1s and CD24(+) cDC2s, resulting in suppressed antigen uptake, increased surface CD86 expression, and rapid migration to

the LDLN. Meanwhile, some EPS-exposed CD24(+) cDC2s remain within the lung after taking up less antigen than their non-EPS exposed counterparts, but the majority of cDCs remaining in the lung 48 hours after EPS treatment were not exposed to EPS, or are derived from recently-arrived pre-cDCs from the bone marrow. Overall, we have found that a single dose of EPS prior to sensitization is sufficient to prevent cDC-mediated antigen translocation to the LDLN, most likely through affecting antigen uptake capacity in lung DCs.

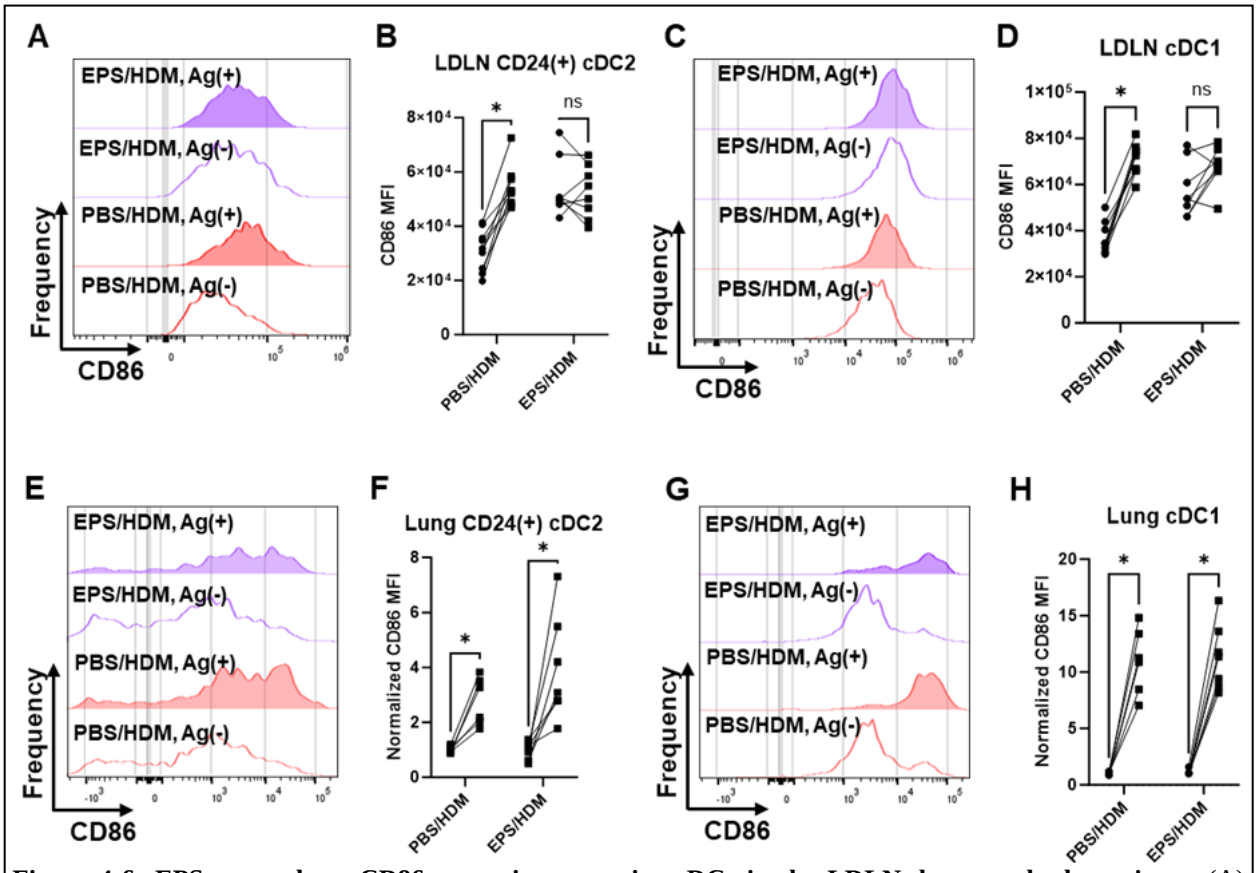

Figure 4-6: EPS upregulates CD86 on antigen-negative cDCs in the LDLN, but not the lung tissue. (A) Representative histograms of CD86 on CD24(+) cDC2s in the LDLN. Individual samples are split into cells negative (open histogram) and positive (shaded histogram) for HDM-AF647. (B) CD86 median fluorescence intensity (MFI) on CD24(+) cDC2s from the LDLN of PBS/HDM- or EPS/HDM-treated mice treated as in Figure 4-3. HDM-positive and HDM-negative CD24(+) cDC2s from the same sample are connected by a line. (C) Representative histograms of CD86 on CD103(+) cDC1s in the LDLN, as in (A). (D) CD86 MFI on LDLN cDC1s from PBS/HDM- or EPS/HDM-treated mice, as in (B). (E-H) CD86 MFI on cDCs in the lungs of PBS/HDM- or EPS/HDM-treated mice. Layout is identical to (A)-(D). Due to variable MFI in CD86 across experiments, lung CD86 was normalized to PBS/HDM, antigen-negarive cDC2s within each subset. *, $p < 0.01$ by multiple unpaired t tests.

Lung DC subsets segregate based on transcriptional identity.

We have demonstrated that EPS induces a maturation-like phenotype in BMDCs and migratory lung cDCs; however, we do not know the mechanism by which EPS imparts this phenotype on these DCs. To understand how EPS affects transcriptional activity in DCs, we performed a bulk RNA-seq on sorted DCs from mice given EPS or PBS (Figure 4-7A). In parallel, we performed bulk RNA-seq on mice treated with EPS or PBS and then sensitized with HDM (Figure 4-7B). Eighteen hours after intratracheal instillation of PBS, EPS, or HDM, lungs were digested and CD11c(+) cells were enriched via magnetic separation. DCs were sorted from CD11c-enriched cell fractions via FACS. DCs were identified as lineage-negative, CD11c(+), MHCII(+). From the total DC gate, three subpopulations of DCs were sorted from each sample. First, CD103(+) cDC1s were separated from CD11b(+) cDC2s. Next, CD11b(+) cDC2s were split into a CD88(+) monocyte-derived DC (MoDC) population, and a CD88(-) cDC2 population. CD88 was chosen as our MoDC marker based on previous work that identified MoDCs as CD88(hi) (Nakano et al., 2013).

After sorting, each DC subpopulation underwent RNA purification, after which samples were sent for library preparation and RNA sequencing. After sequencing, alignment, post-alignment filtering, and normalization of transcript counts, all samples were visualized using Principal Component Analysis (PCA). Strikingly, 79% of the variance across samples was explained by PC1, which corresponded to the DC subset of each sample: cDC1 samples clustered to the left, with cDC2s in the middle and MoDCs to the far right (Figure 4-7C). Within each DC subtype, samples clearly clustered by treatment on PC2, which accounted for 15% of the variance (Figure 4-7C).

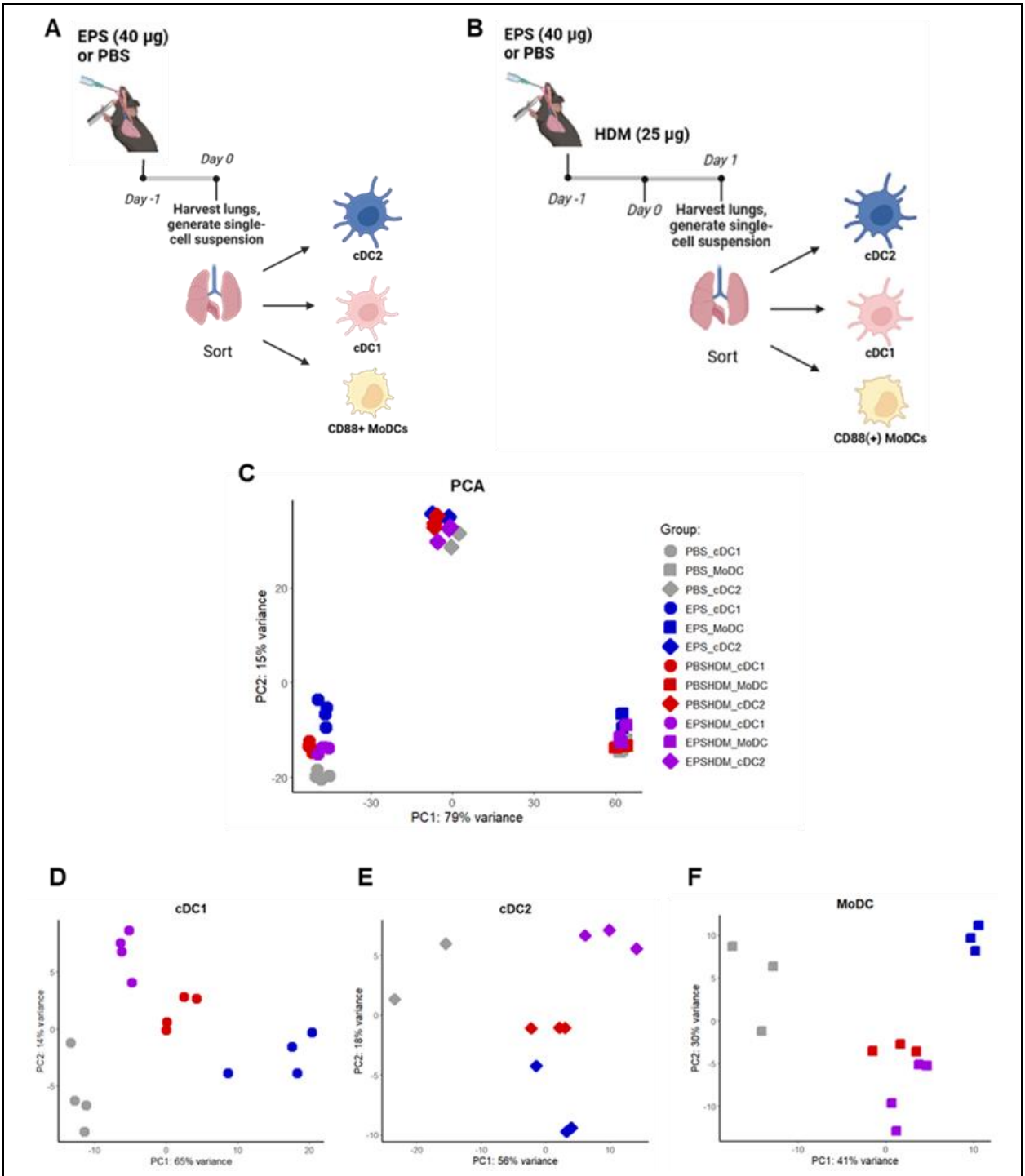

Figure 4-7: Lung DC subset informs transcriptional identity. (A) Experimental schematic for treatment with EPS or PBS and subsequent DC isolation and sorting. (B) Experimental schematic for EPS or PBS pretreatment, followed by HDM sensitization. Eighteen hours after sensitization, DCs enriched and sorted from the whole lung. (C) Principal component analysis (PCA) of all DC subsets and treatment groups. (D) PCA of cDC1s from mice treated with PBS, EPS, or LPS. (E) PCA of cDC2s as in (C). (E) PCA of CD88(+) MoDCs as in (C). Gray: PBS alone; Blue: EPS alone; Red: PBS and then HDM; Purple: EPS and then HDM. Diagrams in (A) and (B) generated in Biorender.

