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RESEARCH ARTICLE

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Neutrophilia in severe asthma is reduced in Ormdl3 overexpressing mice

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Abstract

Genome-wide association studies have linked the ORM (yeast)–like protein isoform 3 (ORMDL3) to asthma severity. Although ORMDL3 is a member of a family that negatively regulates serine palmitoyltransferase (SPT) and thus biosynthesis of sphingolipids, it is still unclear whether ORMDL3 and altered sphingolipid synthesis are causally related to non-Th2 severe asthma associated with a predominant neutrophil inflammation and high interleukin-17 (IL-17) levels. Here, we examined the effects of ORMDL3 overexpression in a preclinical mouse model of allergic lung inflammation that is predominantly neutrophilic and recapitulates many of the clinical features of severe human asthma. ORMDL3 overexpression reduced lung and circulating levels of dihydrosphingosine, the product of SPT. However, the most prominent effect on sphingolipid levels was reduction of circulating S1P. The LPS/OVA challenge increased markers of Th17 inflammation with a predominant infiltration of neutrophils into the lung. A significant decrease of neutrophil infiltration was observed in the *Ormdl3* transgenic mice challenged with LPS/OVA compared to the wild type and concomitant decrease in IL-17, that plays a key role in the pathogenesis of neutrophilic asthma. LPS decreased survival of murine neutrophils, which was prevented by co-treatment with S1P. Moreover, S1P potentiated LPS-induced chemotaxis of neutrophil, suggesting that S1P can regulate neutrophil survival and recruitment following LPS airway inflammation. Our findings reveal a novel connection between ORMDL3 overexpression, circulating levels of S1P, IL-17 and neutrophil recruitment into the lung, and questions the potential involvement of ORMDL3 in the pathology, leading to development of severe neutrophilic asthma.

KEYWORDS
neutropenia, ORMDL3, severe asthma, sphingosine-1-phosphate

Abbreviations: BALF, bronchoalveolar lavage fluid; DHS1P, dihydrosphingosine-1-phosphate; FACS, fluorescence-activated cell sorting; GWAS, genome-wide association studies; HDM, house dust mite; Ig, immunoglobulin; IL, interleukin; LPS, lipopolysaccharide; MPO, myeloperoxidase; ORMDL3, ORM (yeast)-like protein isoform 3; OVA, ovalbumin; S1P, sphingosine-1-phosphate; S1PR, sphingosine-1-phosphate receptor; SPT, serine palmitoyltransferase; TG, transgenic; TLR4, Toll-like receptor 4; UPR, unfolded protein response; WT, wild type.

1 | INTRODUCTION

Asthma, a syndrome with substantial heterogeneity, is a chronic airway inflammatory disease in which exposure to allergens, environmental pollutants, or viruses causes intermittent attacks of breathlessness, airway hyperreactivity, wheezing, and coughing. It is one of the most prevalent diseases and is increasing throughout the world, especially in industrialized urban populations.[1,2] Approximately 10% of patients with asthma have a distinct, severe phenotype.[3,4] Unlike the Th2-driven, eosinophil inflammatory responses commonly observed in patients with milder diagnoses, severe asthma is associated with a more predominant neutrophil inflammation and high interleukin-17 (IL-17) levels.[3,4] Unfortunately, patients with this non-Th2 neutrophilic asthma show poor responses to the currently available anti-inflammatory and traditional glucocorticoid therapies.[5,6]

There is a strong genetic component to asthma, and numerous genome-wide association studies (GWAS) have convincingly and repeatedly identified an association of the 17q21 locus and within it the ORM (yeast)-like protein isoform 3 (*ORMDL3*) gene that has received the greatest attention for functional assessment with asthma in a number of ethnically diverse populations.[7–14] This locus influences disease severity and the frequency of human rhinovirus–initiated exacerbations.[13,15] ORMDL3 is also upregulated in allergic mice challenged with ovalbumin (OVA), house dust mite (HDM), or *Alternaria alternata*.[16–18] However, little is still known about the molecular mechanism by which ORMDL3 is linked to asthma.

ORMDL3 is a member of a family of three ER-localized proteins that are highly homologous with the yeast ORM orthologs.[19] Extensive studies in yeast and mammalian cells have demonstrated that these proteins bind to and inhibit SPT, the rate-limiting enzyme in the de novo biosynthesis of the bioactive sphingolipid metabolite ceramide.[19–21] Recent high-resolution cryo-EM studies suggested that ORMDL3 is located in the center of the SPT complex and stabilizes it,[22] thereby blocking binding of the substrate to SPT.[23] Moreover, it was suggested that ceramide acts as a feedback inhibitor of SPT through interaction with ORMDL proteins.[24] Previously, we reported that ceramide is elevated in lungs of allergic asthmatic mice and humans and that suppression of ceramide elevation suppresses airway hyperreactivity and cytokine production in allergic mice.[16] Because of the ER localization of ORMDL3, several other proposed mechanisms have focused on ER stress and the unfolded protein response (UPR)[25,26] effect on calcium metabolism[27,28] and influencing cytokines/chemokines[25] and adhesion molecules.[29]

How ORMDL3 contributes to asthma pathogenesis is still not well-understood, and conflicting results were reported in studies with transgenic *Ormdl3* overexpressing mice. Increased airway responsiveness and airway remodeling were observed in transgenic mice overexpressing human ORMDL3, potentially through ATF6 target genes, such as sarcoendoplasmic reticulum calcium ATPase that increases airway smooth muscle cell proliferation and contractility.[28,30,31] In contrast, no differences in the severity of all key asthma parameters were found in another line of *Ormdl3* transgenic mice challenged with several allergens including OVA, HDM, and *Alternaria* in acute and chronic asthma models, even though challenge with these allergens led to increased lung ceramide.[17] It was concluded that ORMDL3 regulates ceramide levels, but genetically interfering with Ormdl3 expression does not result in altered allergen-driven asthma in mice.[17]