PCA performed on individual DC subsets revealed clear separation by treatment on both PC1 and PC2, most strikingly in MoDCs: DCs from PBS-treated mice were most distinct from DCs from EPS-treated mice, with HDM-treated DCs falling between PBS and EPS DCs (Figure 4-7D-F). Even cDC2s, which did not cluster as neatly as MoDCs, still had 56% of variance explained by treatment (Figure 4-7E). Altogether, these PCA analyses demonstrate that DC subtype plays a critical role in transcriptional identity. Henceforth, analysis of the effects of EPS on DCs is split by DC subtype, with a particular focus on cDC2s.

EPS modulates expression of immune-associated genes by cDC2s in the context of HDM sensitization.

To understand how EPS affects the function of HDM-sensitized cDC2s, we directly compared the transcriptomes of cDC2s from EPS/HDM-treated and PBS/HDM-treated mice. EPS pretreatment increased expression of 33 genes with a log2FC > 1.5 relative to PBS/HDM cDC2s (Figure 4-8A). These 33 genes were passed to the Metascape gene annotation platform (Zhou et al., 2019) to determine which pathway(s), if any, are enriched in genes upregulated by EPS/HDM. Top GO pathways enriched in the gene set included “inflammatory response” (GO:0006954), “regulation of chemotaxis” (GO:0050920), and “negative regulation of cytokine production” (GO:0001818) (Figure 4-8B). Notable genes within the “inflammatory response” pathway included Acod1, an itaconate-producing enzyme that inhibits LPS-induced innate cytokines in BMDMs and aids in antigen processing (Wu et al., 2020), and the neutrophil chemoattractant Cxcl3 (Figure 4-8C). Excitingly, the genes upregulated in the “negative regulation of cytokine production” pathway, such as Hmox1 and Gpnmb (DC-HIL), have been demonstrated to induce the immunomodulatory cytokine IL-10 and prevent T cell activation, respectively (Chung et al.,

2007; Lee and Chau, 2002) (Figure 4-8D). These highlighted genes have exciting implications for EPS as an anti-inflammatory modulator of cDC2s.

EPS treatment before sensitization also resulted in significant downregulation of 36 genes in cDC2s relative to PBS/HDM-treated controls (Figure 4-9A). Metascape analysis of these 36 genes revealed a significant enrichment for the KEGG pathway "cytokine-cytokine receptor interaction", which contained eight of the 36 input genes (Figure 4-9B). These eight genes fell into three obvious functional subgroups: NFκB-driven innate cytokines (Figure 4-9C), costimulatory molecule ligands (Figure 4-9D), and chemokines (Figure 4-9E). The costimulatory ligands in Figure 4-9D were particularly exciting because they have been previously implicated in control of adaptive immune responses; specifically, CD70 engagement by CD27 on effector T cells provides a pro-proliferative signal for T cell activation(Arroyo Hornero et al., 2020) and OPG (encoded by Tnfrsf11b) is required for B cell maturation(Yun et al., 2001).

Taken together with Figure 4-8, differential gene analysis of cDC2s from EPS/HDM-treated mice versus PBS/HDM-treated controls revealed that EPS preexposure modulates expression of immune-associated genes. Namely, EPS/HDM cDC2s upregulate genes associated with negative regulation of cytokine production (Hmox1, Gpnmb) while suppressing expression of NF-κB-associated cytokines (Ifnb1, Il12b), chemokines (Ccl2, Ccl4, Ccl7, Ccl22) and costimulatory ligands (Tnfrsf11b, Cd70).

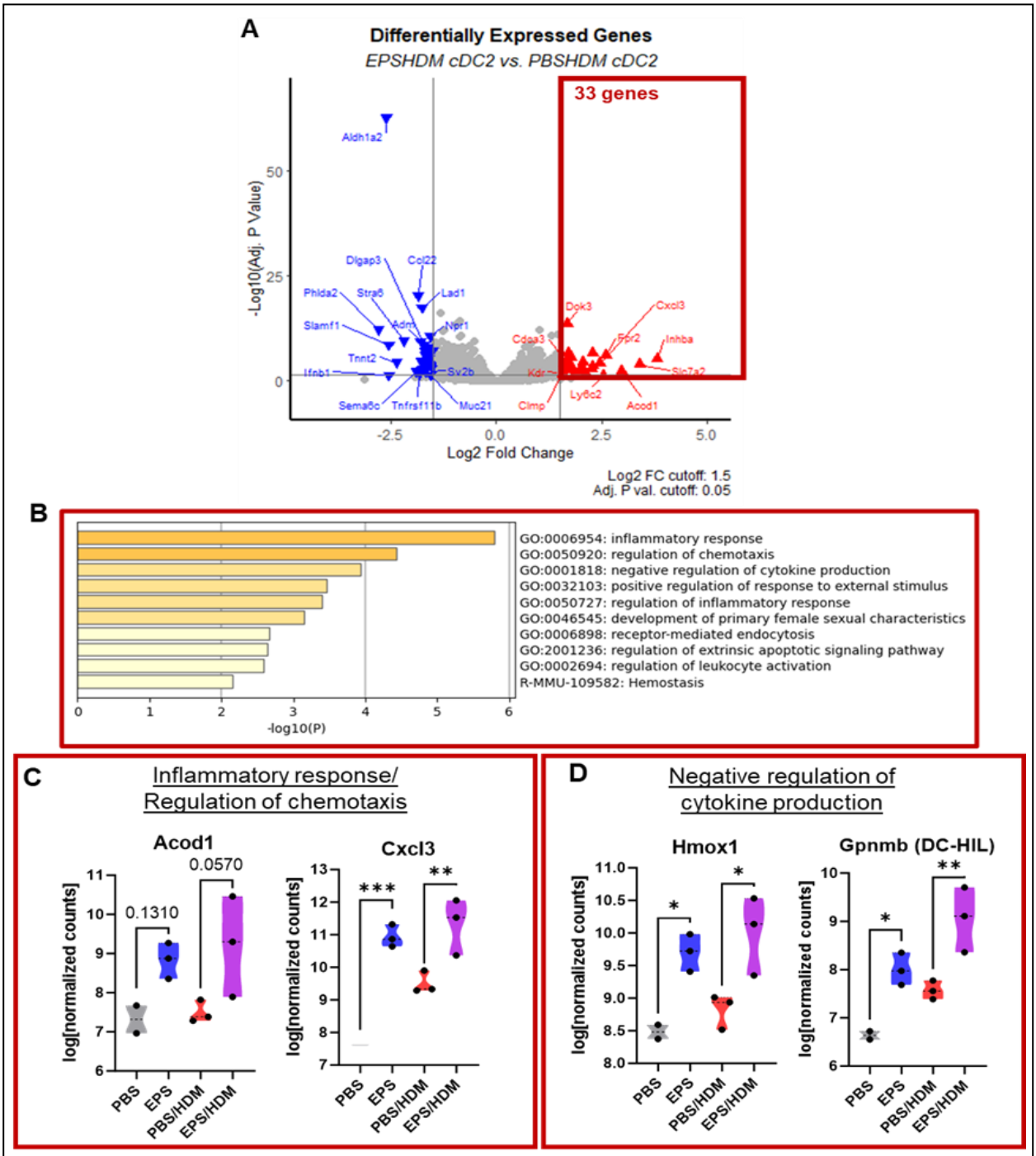

Figure 4-8: EPS upregulates genes associated with inflammation and negative regulation of cytokines. (A) Volcano plot displaying differential gene expression in cDC2s from PBS/HDM- and EPS/HDM-treated mice. Genes that are greater than |1.5| log2 fold change and less than a 0.05 p-value are indicated in colored triangles. (B) Metascape analysis of the 33 genes upregulated in EPS/HDM cDC2s relative to PBS/HDM cDC2s. (C) Violin plots of selected genes from the top two GO pathways in (B). (D) Violin plots of select genes from the "negative regulation of cytokine production" GO pathway. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by one-way ANOVA with Sidak's multiple comparisons test.