Recently, we identified a strong association between increased levels of long-chain ceramide species in bronchoalveolar lavage fluid (BALF) with neutrophilic lung inflammation, asthma severity, and resistance to corticosteroids in patients with severe asthma.[18] Moreover, treatment of HDM-challenged mice with the SPT inhibitor myriocin or fumonisin B1, an inhibitor of ceramide synthases, not only prevented ceramide elevation but also markedly suppressed HDM-induced neutrophilia, even when these inhibitors were only administered during the late asthmatic responses.[18] Furthermore, increasing lung ceramide by intranasal instillation of C16:0 ceramide significantly increased numbers of neutrophils in the BALF.[18,32] Because these data suggest a role for ceramide elevation in allergen-initiated lung neutrophil recruitment, we were interested in examining the effects of ORMDL3 overexpression in a preclinical model of allergic lung inflammation that is predominantly neutrophilic and recapitulates many of the clinical features of severe human asthma.[3,4] Surprisingly, we found that challenged global Ormdl3 overexpressing transgenic mice had less neutrophil infiltration and IL-17 associated with reduced circulating levels of S1P. Our findings introduce a novel connection between ORMDL3 overexpression, circulating levels of S1P, and neutrophil recruitment into the lung and question the potential involvement of ORMDL3 in the pathology underlying the development of severe asthma.

2 | MATERIALS AND METHODS

2.1 | Mice

Ormdl3 transgenic mice were generated by knocking in an *Ormdl3* transgene by homologous recombination to the Rosa26 locus in embryonic stem cells. The Rosa26 locus provides an open chromatin configuration conducive for modest gene expression in all tissues,[33] and the

strong ubiquitous promoter, the chicken β-actin CAG promoter, provides a higher level of transgene expression.[34] The transgene consisted of the CAG promoter followed by a lox-STOP-lox (LSL) cassette, the full-length mouse 3XFlag-Ormdl3 cDNA, and the SV40E pA. The lox-STOP-lox was placed between the promoter and coding sequence for ORMDL3 to prevent expression in the absence of Cre. To create the targeting vector, the transgene was flanked by Rosa26 5′ and 3′ homology arms, and a diphtheria toxin (DTA) minigene was added to one end of the targeting vector (Figure 1A). After germline transmission of the targeted allele was obtained, the transgenic mice were crossed with an EIIA-cre mouse[35] to delete the LSL cassette and universally express the transgene (Figure 1A). These mice were then back-crossed to C57BL/6J mice seven times. Littermate control mice and comparable proportions of male and female mice were used for all transgenic experiments. No sex-dependent differences were observed in experiments. Mice were housed in the animal care facilities at Virginia Commonwealth University under standard temperature, humidity, and timed light conditions and provided with standard rodent chow and water ad libitum. The Institutional Animal Care and Use Committee at Virginia Commonwealth University approved all animal protocols and procedures.

2.2 | Neutrophilic asthma model

Both male and female mice (8–12 weeks of age) were sensitized intranasally with 5 μg of LPS from *Salmonella enterica* (Sigma, catalog no. L6511) and 100 μg of OVA (Sigma, catalog no. A5503) dissolved in 40 μL of PBS on day 1. Mice were boosted and challenged with 1 μg of LPS and 100 μg of OVA in 40 μL of PBS on day 7 and days 17–20. On day 21, mice were humanely euthanized by isoflurane (Figure 1D).

2.3 | Flow cytometry

Following euthanasia, BALF was collected with 1 mL of PBS, and cells were resuspended in fluorescence-activated cell sorting (FACS) buffer containing 2% BSA and 2 mM EDTA in PBS. Cells collected from BALF were stained with a fixable live/dead stain, Zombie Aqua (Biolegend), for 10 min in the dark at room temperature according to the manufacturer's instructions. Cells were then washed and resuspended in FACS buffer, incubated with Fc block anti-mouse CD16/CD32 (clone 93) on ice for 10 min to reduce nonspecific binding, followed by staining with an antibody cocktail (Table 1) in Brilliant Violet Stain Buffer (BD Biosciences). Cells were washed, fixed in the dark at room temperature for 15 min with 3% paraformaldehyde, washed, and resuspended in PBS for analysis on the BD LSRFORTESSA X20 cell analyzer equipped with FACSDIVA 9.0 software (BD Biosciences) at the VCU Flow Cytometry Shared Resource. UltraComp eBeads Plus compensation beads (ThermoFisher) were used to optimize fluorescence compensation settings for multicolor flow cytometric analyses. Cells were first gated for forward (FSC-A) and side (SSC-A) scatter to exclude debris, FSC-A and FSC-H to exclude doublets, and then Zombie-Aqua-negative (live) and CD45-positive for white blood ce+lls. The gating strategy for each cell population was as follows: $CD11b^{+}F4/80^{+}$ macrophages, $CD11c^{-}CD11b^{+}Ly6G^{+}$ neutrophils, $CD11c^{-}CD3^{+}MHCII^{-}$ T cells, $CD11c^{-}B220^{+}MHCII^{+}$ B cells, $CD11c^{+}SiglecF^{-}MHCII^{+}$ dendritic cells, and $CD11c^{-}CD11b^{+}Ly6G^{-}SiglecF^{+}SSChigh$ eosinophils. Data analysis was performed using FlowJo, version 10.7.2 (BD Biosciences).

2.4 | Mass spectrometry measurements of sphingolipid metabolites

Sphingolipid metabolites in blood, plasma, and lung were quantified by liquid chromatography electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS) as previously described.[36] Briefly, 50 μL of whole blood was used for the determination of sphingolipids. To obtain plasma, fresh whole blood was centrifuged (2000× g, 15 min, 4°C), and the supernatant was removed and stored at −80°C for later analysis.[37] Weighed lung tissues were placed into 13 × 100 mm borosilicate tubes with a Teflon-lined cap and 2 mL of CH_3OH and 1 mL of $CHCl_3$. Internal standards (Avanti Polar Lipids, Alabaster, AL) in 10 μL ethanol:methanol:water (7:2:1) as a cocktail of 250 pmol each were added to samples. Standards for sphingoid bases and sphingoid base 1-phosphates were all 17-carbon chain length analogs. Samples were dispersed by sonication at room temperature for 30 s. The single-phase mixtures were incubated at 48°C for 8 h. The extracts were centrifuged and the supernatants transferred to new tubes. The extracts were dried with a speed vac and reconstituted in 0.5 mL of the starting mobile phase solvent for LC-ESI-MS/MS analysis, briefly sonicated and centrifuged, and supernatants transferred to autoinjector vials. Sphingolipids were separated by reverse-phase HPLC using a Supelco 2.1 × 50 mm Ascentis Express C18 column (Sigma) with a binary solvent system at a flow rate of 0.5 mL/min with a column oven set at 35°C. Prior to injection of samples, the column was equilibrated for 0.5 min with a solvent mixture of 95% mobile phase A1 (CH_3OH/H_2O/HCOOH, 58/41/1, v/v/v, with 5 mM ammonium formate) and 5% mobile phase B1 (CH_3OH/HCOOH, 99/1, v/v, with 5 mM