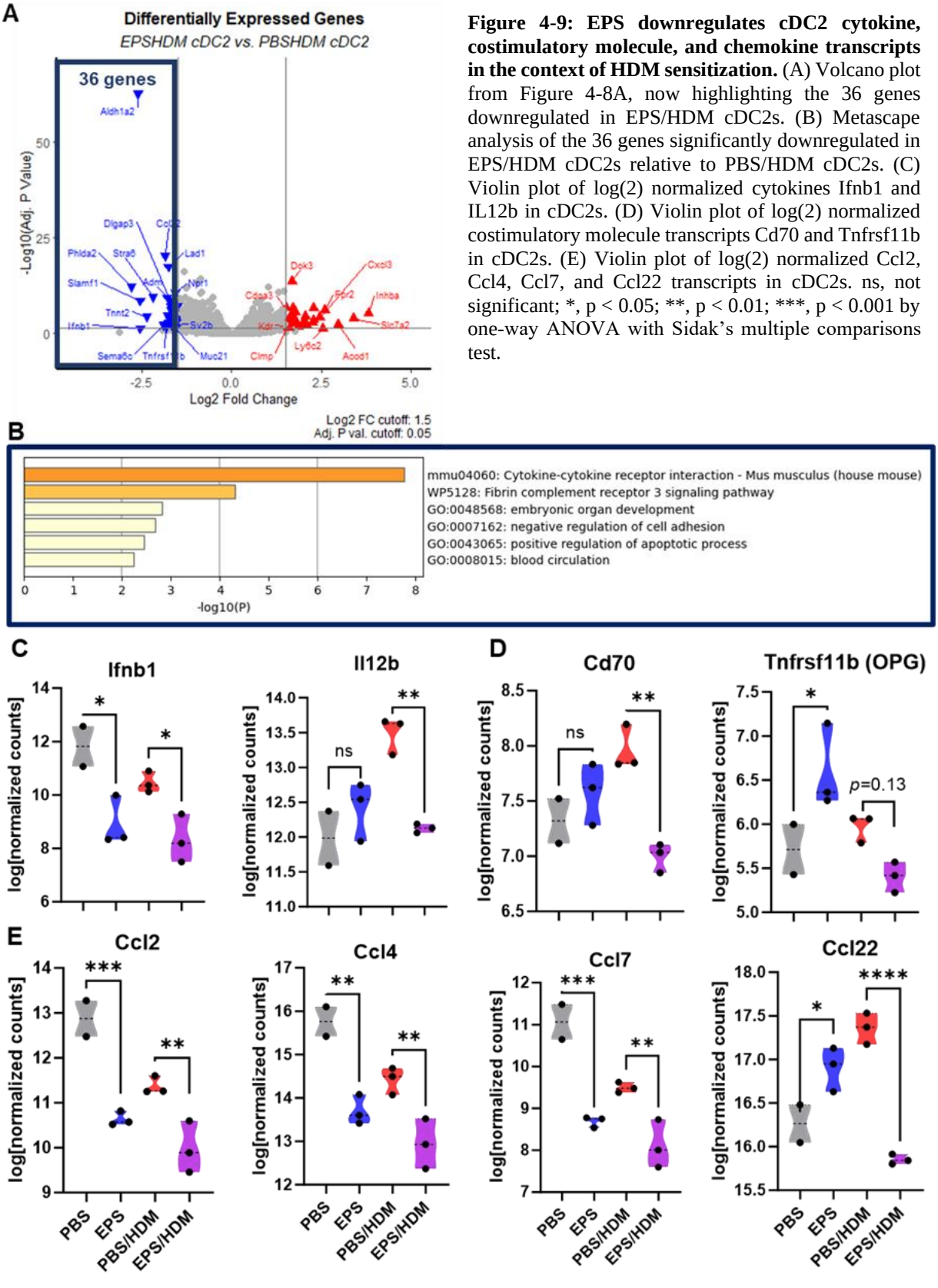

Figure 4-9: EPS downregulates cDC2 cytokine, costimulatory molecule, and chemokine transcripts in the context of HDM sensitization. (A) Volcano plot from Figure 4-8A, now highlighting the 36 genes downregulated in EPS/HDM cDC2s. (B) Metascape analysis of the 36 genes significantly downregulated in EPS/HDM cDC2s relative to PBS/HDM cDC2s. (C) Violin plot of log(2) normalized cytokines Ifnb1 and IL12b in cDC2s. (D) Violin plot of log(2) normalized costimulatory molecule transcripts Cd70 and Tnfrsf11b in cDC2s. (E) Violin plot of log(2) normalized Ccl2, Ccl4, Ccl7, and Ccl22 transcripts in cDC2s. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ by one-way ANOVA with Sidak's multiple comparisons test.

Lung cDC2s exposed to EPS display a broadly immunosuppressive signature.

As we observed distinct differences between cDC2s from EPS/HDM- and PBS/HDM-treated cDC2s, we next wanted to determine if these differences were also observed in cDC2s from mice given EPS alone. In lung cDC2s isolated from EPS-treated mice, 112 genes were upregulated and 138 genes were downregulated (significance set at $p < 0.05$, with a log2 fold change of at least 1.5; Figure 4-10A). Metascape analysis revealed that the 112 genes upregulated in cDC2s from EPS-treated mice were most significantly enriched within the "inflammatory response" GO pathway (Figure 4-10B). This was also the most-significantly enriched GO pathway in cDC2s from EPS/HDM-treated mice, arguing that EPS affects the same transcriptional program(s) in the context of HDM sensitization. Indeed, 19 of the 33 genes upregulated in EPS/HDM cDC2s were also upregulated in EPS cDC2s, including Acod1, Cxcl3, Hmox1, and Gpnmb. Other genes within the "inflammatory response" pathway upregulated by EPS treatment included Il2ra, Saa3 (which encodes an acute phase response protein), and Tnfrsf3 (LTBR). Interestingly, another top pathway enriched in EPS-upregulated genes was the KEGG pathway "cytokine-cytokine receptor interaction"—a pathway that was enriched in genes <u>down</u>regulated in EPS/HDM cDC2s. However, none of the genes within this pathway upregulated in EPS cDC2s were also downregulated in EPS/HDM cDC2s, i.e. there was no overlap between cytokine genes upregulated by EPS and downregulated by EPS/HDM.

Within the 138 genes downregulated by EPS treatment in cDC2s, the most significantly enriched pathways had to do with innate defense against viral infection, i.e. GO pathways "defense response to virus" and "innate immune response" and KEGG pathways "Coronavirus disease – COVID-19" and "Viral protein interaction with cytokine and cytokine receptor" (Figure 4-10C). None of these top enriched pathways were also enriched in the list of genes downregulated in

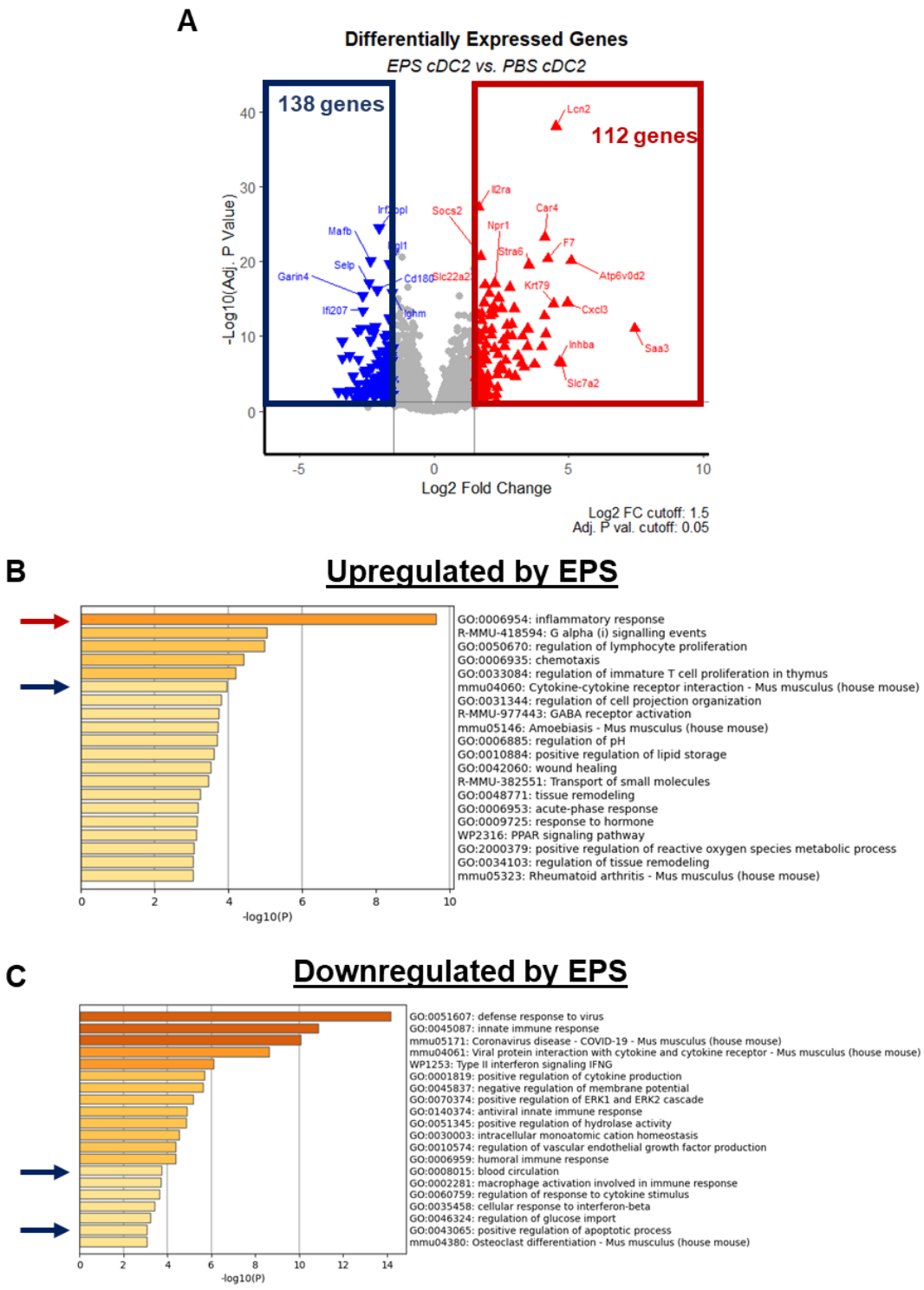

Figure 4-10: EPS induces a broadly immunosuppressive signature in cDC2s. (A) Volcano plot displaying differential gene expression in cDC2s from PBS- and EPS-treated mice. Genes that are greater than |1.5| log2 fold change and less than a 0.05 p-value are indicated in colored triangles. (B) Top pathways enriched in genes upregulated by EPS treatment in cDC2s. (C) Top pathways enriched in genes downregulated by EPS treatment in cDC2s. Red arrows indicate pathways also found in genes upregulated by EPS/HDM; blue arrows are pathways also enriched in EPS/HDM cDC2s.

EPS/HDM cDC2s. Also, only 7 of the 36 genes downregulated in EPS/HDM cDC2s were also downregulated in EPS cDC2s. Genes downregulated by EPS treatment in cDC2s included those encoding innate cytokines TNF-α and IL-6, antiviral proteins RNaseI and Mx2, and costimulatory molecule LIGHT. Of the 7 genes downregulated in EPS/HDM cDC2s and EPS cDC2s, three were chemokine ligands (Ccl2, Ccl4, Ccl7) and the fourth was IFNb1. Thus, EPS alone downregulated a larger swathe of pro-inflammatory mediators in cDC2s than EPS in combination with HDM, but also downregulated transcription of IFN-β and chemokines CCL2, CCL4, and CCL7.

EPS and LPS alone induce similar transcriptional signatures.

Like LPS, EPS is a polysaccharide-containing bacterial product that signals primarily through TLR4(Jones et al., 2014) and is capable of preventing allergic eosinophilia. EPS, however, is hypothesized to use the coreceptor CD11b, rather than MD-2, to activate non-canonical NF-κB signaling and induce expression of anti-inflammatory enzymes such as IDO1 (Zamora-Pineda et al., 2023). Therefore, EPS and LPS should induce distinct transcriptional signatures in DCs. To determine how EPS and LPS affect the transcriptomes of cDC2s, we also treated a cohort of mice with 5 μg of LPS i.t. while other mice received 40 μg of EPS i.t. This dose was chosen based on the observation that a 5 μg dose of LPS can induce tolerance to HDM without appreciable lung injury (Bachus et al., 2019).