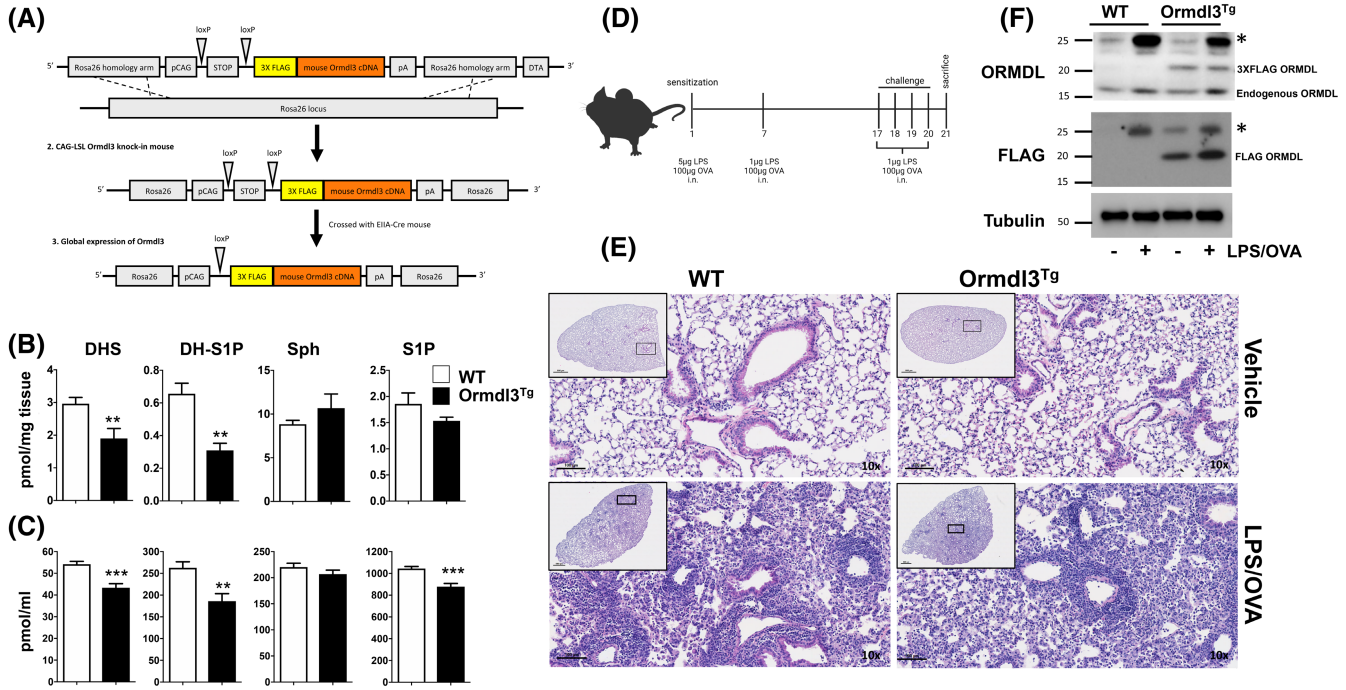

FIGURE 1 Sensitization with LPS/OVA increases ORMDL expression and airway inflammation in global Ormdl3 transgenic mice. (A) Schematic of the strategy used to generate *Ormdl3-Flag* transgenic mice. Ormdl3-Flag transgene was knocked in by homologous recombination to the Rosa26 locus. The transgene consists of the CAG promoter followed by a lox-STOP-lox (LSL) cassette, the full-length mouse 3XFlag-Ormdl3 cDNA, and SV40E pA. The targeting vector is flanked by Rosa26 5′ and 3′ homology arms with a diphtheria toxin (DTA) minigene added to the end. These mice were crossed with an EII-Cre mouse to induce universal expression of the 3X-FLAG-Ormdl3 transgene. (B, C) Sphingolipids were extracted from lung (B) or plasma (C), and levels of dihydrosphingosine (DHS), dihydrosphingosine-1-phosphate (DH-S1P), sphingosine (Sph), and S1P were measured by liquid chromatography–electrospray ionization–tandem mass spectrometry. Data are means ± SEM, $n = 11$ (B), $n = 8, 7$ (C) $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ compared to WT. (D) Schematic showing sensitization and challenge protocol. Wild-type and *Ormdl3-Flag* transgenic ($Ormdl3^{Tg}$) mice were sensitized with vehicle or LPS (5 μg) and OVA (100 μg) intranasally on day 1 and then challenged with LPS (1 μg) and OVA (100 μg) or vehicle on days 7 and 17–20. Mice were sacrificed on day 21. (E) H&E staining of lung sections. Size bars: 100 μm. (F) Proteins in lung lysates were analyzed by Western blotting with anti-ORMDL and anti-FLAG antibodies. Tubulin was used as a loading control. Asterisk indicates non-specific band.

TABLE 1 Antibodies used.

Target	Conjugate	Company	Clone	Product No.
Live/dead	Zombie Aqua	Biolegend		423101
CD45	APC-Fire750	Biolegend	30-F11	103154
CD3e	BUV737	BD	145-2C11	612771
B220	BUV737	BD	RA3-6B2	612838
CD11b	PE/Cy7	Biolegend	M1/70	101215
MHCII	BV421	Biolegend	M5/114.15.2	107631
Ly6G	FITC	Biolegend	1A8	127605
SiglecF	AF647	BD	E50 2440	562680
CD11c	BV711	Biolegend	N418	117349
F4/80	PE	Biolegend	BM8	123110

ammonium formate), and after sample injection, the A1/B1 ratio was maintained at 95/5 for 2.25 min, followed by a linear gradient to 100% B1 over 1.5 min, which was held at 100% B1 for 5.5 min, followed by a 0.5 min gradient return to 95/5 A1/B1. The column was re-equilibrated with 95:5 A1/B1 for 0.5 min before each run. The HPLC column was coupled to a Sciex 5500 quadrupole/linear ion trap (QTrap; SCIEX Framingham, MA) operating in triple quadrupole mode. Q1 and Q3 were set to pass molecularly distinctive precursor and product ions (or a scan across multiple *m/z*

in Q1 or Q3), using N_2 to collisionally induce dissociations in Q2 (which was offset from Q1 by 30–120 eV). The temperature of the ion source was set at 300°C.

2.5 | Lung cytokine and chemokine analysis

The BioLegend LEGENDplex Mouse Inflammation Panel (13-plex) and LEGENDplex Mouse Proinflammatory Chemokine Panel (13-plex) were used to measure pro-inflammatory cytokines and chemokines. Assays were performed according to the manufacturer's protocol with modifications in sample preparation. Briefly, lung tissue was homogenized by freeze–thaw cycles and mechanical disruption in ice-cold assay buffer (10 µL/mg tissue). The supernatant was used to analyze 13 cytokines, including IL-1α, IL-1β, IL-17A, MCP-1, IFN-γ, and TNF-α, simultaneously according to the manufacturer's instructions. Similarly, 13 chemokines, including MCP-1 (CCL2), RANTES (CCL5), IP-10 (CXCL10), Eotaxin (CCL11), TARC (CCL17), MIP-1α (CCL3), MIP-1β (CCL4), MIP-3α (CCL20), and KC (CXCL1), were also measured. Data were acquired on the BD LSRFORTESSA X20 cell analyzer equipped with FACSDIVA 9.0 software (BD Biosciences) and analyzed with the LEGENDplex Data Analysis Software Version 8.0 (BioLegend). Results were normalized to lung lysate protein levels measured with the Pierce BCA Protein Assay Kit (Thermo Scientific).

2.6 | Neutrophil attachment and chemotaxis assays

Primary neutrophils and epithelial cells were isolated from the lungs of wild type and *Ormdl3* transgenic mice using magnetic bead cell selection for Ly6G-positive or CD326 (EpCAM)-positive cells, respectively, exactly as we previously described.[18] Epithelial cells were stimulated for 20 h with vehicle or LPS (200 ng/mL), and the neutrophil attachment assay was performed as previously described.[18] Transwell inserts (pore size: 5.0 µm; Sarstedt) were used to measure the migration of neutrophils and pre-coated with laminin. 1.5×10^5 cells were seeded in the top well in serum-free DMEM and placed on wells containing 100 nM fMLP with S1P (100 nM) or LPS (200 ng/mL) in the lower chamber. The chambers were incubated at 37°C in 5% CO_2 for 1 h. Subsequently, migrated neutrophil numbers in lower chambers were determined by removing all cells from the upper chamber and filling the bottom chamber with 100 µL of cell dissociation solution containing 2 µg/mL Calcein-AM (Life Technologies, CA). The re-assembled chamber was incubated for an additional 1 h at 37°C. Migrated cells in the fluid of the bottom chambers were quantified by measuring fluorescence of Calcein at 485 nm excitation, 520 nm emission with a TECAN Infinite M1000 fluorescence plate reader (Männedorf, Switzerland).