Differential gene expression analysis of cDC2s from LPS-treated mice revealed dramatic transcriptome changes: LPS significantly increased the expression of 265 genes above cDC2s from PBS-treated mice, compared to 112 genes upregulated by EPS alone (Figure 4-11A). Consistent with LPS's immunomodulatory abilities in the lung, these 265 genes were highly enriched for the GO pathways "negative regulation of immune system process", "positive regulation of response to external stimulus", and "positive regulation of cytokine production" (Figure 4-11B). Genes

within these pathways included transcription factors Irf1, Irf7, and Irf8; complement component C3; and itaconate-producing enzyme Acod1, which was also upregulated in cDC2s from EPS/HDM-treated mice (see Figure 4-8C).

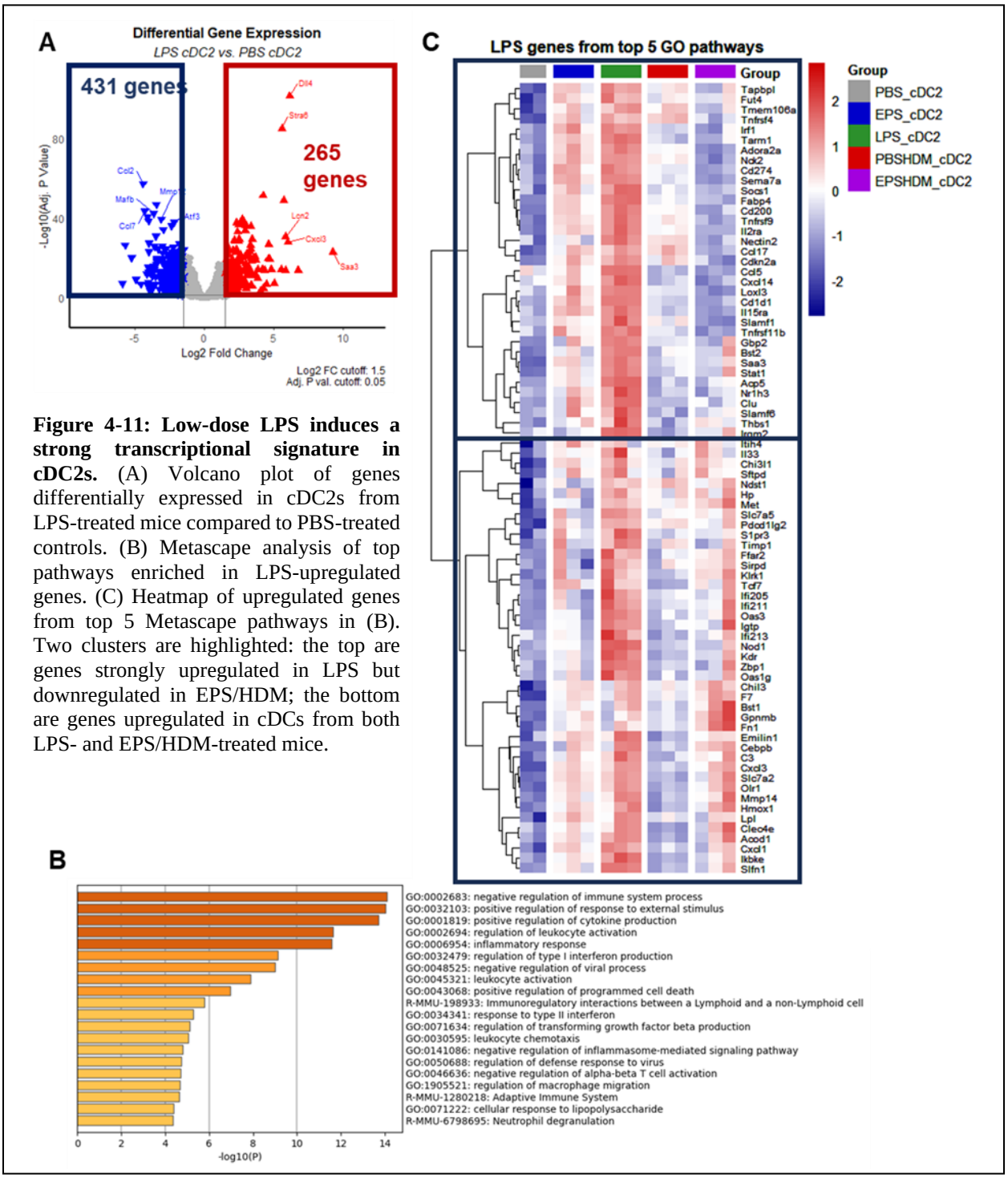

Figure 4-11: Low-dose LPS induces a strong transcriptional signature in cDC2s. (A) Volcano plot of genes differentially expressed in cDC2s from LPS-treated mice compared to PBS-treated controls. (B) Metascape analysis of top pathways enriched in LPS-upregulated genes. (C) Heatmap of upregulated genes from top 5 Metascape pathways in (B). Two clusters are highlighted: the top are genes strongly upregulated in LPS but downregulated in EPS/HDM; the bottom are genes upregulated in cDCs from both LPS- and EPS/HDM-treated mice.

Genes from the top 5 pathways enriched in LPS-upregulated genes were selected to compare to cDC2s from mice treated with EPS, PBS/HDM, and EPS/HDM. Remarkably, genes that were significantly upregulated by LPS were also upregulated by EPS, albeit not to the same level of significance in most cases (compare blue and green groups in Figure 4-11C). Within this subset of LPS-upregulated genes, two main clusters appeared: the top cluster contained genes upregulated slightly by EPS and even more so in LPS, that were significantly downregulated in EPS/HDM cDC2s; and the bottom cluster contained genes upregulated slightly by EPS, more by LPS, and then also upregulated in EPS/HDM cDC2s relative to PBS/HDM cDC2s (Figure 4-11C). This division in LPS-upregulated genes—between those expressed in EPS/HDM cDC2s and those not—suggests that EPS, in conjunction with an inflammatory stimulus such as HDM, activates transcriptional programs both shared with and distinct from LPS.

Genes downregulated by EPS and LPS converge on innate cytokines and chemokines.

Parallel analysis of the genes downregulated by LPS treatment revealed that low-dose LPS downregulated GO and KEGG pathways associated with the inflammatory response, migration, and IL-1 production (Figure 4-12A). Of note, LPS-downregulated genes were only enriched for one pathway involving viral infection, namely the KEGG pathway "coronavirus disease – COVID-19". Of the top 20 pathways enriched in EPS-downregulated genes, four of them were directly related to viral responses (Figure 4-10C). When the top genes downregulated by LPS were visualized via heatmap, it was clear that there were no genes clearly upregulated by EPS and downregulated by LPS relative to PBS controls—if anything, EPS weakly downregulated any genes that were strongly downregulated by LPS. A cluster of note in the middle of the heatmap (Figure 4-12B) included genes downregulated by LPS and EPS/HDM and included the genes for cytokines IL-10, TNF-α, and IFN-β. When quantified, Il10 transcript was only significantly

reduced in cDC2s from LPS-treated mice, although Il10 transcripts trended downward in EPS cDC2s (Figure 4-12C). For Tnf and Ifnb, however, EPS and LPS significantly reduced normalized transcript counts, though LPS treatment consistently reduced transcripts to a greater extent than EPS treatment. Excitingly, cDC2s from EPS/HDM-treated mice had lower transcript counts for TNF-α and IFN-β than cDC2s from PBS/HDM-treated controls (Figure 4-12D,E). As cytokines produced by DCs provide the essential Signal 3 necessary for T cell differentiation, it is easy to postulate that downregulation of cytokines like TNF-α and IFN-β could impact successful T cell priming and differentiation.

As chemokines play a key role in DC migration within the tissue and to secondary lymphoid organs (SLOs), it was also interesting to observe a number of chemokine ligands within the same cluster of genes downregulated by treatment with EPS, LPS, or EPS/HDM. Specifically, the chemokine ligands Ccl2 (Figure 4-12F), Ccl3 (Figure 4-12G), Ccl4, and Ccl7 (Figure 4-12H) were strongly downregulated in cDC2s from mice treated by EPS or LPS alone, or in cDC2s from mice pre-treated with EPS and then sensitized to HDM. CCL2, CCL3, and CCL4 have all been demonstrated to affect Th cell differentiation(Luther and Cyster, 2001); therefore, downregulation of these chemokine ligands by cDC2s in response to EPS or LPS exposure may be a mechanism by which these cDC2s negatively impact T cell priming.

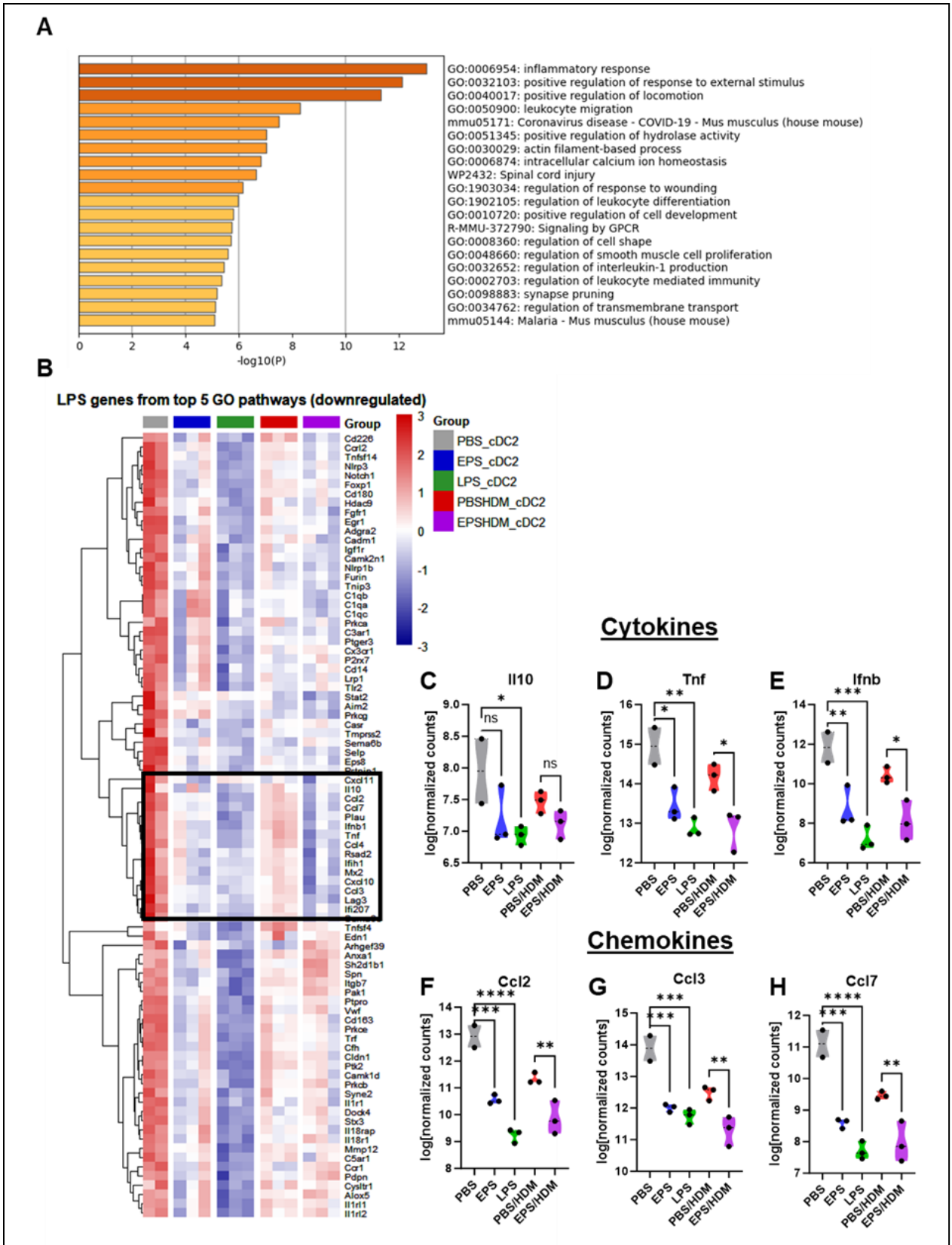

Figure 4-12: LPS and EPS downregulate similar cytokines and chemokines in lung cDC2s. (A) Pathways

Figure 4-12, continued: enriched in genes significantly downregulated in cDC2s from LPS-treated mice relative to PBS controls. Analysis was performed in Metascape. (B) Heatmap of top genes downregulated by LPS in the top 5 pathways depicted in (A). The black box indicates a cluster in which genes are downregulated by EPS, LPS, and EPS in combination with HDM sensitization. (C-E) Quantification of normalized, log-transformed gene counts from cDC2s. The cytokine transcript quantified is indicated in the plot title. (F-H) Quantification of normalized, log-transformed gene counts from cDC3s. The chemokine ligand transcript is indicated in the plot title. Ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ by one-way ANOVA with Sidak's multiple comparisons test.

Discussion

In this study, we found that EPS dysregulates several cDC functions to prevent optimal Th cell priming and differentiation. EPS-cocultured BMDCs are less capable of inducing antigen-specific proliferation, take up less antigen, and display more CD86 on their cell surface relative to control BMDCs. We also found migratory DCs from EPS-pretreated mice were less positive for fluorescent antigen and upregulated CD86, independent of their antigen positivity. This decrease in antigen-positive migDCs was not due to retention within the lung, as lung DCs did not harbor more antigen in EPS-treated mice, and the number of DCs in the lungs of EPS/HDM-treated lungs was comparable to their PBS/HDM-treated counterparts.

To understand how EPS affects the transcriptome of lung DCs, we performed bulk RNA-sequencing on sorted lung DCs. Within the cDC2 subset, EPS treatment followed by sensitization to HDM upregulated genes associated with chemotaxis and negative regulation of cytokine production while simultaneously downregulating innate cytokines, chemokine ligands, and some costimulatory molecules. cDC2s exposed to EPS alone contained more differentially expressed transcripts associated with regulating lymphocyte proliferation, cell migration, and antiviral defense, suggesting that EPS induces a broadly immunosuppressive signature that acts to oppose subsequent immune insults such as HDM. Compared to LPS, EPS alone affected the differential expression of fewer genes, even at an eightfold higher dose. However, both EPS and LPS

downregulated expression of key cytokines TNF-α and IFN-β in lung cDC2s, as well as chemokines CCL2, CCL3, CCL4, and CCL7.

Previous studies by the Knight group have demonstrated that EPS prevents generation of an effective CD4 T cell response both *in vitro* and *in vivo* (Kalinina et al., 2021; Paynich et al., 2017; Zamora-Pineda et al., 2023). While the *in vitro* T cell proliferation studies in Figure 4-1 may seem redundant to their work, BMDCs react differently to different stimuli, and none of the Knight group's *in vitro* studies used allergen as an adjuvanting agent. Therefore, it was important to confirm that the anti-T cell proliferation effect seen by EPS previously could also be extended to allergen sensitization.

Exposure to EPS resulted in attenuated antigen uptake both in BMDCs (Figure 4-2) and in LDLN DCs (Figure 4-4). While the defect in cDC2 antigen uptake in the lung seemed modest, this is likely because many cells that have seen EPS and/or HDM had already migrated to the LDLN. Previous work from the Sperling lab has demonstrated that antigen-positive migDCs appear in the LDLN as early as 18 hours post-sensitization (Camacho et al., 2022). Additionally, the upregulation of CD86 in antigen-negative, LDLN cDC2s (Figure 4-6B) mirrors the higher CD86 MFI on BMDCs exposed to EPS alone (Figure 4-2E). Therefore, it is likely that EPS treatment results in migration of cDCs from the lung to the LDLN, even in the absence of antigen. Although we did not observe an increase in LDLN CD24(+) cDC2s in EPS/HDM-treated mice (Figure 4-5A), the high turnover of migDCs in secondary lymphoid organs (Kamath et al., 2002) may have rendered the influx of migDCs in EPS-treated mice undetectable. DC migration studies using administration of PKH26, such as studies performed by Si et al(Si et al., 2023), would clearly answer if EPS induces an early wave of DC migration prior to sensitization with allergen. If EPS temporarily depletes lung DCs, this could explain why fewer migDCs are positive for antigen: a

proportion of the DCs in the LDLN migrated after EPS exposure but prior to sensitization, leaving a smaller pool of DCs to pick up antigen in the lung tissue and carry it to the LDLN.

While EPS clearly signals through TLR4(Paynich et al., 2017), it is less clear what other components are required for EPS to exert its anti-inflammatory effects. A recent collaborative study between the Knight lab and our own demonstrated that EPS activates both the canonical and non-canonical NF-κB pathways downstream of TLR4, and downstream IDO expression is necessary for EPS-treated BMDCs to suppress T cell proliferation(Zamora-Pineda et al., 2023). This mechanism, while possible in our model, is likely not the only way in which EPS inhibits Th2 inflammation through DCs; as such, we decided to perform bulk RNA-sequencing of DCs sorted from the lungs of EPS-treated mice. We first focused on differential gene expression between cDC2s from EPS/HDM- and PBS/HDM-treated cDC2s, as our central question was how EPS affects transcription and, in turn, DC function in the context of HDM sensitization. Despite the profound protection against eosinophilia observed in EPS/HDM-treated mice in the full model, only 69 genes were differentially regulated between cDC2s from PBS/HDM- and EPS/HDM-treated mice. This could be for a number of reasons: first, EPS-mediated changes to the transcriptome could be antagonized by PBS/HDM sensitization. In support of this, a number of genes differentially regulated by EPS treatment—Cxcl3, Gpnmb, Tnf, Ccl2, Ccl3, and Ccl7, for example—are also up- or downregulated by HDM sensitization (volcano plots of normalized, log2-transformed transcript counts are in Figures 4-9 and 4-12). Second, DCs most significantly impacted by EPS treatment could have migrated from the lung to the LDLN, which was not harvested with the lungs in these studies. Although the ideal experiment would have been to compare the transcriptomes of lung and LDLN cDC2s after EPS treatment, the low number of

DCs within a single LDLN would have required pooling of multiple samples and an increase in sample size per group, which was cost- and labor-prohibitive.

Within the genes differentially regulated by EPS/HDM treatment relative to PBS/HDM, several immunomodulatory pathways were identified. The top GO pathway in upregulated genes was “inflammatory response”, which is to be expected from introduction of a foreign polysaccharide such as EPS. The second most-enriched pathway was “regulation of chemotaxis”, which is consistent with the hypothesis that EPS induces migration of lung cDCs. The third, “negative regulation of cytokine production”, included Hmox1, which encodes for the iron-scavenging enzyme heme-oxygenase 1 (HO-1). As an inducible antioxidant protein, HO-1 plays an important role in producing anti-inflammatory metabolites in the lung tissue after insult (Fredenburgh et al., 2007). In the context of pulmonary allergy, HO-1 upregulation in DCs resulted in an increase in lung antigen-specific Tregs and reduced eosinophilia and neutrophilia after OVA challenge (Wong et al., 2016). While EPS does decrease eosinophilia in our model, it does not affect neutrophilia or total Treg numbers; this does not rule out that HO-1 upregulation downstream of EPS could be protective in the context of HDM-mediated inflammation. A second gene within the same pathway was Gpnmb (DC-HIL), which has been demonstrated to downregulate IL-2 and prevent entry of T cells into the cell cycle after stimulation (Chung et al., 2007). It is unlikely that either of these mediators are solely responsible for EPS’s anti-inflammatory effects; however, further studies are required to determine their relative importance in preventing eosinophilia.

Excitingly, EPS also significantly downregulated genes associated with cytokine-cytokine receptor interactions. The genes encoding TNF-α and IFN-β (Figure 4-12) were both downregulated in cDC2s from EPS/HDM-treated mice, which is interesting to note considering

that both genes can be upregulated by TLR4 stimulation by LPS (Sheikh et al., 2014; Wall et al., 2009). DC-derived chemokines CCL2, CCL3, CCL4, and CCL7 were also reduced in cDC2s from EPS/HDM-treated mice. Less is known about the exact chemokine(s) responsible for attracting naïve CD4 T cells to DCs and favoring Th2 cell differentiation. It is plausible that EPS, through modulating these chemokines, could also influence the magnitude of the Th response to HDM sensitization.

How does EPS compare to low-dose LPS, which can prevent allergic inflammation in adult mice (Bachus et al., 2019) and has been implicated in the Farm Effect(von Mutius et al., 2000)? Just 5 μg of LPS significantly changed expression of almost 700 genes in cDC2s, as compared to EPS significantly changing expression of 250 genes in cDC2s. Genes upregulated or downregulated by EPS were often even more markedly upregulated/downregulated by LPS (see heatmaps in Figures 4-12B and 4-11C). To truly dissect the differences between the ability of EPS and LPS to suppress allergic responses, an ideal control group would be the inclusion of an LPS/HDM group in our RNA-seq studies. However, this dissertation is not focused on the mechanisms by which LPS prevents allergic inflammation to HDM, and so we focused on comparing EPS and LPS treatment in cDC2s.