2.7 | RNA isolation and QPCR

Total RNA was extracted with TRIzol (#15596026, ThermoFisher) and reverse-transcribed with the High-Capacity cDNA Reverse Transcription Kit (#4368814, ThermoFisher). mRNA expression was determined by mixing cDNA with the following primer pairs (5′-3′): Ormdl1 for CATAATCTGGGGATGTATGTG and rev CTTCCGAGAAGATGTAAACTG; Ormdl2 for AACAA CAAGCCTGAAGTTAG and rev agcatgtagccctaattttg; Ormdl3 for TATAGTGCTGTACTTCCTCAC and rev GAATGAGCACAGTCATCAAG; TATA-binding protein (TBP) for TTCCAAAACTCCGGGTAGGC and rev AACCGATTCCGCACAGTCTT; Tubulin, Beta 1 Class VI (Tubb) for AAGCCTACGGTAAGAAGTATG and rev CCATGAACAAAACTGTCAGG; 18 S for AGTCCCTG CCCTTTGTACACA, rev GATCCGAGGGCCTCACT AAAC and probe CGCCCGTCGCTACTACCGATTGG along with either *Power*SYBR Green PCR Master Mix (#4367659, ThermoFisher) or TaqMan Universal PCR Master Mix (#4324018, ThermoFisher). cDNA was amplified and measured using a CFX Opus 96 Real-Time PCR Detection System (#12011319, BioRad). Relative changes in gene expression were calculated using the ΔΔCt method, normalized to the geometric mean of the expression of 18 S ribosomal RNA, Tubb, and TBP.

2.8 | Western blot analysis

Total protein from lung homogenates was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific), and aliquots were loaded and separated on 15% SDS-PAGE gels. The PierceG2 Fast Blotter system (Thermo Scientific) was used to transfer protein onto 0.2-µm nitrocellulose membranes, and the blots were incubated with the following antibodies: anti-ORMDL3 (1:1000; #ABN417, Millipore), anti-FLAG (1:1000; #F1804; Sigma), and anti-tubulin (1:20 000; #2146 S, Cell Signaling Technology). Peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000; Jackson ImmunoResearch) and chemiluminescent substrate (Pierce) were used to visualize protein bands.

2.9 | H&E staining and immunohistochemistry

Lung tissue was fixed in 1:10 formalin dilution (Fisherbrand), paraffin-embedded, and then sectioned and stained with hematoxylin and eosin (H&E). For immunofluorescent staining, cryosections were fixed with ice-cold methanol for 15 min at −20°C. Slides were washed three times with PBS and then blocked and permeabilized with PBS containing 5% normal donkey serum, 1% BSA, and 0.4% Triton-X100 (buffer A) for 1 h at room temperature. Afterward, sections were incubated overnight with anti-Ly6G (BioLegend, clone 1A8, #127601) 1:200 or anti-MPO (R&D Systems, #AF3667-SP) 1:200 in buffer A at 4°C. After washing for 5 min with PBS three times, sections were stained with anti-rat AlexaFluor488 or anti-goat AlexaFluor488 1:300 in buffer A for 2 h. Sections were washed three times for 5 min with PBS and stained with 1 μg/mL DAPI for 15 min at room temperature. Afterward, sections were washed with PBS three times for 5 min and mounted with CC/Mount on coverslips. Epifluorescence microscopy was performed with a BZ-X810 Keyence fluorescence microscope

2.10 | Statistics

Statistical significances were determined with an unpaired 2-tailed Student *t* test for comparison of two groups or by ANOVA for multiple comparisons using GraphPad Prism 7.0 software (San Diego, Calif). All experiments were first tested for normality using the Shapiro–Wilk statistical test, and non-normality distributed data were analyzed with Kruskal–Wallis or Mann–Whitney tests. *p* values less than .05 were defined as significant.

3 | RESULTS

3.1 | Sensitization with LPS/OVA increases ORMDL expression and airway inflammation in global *Ormdl3* transgenic mice

To assess the impact of ORMDL3 on neutrophilic inflammation and its contribution to asthma exacerbation, we generated global *Ormdl3-Flag* transgenic mice (Figure 1A). As ORMDL3 inhibits SPT and negatively regulates de novo sphingolipid biosynthesis, we first assessed the effect of *Ormdl3* overexpression on alterations of sphingolipid metabolite levels. Overexpression of *Ormdl3* reduced lung dihydrosphingosine (DHS) produced from the reduction of 3-ketosphinganine, the direct product of SPT activity.[38] In addition, dihydro-S1P, the phosphorylated product of DHS, was also reduced (Figure 1B). The reductions in DHS and dihydro-S1P are consistent with a role for ORMDL3 in blocking SPT.[19,21] Similar to previous reports with other transgenic mice,[17,39] there were no major changes in lung levels of other sphingolipids, including sphingosine, dihydroceramide, and ceramide. Levels of DHS and dihydro-S1P were also significantly reduced in plasma (Figure 1C) and whole blood of *Ormdl3-Flag*[18] transgenic mice (Figure S1). Like in other *Ormdl3* transgenic mice,[17,39] overexpression of *Ormdl3-Flag* also greatly reduced blood levels of S1P (Figure S1). It was of interest to determine whether overexpression of *Ormdl3-Flag* caused compensatory changes in its paralogs *Ormdl1* or *Ormdl2*. However, while *Ormdl3* transcripts increased by 15-fold in lungs of *Ormdl3-Flag* transgenic mice, there were no significant changes in *Ormdl1* or *Ormdl2* expression compared to WT (Figure S2A). Moreover, consistent with a previous study,[17] airway hyperresponsiveness (AHR) in response to increasing doses of methacholine was not significantly different between WT and transgenic mice (Figure S2B).