Chapter 5: Discussion

In this dissertation, I have discovered the probiotic potential of inhaled exopolysaccharide (EPS) from the common hay bacterium *B. subtilis*. As the Old Friends Hypothesis posits, exposure to fewer environmental and commensal microbes has resulted in a population that is more sensitive to self and environmental antigens; therefore, this study provides a proof-of-concept in which adding back an environmental bacterial product renders the host less responsive to allergic sensitization. First, I demonstrated that EPS from *B. subtilis*, when given intratracheally around the time of sensitization to HDM, protects against mucus hypersecretion, eosinophilia, and Th2

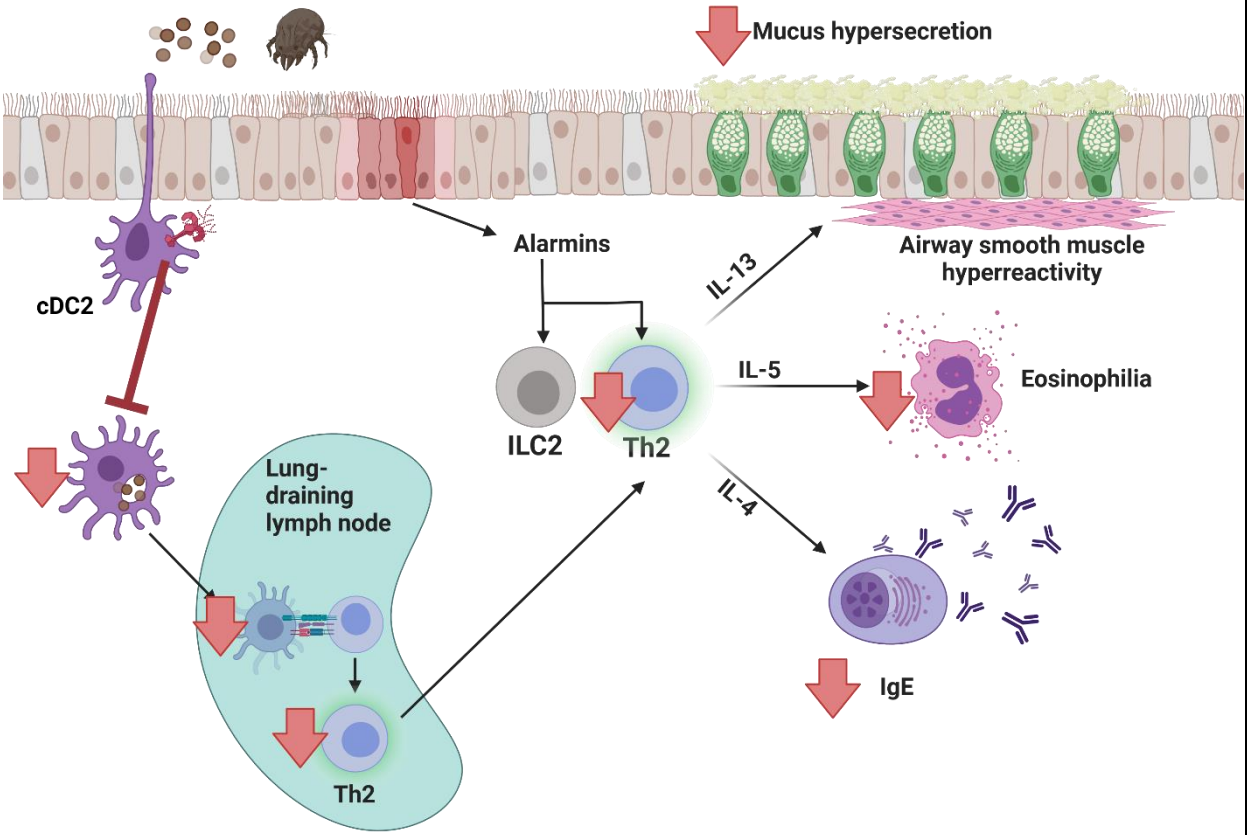

Figure 5-1: Summary of how EPS modulates cellular responses to allergen. EPS pre-treatment engages with TLR4 on cDC2s (red receptor-ligand complex) to prevent subsequent HDM uptake in the lung. Upon migration to the LDLN, these antigen-negative cells are deficient at T cell priming, thereby reducing the total number of Th2 cells generated to HDM. The reduced number of total Th2 cells induced in response to HDM results in fewer eosinophils, lower IgE titers, and reduced mucus secretion. Image generated in Biorender.

cell accumulation in the lungs in a TLR4-dependent manner (see Figure 5-1). While protection is optimal when EPS is given around the time of sensitization, EPS administration prior to challenge also suppresses lung and BAL eosinophilia and increases the number of AMs, an important homeostatic indicator cell in the lung (Chapter 3).

Second, I demonstrated that EPS prevents successful T cell proliferation *in vitro*, and that EPS dampens antigen uptake capacity by myeloid cells *in vitro* and *in vivo*. Additionally, EPS treatment upregulated CD86 on DCs that had migrated from the lung to the lung-draining lymph node, regardless of whether these DCs had successfully internalized antigen. From these data, we hypothesized that EPS was inducing DC maturation. Closer analysis of sorted lung DCs by RNA-seq revealed that cDC2s from EPS-treated mice enhanced expression of cytokine production antagonists, while downregulating multiple pathways implicated in T cell priming. Namely, EPS pretreatment in HDM-sensitized cDC2s downregulated cytokines downstream of TLR4 such as IFN-β and TNF-α; costimulatory molecules CD70 and OPG; and chemokines such as MIP-1-α (CCL3), and MCP1 (CCL2). As such, it appears that EPS is not simply inducing DC maturation; rather, it is generating a DC population that has some markers of maturation, but also have potential immunomodulatory function. Taken together, these data reveal a novel immunomodulatory role for EPS in the prevention of allergic inflammation and provide insight as to how a bacterial product can prevent induction of responses to allergen *in vivo*.

In previous studies on the immunomodulatory effects of EPS, EPS is delivered systemically via intraperitoneal or intravenous injection (Jones et al., 2014; Kalinina et al., 2021; Paik et al., 2019; Paynich et al., 2017; Zamora-Pineda et al., 2023). As exposure to a farm environment is protective against the development of asthma and allergies, we hypothesized that EPS could act as an anti-allergic agent through respiratory exposure. Indeed, EPS treatment effectively prevents

lung eosinophilia (Chapter 3), and intratracheal EPS clearly impacts cDCs residing within the lung. Our studies therefore provide an example in which local, not systemic, immunomodulation using a probiotic product protects against pulmonary allergy.

LPS (endotoxin), a microbe-associated molecule associated with the Farm Effect, has been demonstrated to dampen allergic responses *in vivo*, primarily through preventing chemokine release by epithelial cells (Schuijs et al., 2015). In large doses, endotoxin can cause acute lung injury in mice and is often used as a model for acute respiratory distress syndrome (ARDS) (Matute-Bello et al., 2008). Furthermore, sufficiently low doses of LPS can promote Th2 responses *in vivo* (Eisenbarth et al., 2002; Hammad et al., 2009; Tan et al., 2010) and some studies have found a positive association between endotoxin in household dust and asthma prevalence (Michel et al., 1996; Thorne et al., 2005). The lack of agreement between studies as to what dose of endotoxin is protective against or induces allergy suggest that the pulmonary immune system is uniquely sensitive to endotoxin dose and this sensitivity could be affected by other microbial exposures, i.e. the microbiota in different facilities (for mouse studies) or rural environments (for human studies).

In contrast with previously published work on LPS, we found that EPS alone did not significantly affect mucus secretion, total lung or BAL cell number, or accumulation of any cell subset in the BAL or lung (Chapter 3). As we gave a relatively large dose (40 μg) of EPS directly to the lung, the lack of toxicity indicates that EPS can be tolerated in repeated, high doses. Therefore, use of EPS as a prophylactic agent to prevent allergic sensitization is more attractive than the possibility of giving LPS. The use of a probiotic-derived agent, rather than the probiotic itself, is also attractive because it excludes the possibility of bacterial outgrowth and possible

opportunistic infection. For example, *A. lwoffii* from cow sheds can prevent allergic inflammation, but can also act as an opportunistic pathogen in children (Hu and Robinson, 2010).

Besides being well-tolerated, EPS also has exciting implications as a therapeutic agent to stop or reverse allergic sensitization *in vivo*. Studies using EPS treatment around the time of challenge with HDM demonstrated that EPS can improve lung AM cell counts, thereby driving down the eosinophil-to-alveolar macrophage ratio (Figure 3-9). However, Th2 cells was not reduced in mice given EPS only prior to challenge. Therefore, the increase in AMs relative to eosinophils may be due to an EPS-mediated effect on AMs directly, rather than preventing Th2 differentiation and recruitment (see Question 1, Figure 5-2). Consistent with this possibility, AMs express abundant quantities of TLR4 (Rayees et al., 2020). If EPS can affect lung AMs directly—potentially by expanding AMs and enabling them to generate an anti-inflammatory environment—then EPS has potential to suppress already-established type 2 inflammation. Our laboratory is uniquely equipped to address whether EPS can affect already-established memory responses to allergen, as previous published work dissected the relative contributions of IRF4-dependent cDC2s to acute and memory responses (Camacho et al., 2022). We would be able to use many of the same techniques and memory HDM models to address whether EPS is capable of protecting against subsequent challenges to HDM. These future studies could also unveil as-yet unappreciated roles for EPS in suppressing asthmatic exacerbations.