The presence of endotoxin lipopolysaccharide (LPS), a component from the outer cell membrane of Gram-negative bacteria, during ovalbumin (OVA) sensitization has been reported to cause neutrophilic airway inflammation in mice, in contrast to the eosinophilic airway inflammation provoked by OVA/Alum.[40–42] To this end, *Ormdl3-Flag* transgenic and wild type mice were sensitized intranasally with 5 μg of LPS and 100 μg of OVA on day 1 and then subsequently boosted and challenged on days 7 and 17–20, respectively, with 1 μg of LPS and 100 μg of OVA (Figure 1D). Strong infiltration of immune cells was detected in the lungs of LPS/OVA challenged transgenic mice, visualized by H&E staining (Figure 1E).

ORMDL3 protein shares over 80% identity with ORMDL1 and ORMDL2,[21] limiting the specificity of current antibodies. The FLAG-tag epitope ensured specific detection of ORMDL3 with anti-Flag antibody. In accordance with previous reports,[16–18] we observed that LPS/OVA also induced an increase in expression of endogenous ORMDL3 in the lungs (Figure 1F). LPS/OVA challenge also enhanced the expression of epitope tagged ORMDL3 detected by anti-Flag or anti-ORMDL antibodies (Figure 1F), suggesting that allergen challenge increased ORMDL3 protein level. Although ORMDL3 is an asthma susceptibility gene, ORMDL3-Flag expression was relatively low in lung tissue, compared with other tissues, for example, liver and adipose tissues (Figure 1F, and data not shown) where *Ormdl3* mRNA expression is much more abundant.[17]

3.2 | Neutrophilic inflammation is reduced in *Ormdl3* transgenic mice

Because we were interested in the effects of *Ormdl3* overexpression in neutrophil inflammation, flow cytometry was utilized to distinguish between different immune cell populations infiltrating lungs after LPS/OVA challenge, following the gating strategy depicted in Figure 2A. Consistent with previous studies,[40–42] neutrophils were the most abundant cell type measured in the BALF (Figure 2B) of challenged mice, with relatively lower levels of eosinophils, indicative of a non-Th2 neutrophil dominant phenotype. LPS/OVA also increased T- and B-lymphocytes, dendritic cells, and macrophages in the BALF (Figure 2C–F), albeit to a much lesser extent than the increase in neutrophils. Surprisingly, there was a significant decrease of neutrophils in the *Ormdl3* transgenic mice challenged with LPS/OVA compared to the wild-type challenged mice. Neutrophils were the only cell population that was significantly different between these two groups (Figure 2B). Moreover, immunofluorescent staining of lung sections for the neutrophil-specific marker Ly6G[43] also revealed intense staining after LPS/OVA exposure, which was reduced in *Ormdl3* transgenic challenged mice (Figure 3A). Similarly, more pronounced staining of myeloperoxidase (MPO) found predominantly in neutrophil granules, a marker of their activation, was observed in wild type mice challenged with LPS/OVA compared to *Ormdl3* transgenic challenged mice (Figure 3B). These data suggest that *Ormdl3* overexpression reduces the influx of neutrophils into lung airways after LPS/OVA challenge.

3.3 | *Ormdl3* overexpression reduces cytokine IL-17A in lungs of challenged mice

It was of interest to investigate how overexpression of *Ormdl3* reduced neutrophilic airway inflammation in LPS/OVA-challenged transgenic mice. In patients with neutrophilic asthma, high neutrophil count is associated with increased proinflammatory cytokine/chemokine release.[44] Therefore, we next measured levels of a panel of cytokines in lung tissue known to be associated with the neutrophilic inflammation.[45,46] Cytokines IL-17A, TNF-α, and IFN-γ as well as IL-6 and IL-1α were all markedly increased in LPS/OVA challenged mice (Figure 4). LPS/OVA challenge did not enhance IL-1β levels and slightly but not significantly increased IFN-β levels, and the cytokines IL-23, IL-10, IL-12p70, and IL-27 were below the detection limits (data not shown). Interestingly, only IL-17A was significantly reduced in challenged transgenic mice compared to challenged WT mice (Figure 4). IL-17 plays a key role in the pathogenesis of neutrophilic asthma by inducing secretion of chemoattractants, cytokines, and chemokines, leading to recruitment and activation of neutrophils.[42,45] These results suggest that *Ormdl3* overexpression reduces LPS/OVA-induced IL-17A production, which is responsible, at least in part, for the attenuation of LPS/OVA-induced airway neutrophilia.

We also examined whether *Ormdl3* overexpression differentially affects LPS-mediated increase of chemokines in the airways. In response to LPS/OVA, the airways secreted multiple cytokines with some functional overlap including CCL3/MIP-1α and CXCL1/KC to attract neutrophils; CCL20/MIP-3α, which is strongly chemotactic for lymphocytes and weakly attracts neutrophils; and CCL2/MCP-1 and CCL4/MIP-1β to chemoattract monocytes[47] (Figure 5). CCL2 also acts as a neutrophil chemokine during LPS-mediated lung inflammation.[48] In addition, LPS/OVA challenge also increased the T-cell chemokines IP-10/CXCL10, CCL20/MIP-3α, Mig/CXCL9, and RANTES/CCL5 and the B cells chemokine CXCL13/BLC (Figure 5). As expected, chemokines commonly associated with an eosinophilic Th2 response, TARC/CCL17 and eotaxin/CCL11, were lower in LPS/OVA challenged mice. Although CXCL1, CCL3, and CCL5 were reduced in allergen challenged Ormdl3 transgenic mice, these did not reach statistical significance. However, CCL2 that is known to recruit Th17 to the lungs in a murine asthma model[48,49] was significantly reduced in transgenic compared to WT mice challenged with LPS/OVA (Figure 5). Likewise, CXCL13/BLC was significantly reduced in the transgenic mice compared to WT mice, which correlates with the decrease in IL-17A, as multiple studies have reported that IL-17 enhances the migration of B cells during asthma by inducing production of CXCL13/BLC.[50,51]