In Chapter 4, I focused on EPS's activity on cDCs, as cDC2s are central for the development of productive Th2 responses (Williams et al., 2013). In the LDLN, we observed that fewer migratory cDC1 and cDC2 from EPS-treated mice contained fluorescent HDM (Figure 4-4) and had upregulated surface CD86, regardless of their antigen positivity (Figure 4-6). Subsequent transcriptional studies of lung DCs focused on cDC2s isolated from the lungs. Future

transcriptional analysis of lung cDC1s will be performed, as cDC1s have been implicated in preventing allergic inflammation *in vivo*. For example, cDC1s induce production of IL-12 and

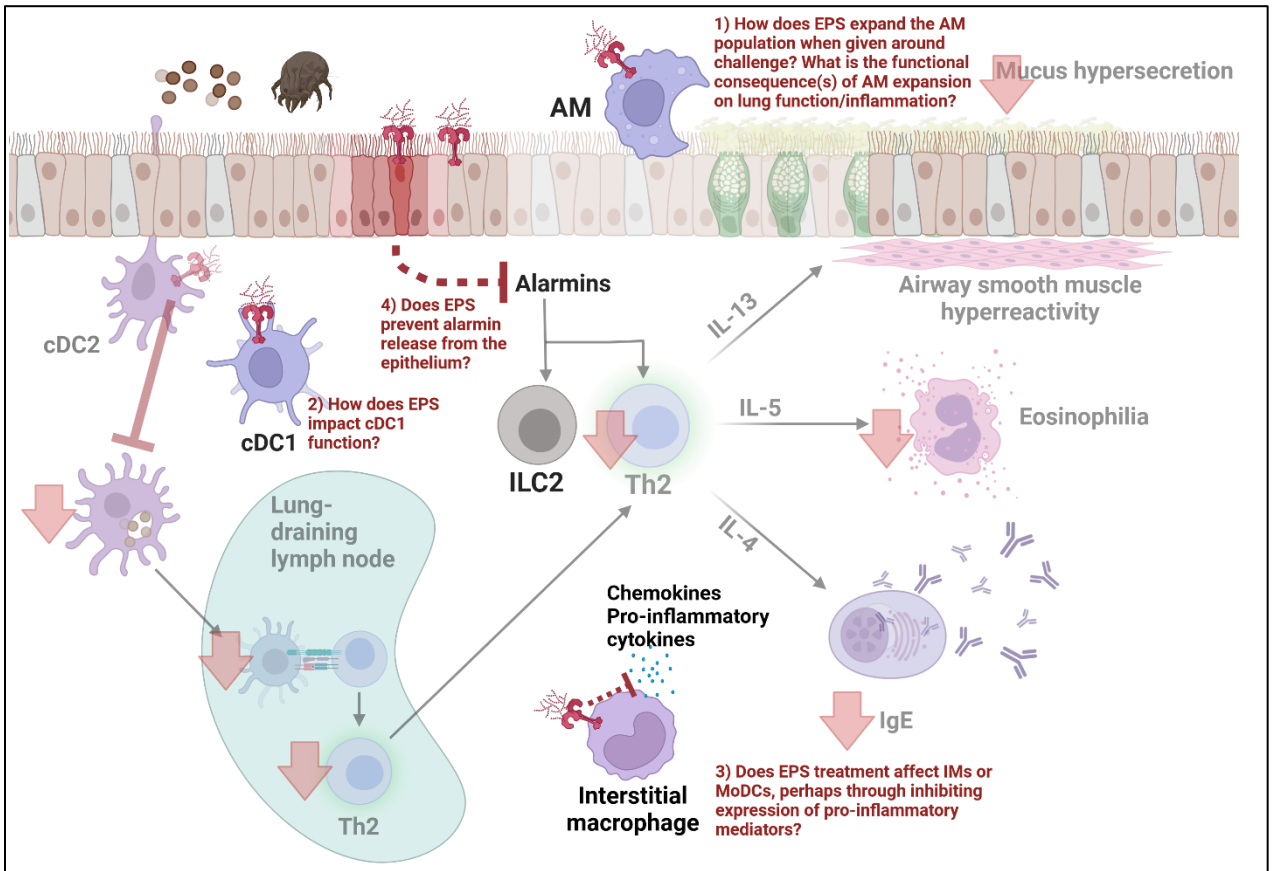

Figure 5-2: Outstanding questions on the effects of EPS on the lung environment. We have already demonstrated that EPS prevents successful migration of antigen-positive cDCs to the lung-draining lymph node (LDLN); however, numerous cell types other than DCs express TLR4, including alveolar macrophages (Question 1), cDC1s (Question 2), monocytes and monocyte-derived DCs (Question 3), and epithelial cells (Question 4). After sensing EPS, some or all of these cell types may work in concert with cDC2s to dampen responses to allergen. Diagram generated in Biorender.

generate counterbalancing Th1 immunity to restrain Th2/Th17 inflammation in response to allergen (Conejero et al., 2017). In our study, we did not observe an increase in Th1 cells after sensitization and challenge with HDM in the lung or LDLN (Figures 3-5 and 3-6), which argues that EPS is not suppressing Th2 responses by inducing compensatory Th1 responses. Another mechanism by which cDC1s can prevent allergic inflammation is through induction of Tregs, as is seen in mice treated with OM-85 prior to administration of allergen(Pivniouk et al., 2022). While

we did not see an increase in total Tregs after EPS treatment, this does not rule out that EPS is affecting cDC1 function to generate Tregs that function better on a per-cell basis (see Question 2, Figure 5-2). To test this, one could imagine isolating Tregs from EPS/HDM- or PBS/HDM-treated mice to perform functional analysis *in vitro*. Further investigation of the transcriptomes of cDC1s from EPS/HDM-treated mice will be necessary to determine if EPS is also exerting its anti-inflammatory effects through cDC1s.

Previous work has found that monocyte-derived dendritic cells (MoDCs) are poor at processing and presenting antigen to naïve T cells; however, they are capable of inducing T cell proliferation if given pre-processed peptide (Bosteels et al., 2020). MoDCs are primarily thought to secrete chemokines that attract inflammatory cells such as eosinophils, neutrophils, monocytes, and T cells to the lung (Tjota et al., 2017). As monocytes also express TLR4, they may also interact with EPS and downregulate chemokines otherwise upregulated in response to HDM (see Question 3, Figure 5-2). In our transcriptomic studies, MoDCs from mice treated with EPS/HDM and HDM alone clustered together by PCA (Figure 4-7F), which implies that EPS given i.t. results in only minor transcriptional changes to MoDCs. Closer inspection of the transcriptomes of MoDCs from EPS/HDM and PBS/HDM treated mice merits further investigation. If EPS also suppresses allergic inflammation through MoDCs, I anticipate that chemokines important in recruiting eosinophils, such as CCL19 and CCL21 (Tjota et al., 2017), will be downregulated in MoDCs from EPS/HDM-treated mice.

The broad array of allergens—from the hundreds of proteins, lipids, and carbohydrates present in inhaled house dust mites to small molecules like antibiotics and heavy metals—means that there are very few common denominators in initiation of a Th2 response. The primary similarity put forward by Kopp et al is tissue perturbation, combined with an adjuvant, that releases

both PAMPs and DAMPs at the site of allergen exposure (Kopp et al., 2023). Therefore, identifying a causal set of gene(s) modulated by EPS that attenuates a pro-Th2 program in cDC2s is difficult. Highlighted in Figure 5-3 are gene products differentially expressed in EPS/HDM cDC2s, and their potential consequences on DC function.

Costimulation/Coinhibition. We have observed in BMDCs and *ex vivo* LDLN DCs that EPS treatment dramatically increased surface CD86 (Figures 4-2E, 4-6A-D). Upregulation of costimulation and downregulation in antigen uptake is associated with DC activation; therefore, we hypothesized that EPS was inducing DC activation in the absence of antigen, thereby preventing lung DCs from taking up antigen and priming Th2 cells in the LDLN. To clarify the exact way(s) in which EPS modulates DC function, we performed transcriptomic analysis of cDCs

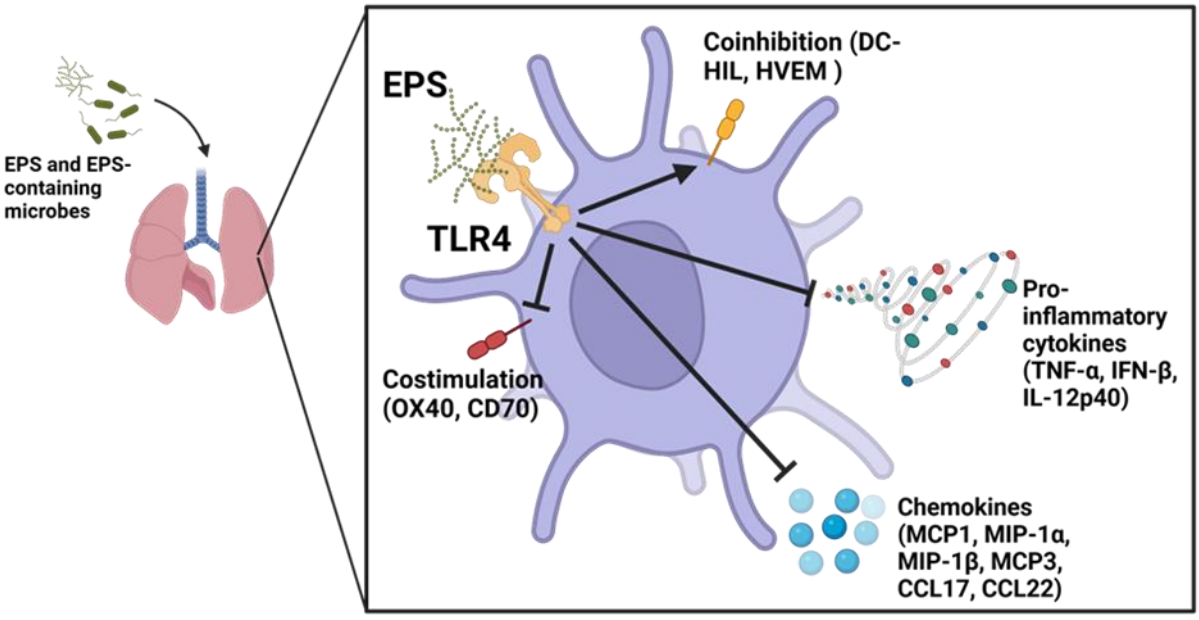

Figure 5-3: Transcriptional responses altered by EPS treatment include multiple pathways important in T cell activation and inflammation. The transcriptomes of cDC2s isolated from EPS/HDM-treated mice were enriched for coinhibitory molecules DC-HIL and HVEM, while some costimulatory molecules implicated in driving Th2 differentiation, such as OX40L and CD70, were downregulated. In addition to costimulatory pathways, cytokines immediately downstream of TLR4, such as TNF-α, IL-1β, and IL12p40 were also downregulated in cDC2s from EPS/HDM-treated mice. Finally, we observed an array of chemokine transcripts downregulated by EPS, including the myeloid chemoattractants MCP1, MIP-1-α, and MCP3 as well as the T cell chemoattractants CCL17 (data not show) and CCL22. Together, these transcriptional changes render cDC2s less capable of inducing Th2 responses to allergens. Diagram generated in Biorender.

isolated from the lungs of EPS/HDM- and PBS/HDM-treated mice. Rather than observing a dramatic increase in costimulatory molecules as would be expected in DC maturation, we observed selective up- and down-regulation of specific costimulatory pathways. Specifically, the costimulatory molecules OX40L (data not shown) and CD70 (Figure 4-9F) were both downregulated in cDC2s from EPS/HDM-treated mice. OX40L is hypothesized to be one of the costimulatory molecules capable of inducing Th2 polarization, as OX40-deficient mice have attenuated Th2 responses to allergen(Gracias et al., 2021). Downregulation of OX40L and costimulatory molecule CD70, therefore, may be another way in which EPS prevents effective Th2 polarization *in vivo* (Figure 5-3). To confirm that EPS downregulates these costimulatory molecules at the protein level, we plan to add OX40L and CD70 to the dendritic cell panel. If these molecules are indeed downregulated, we hope to investigate further which transcriptional program(s) they have in common, which may lead us to druggable targets for preventing allergic inflammation *in vivo*.

Antigen-presenting cells can also upregulate coinhibitory molecules on their surface, to attenuate T cell proliferation and survival. Two of these coinhibitory molecules, HVEM and DC-HIL, are upregulated in cDC2s from EPS/HDM-treated mice relative to cDC2s from HDM-treated mice (Figure 4-8D). DC-HIL has long been appreciated as a negative regulatory of T cell activation(Chung et al., 2007), and HVEM can interact with ligand BTLA to suppress TCR signaling(Rodriguez-Barbosa et al., 2019). The downregulation of costimulatory OX40L and CD70, in concert with upregulation of DC-HIL and HVEM, could therefore shift EPS-treated DCs to inhibit T cell responses, rather than promote them (Figure 5-3).

Proinflammatory cytokines downstream of NF-κB. In our RNA-seq analysis of cDC2s, we observed downregulation of pro-inflammatory cytokines downstream of TLR4 such as TNF-α,

IFN-β, and the beta subunit of IL-12. Genetic polymorphisms in the *TNFA* locus have been associated with asthma heritability in a Japanese population(Noguchi et al., 2002) and HDM-induced hyperreactivity in tracheal cultures could be partially inhibited by TNF-α inhibitors; therefore, TNF-α could be a central player in the induction of allergic inflammation that is otherwise blocked by EPS treatment. Consistent with this idea, TNF-α is induced in DCs after HDM treatment. However, TNF-α in DCs is largely induced by allergen Der p 2 engagement with C-type lectin receptor DC-SIGN, rather than TLR4(Hsu et al., 2010). Both DC-SIGN and TLR4 are upstream of NF-κB signaling(Svajger et al., 2010); therefore, it is possible that, by altering the TLR4/NF-κB signaling axis, EPS also interferes with other pathways that use NF-κB signaling such as DC-SIGN. If this is the case, EPS interference with C-type lectins and other phagocytic receptors could explain why BMDCs and cDCs exposed to EPS are deficient at antigen uptake.

Chemokines. The chemokines CCL17 and CCL22 are expressed by DCs and bind to CCR4, which is enriched in Th2 cells (Luther and Cyster, 2001). cDC2s from EPS/HDM-treated mice downregulate Ccl22 transcripts (Figure 4-12), potentially resulting in inferior DC recruitment of Th cells. Other chemokines downregulated by EPS in HDM-sensitized mice included CCL2 (MCP1), CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL7 (MCP3), all of which are known myeloid cell chemoattractants (Kohli et al., 2022). Altogether, EPS treatment appears to suppress expression of pro-T and pro-myeloid chemokines.

We initially assumed that EPS preventing antigen uptake and upregulating CD86 at the protein level was due to premature DC activation, thereby depleting the immature DC pool by the time allergen was administered to the lungs. However, the number of cDCs in the lung after EPS treatment was not significantly changed. Therefore, we revised our model from EPS inducing DC maturation, to EPS inducing immunosuppressive or tolerogenic DCs. This model is in agreement

with a recent publication from the Knight group, in which EPS-treated BMDCs upregulate the immunosuppressive enzyme IDO1 (Zamora-Pineda et al., 2023). We did not detect high levels of IDO1 in our RNA-seq dataset, likely because the minimum transcript count of 800 was too stringent to detect *Ido1* transcripts. We may consider deeper sequencing in future work to identify rarer transcripts that are still significantly differentially regulated by EPS treatment.

A recent publication by the Merad group (Maier et al., 2020) identified a subset of lung DCs they called mregDCs, or "mature DCs enriched in immunoregulatory molecules". These mregDCs were found within the canonical cDC1 and cDC2 populations, expressed PD-L1, PD-L2, and IL-12β, and suppressed anti-tumor responses in the lung. Among the genes upregulated by mregDCs were CD86 and Relb, a central player in the non-canonical NF-κB signaling pathway which EPS also induces (Zamora-Pineda et al., 2023). This led us to hypothesize that these same mregDCs are being induced by EPS. However, mregDCs were associated with tumor antigen uptake in the Merad study, while EPS suppressed antigen uptake in cDC1 and cDC2 (Chapter 4). Another important difference is the upstream signaling required for each DC phenotype: EPS signaling requires TLR4 (Chapter 3), while TRIF/MyD88 signaling is dispensable for generating mregDCs (Maier et al., 2020). Perhaps the most conclusive evidence that EPS is not inducing mregDCs is that mregDCs upregulated Th2 response genes such as *Ccl22*, *Tnfrsf4*, and *Il4il*; in Chapter 4, we found that two of these genes were <u>down</u>regulated by EPS, rather than upregulated. Therefore, the cDC2s induced by EPS are a separate immunosuppressive DC subtype independent of mregDCs.

Future directions

The dramatic reduction in antigen-positive migDCs after EPS-pretreatment (Figure 4-4) implicates sub-optimal DC antigen presentation in the Th2 defect seen in EPS-treated mice after

sensitization and challenge (Chapter 3). To get at the transcriptional program(s) affected by EPS in these migratory DCs, it would be ideal to perform RNA-seq on migDCs that have already reached the lymph node one day after HDM sensitization. This experiment was not performed due to the low DC count from each LN ($<10^5$ in PBS/HDM-treated mice; see Figure 4-5), which would necessitate pooling of multiple mice. Because we see transcriptomic changes in the lung cDC compartment—even with minor changes to antigen uptake in the lung (Figure 4-4)—we are now confident that EPS has induced a different transcriptional signature in DCs that have already moved to the LDLN. As such, we would like to confirm that LDLN mig-cDC2s from EPS/HDM-treated mice also express fewer pro-inflammatory cytokines (TNF-α, IFN-β, IL-12b), fewer chemokines (MIP-1α/β, CCL17/CCL22), fewer costimulatory molecules (OX40L, CD70), and more anti-inflammatory proteins (HO-1, DC-HIL) like their counterparts in the lung. This would be another line of direct evidence that EPS is preventing successful initiation of a Th2 response if given before HDM.

Perhaps one of the largest questions left unanswered by this work is how EPS treatment specifically affects Th2 priming through DCs, i.e. is DC sensing directly by EPS sufficient to prevent allergic inflammation, or is EPS sensed by other cells within the lung environment? Endotoxin-induced hyporesponsiveness to allergen depends on TLR4 on epithelial cells, which become hyporesponsive to allergen through upregulation of the ubiquitin-editing enzyme A20 and downregulate key chemokines such as CCL20 and GM-CSF to prevent DC infiltration (Schuijs et al., 2015). The expression of TLR4 on epithelial cells and macrophages, as previously discussed, likely results in EPS affecting the transcriptional profiles of these cells concurrent with, and possibly upstream of, DC sensing of EPS (see Questions 1 and 4, Figure 5-2). To address the effects of EPS on whole lung cells, we plan to perform single-cell RNA sequencing (scRNA-seq)

on total cells isolated from the lungs of mice treated with EPS, HDM, and EPS/HDM according to the treatment schedule used for our bulk RNA-sequencing experiments. If, as we anticipate, cells besides DCs are transcriptionally altered by EPS treatment, we can investigate the necessity of EPS sensing on these cells using conditional TLR4 knockout mice.

The Farm Effect is only protective against allergy and asthma if the subject is exposed to a farm environment during pregnancy or within the first two years of life(Riedler et al., 2001). As such, agents implicated in the Farm Effect are also being explored as prenatal supplements in animal models. Prenatal and perinatal probiotics have shown promise in preventing allergic inflammation in offspring by inducing mucosal-homing Tregs(Mincham et al., 2018) or downregulating TLR expression in placental tissue, rendering the offspring less sensitive to allergen(Conrad et al., 2009). The high tolerance to intratracheal EPS seen in our studies makes EPS an ideal candidate for testing whether exposure to pregnant mothers could prevent allergic sensitization in offspring. The large number of transcriptional changes in EPS-treated cDC2s, even in the absence of HDM (Figure 4-10), implies that EPS alone could be "re-programming" DCs to become refractory to allergic stimuli. If EPS is capable of crossing the fetal-maternal barrier, it could also modulate offspring DC function or myelopoiesis—as is seen in the offspring of mothers given the bacterial product OM-85(Mincham et al., 2018)—and render offspring tolerant to subsequent allergen exposure.

While we have demonstrated that EPS is a powerful immunomodulatory agent on its own, *B. subtilis* also modulates barrier integrity to improve nutrient absorption and prevent enteric infection. As such, it may be advantageous to study the anti-inflammatory potential of whole *B. subtilis* in allergic disease models. It has been established that intragastric administration of *B. subtilis* protects against allergic eosinophilia (Rosalinda Monroy Del Toro, 2023; Swartzendruber

et al., 2019). Tthe possibility of pulmonary *B. subtilis* providing the same protection against allergic eosinophilia is currently unexplored. *B. subtilis* colonization in the nose has been described in rural populations (Piewngam et al., 2018). It is possible that *B. subtilis*, a widespread soil-associated bacterium, is already preventing allergic inflammation in rural populations, but its lower prevalence in the GI tract relative to other microbes has masked it from identification in microbiome studies of the Farm Effect.

Conclusion.

Early-life exposure to commensal and environmental microbes can protect against the development of asthma and atopy, a phenomenon known as the Farm Effect. Understanding how these microbial exposures render the immune system refractory to allergens could lead to prophylactic and/or therapeutic interventions, reducing the morbidity and mortality associated with allergic asthma. In this thesis, I show for the first time that inhaled exopolysaccharide (EPS) from the environment-associated bacterium *Bacillus subtilis* significantly attenuates mucus hypersecretion, eosinophilia, and CD4 T cell accumulation in a clinically relevant murine model of allergic asthma. Mechanistically, EPS prevents the generation of pathogenic Th2 cells through affecting the capacity of DCs to phagocytose allergen and shuttle it to the LDLN for effective T cell priming. I then demonstrate that EPS affects transcription of key DC effector proteins, including costimulatory molecules, pro-inflammatory cytokines, and chemokines important for T cell-DC interactions. Altogether, these studies provide a mechanism through which a novel probiotic product can suppress allergic inflammation prophylactically and demonstrates the potential of EPS as a therapeutic agent in treating allergic asthma.

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