3.4 | Role of S1P in neutrophil recruitment and survival

Because circulating levels of the bioactive sphingolipid mediator S1P were significantly reduced in *Ormdl3* transgenic mice (Figure 1C) and it has been suggested that S1P might influence survival and trafficking of neutrophils,[52–56] we next sought to examine whether deficiency of circulating S1P influenced the ability of neutrophils to migrate to the site of inflammation. As expected, survival of murine neutrophils was decreased by LPS treatment in a time-dependent manner (Figure 6A). Consistent with the ability of S1P to promote human neutrophil survival,[56] S1P treatment suppressed the effects of LPS and enhanced murine neutrophil survival (Figure 6A). Treatment of primary lung epithelial cells with LPS slightly increased neutrophil adhesion to an epithelial monolayer (Figure 6B).

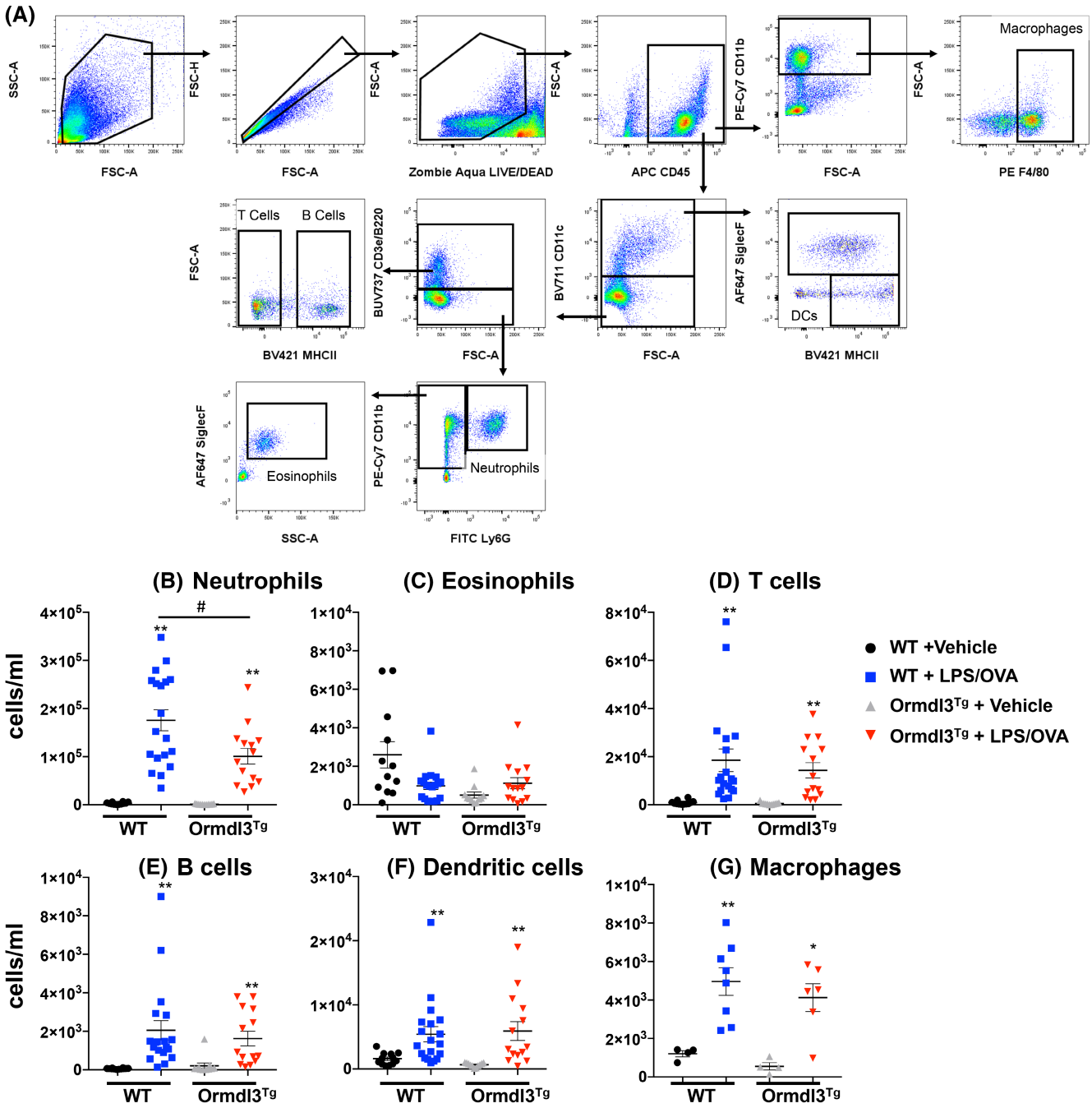

FIGURE 2 Neutrophilic airway inflammation is reduced in LPS/OVA sensitized *Ormdl3* transgenic mice. (A) Gating strategy for FACS analyses of infiltrating immune cell populations in BALF. Representative flow cytometric analysis of neutrophils ($CD11b^+Ly6G^+$), T cells ($CD3^+MHCII^-$), B cells ($B220^+MHCII^+$), dendritic cells ($CD11c^+SiglecF^-MHCII^+$), macrophages ($CD11b^+F4/80^+$), and eosinophils ($CD11b^+SiglecF^+SSChigh$). The cells were first gated using FSC/SSC plots, followed by doublet exclusion using FSC-A and FSC-H. All cells were gated as living cells and $CD45^+$ for hematopoietic cells. Unstained samples were used to determine autofluorescence and as a negative control for the gating. (B–G) wild type (WT) and global *Ormdl3* transgenic ($Ormdl3^{Tg}$) mice were sensitized and challenged with LPS/OVA. BALF Inflammatory cells were quantified by flow cytometry. Data are means ± SEMs. (B–F) $n = 12$, $n = 19$, $n = 11$, and $n = 14$; (G) $n = 4$, $n = 8$, $n = 4$, and $n = 6$ for WT treated with vehicle, WT treated with LPS/OVA, TG treated with vehicle, and TG treated with LPS/OVA, respectively. $^{**}p < .01$ compared to vehicle-treated mice, $^{\#}p < .05$ compared to WT challenged with LPS/OVA.

Adhesion of neutrophils from *Ormdl3* transgenic mice was similar to that of WT neutrophils and increased by LPS treatment (Figure 6B). Moreover, treatment with LPS significantly increased chemotaxis and S1P had even a stronger effect (Figure 6C). No major differences were observed in chemotaxis of neutrophils from *Ormdl3* transgenic mice compared to neutrophils from wild-type mice toward S1P or LPS. Importantly, S1P potentiated

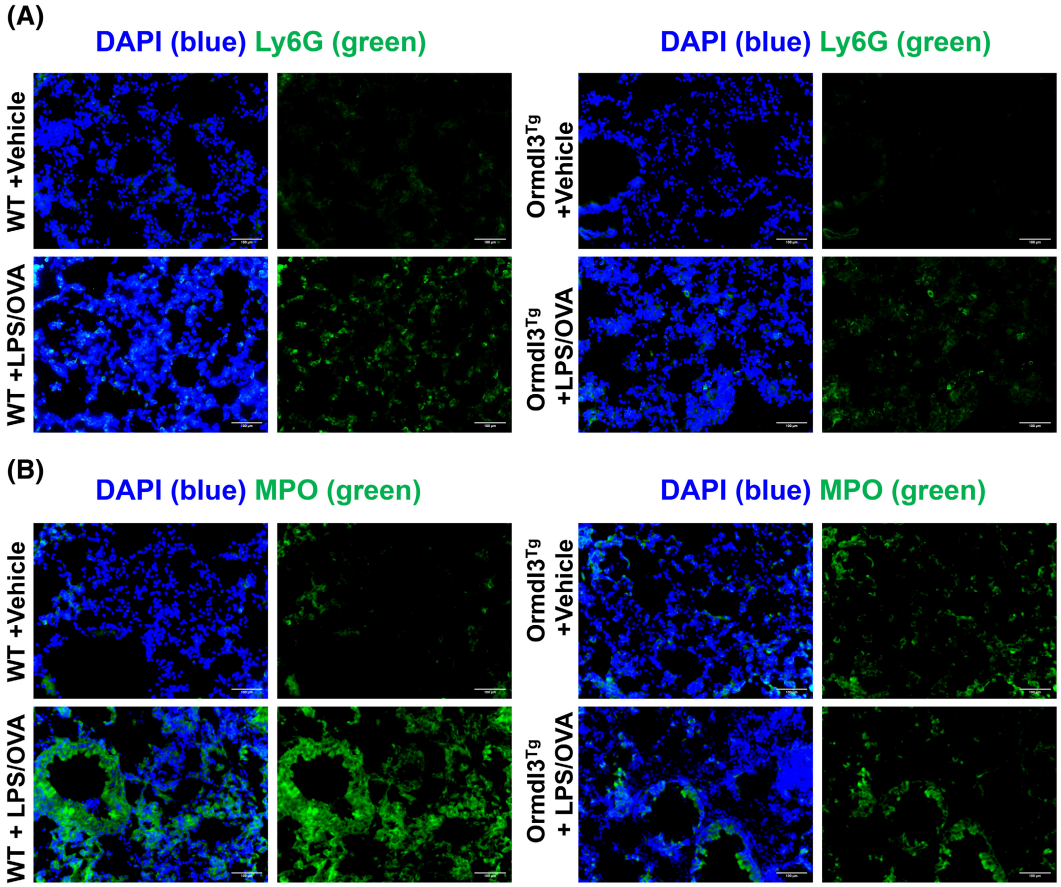

FIGURE 3 Decreased neutrophil recruitment into the lungs of *Ormdl3* transgenic mice. Wild-type (WT) and global *Ormdl3* transgenic ($Ormdl3^{Tg}$) mice were sensitized and challenged with LPS/OVA. Lung sections were stained with (A) anti-Ly6G (green) or (B) anti-MPO antibody (green) and co-stained with DAPI to visualize nuclei (blue). Size bar: 100 μm.

LPS-induced chemotaxis of neutrophils from both wild type and *Ormdl3* transgenic mice (Figure 6C). Taken together, these data suggest circulating S1P can regulate neutrophil survival and recruitment following LPS airway inflammation.

4 | DISCUSSION

Severe asthma has received much attention in recent years due to its difficulty of treatment. The frequent exacerbations of non-allergic/non-eosinophilic asthma and steroid resistance are major causes of asthma-related morbidity.[5,6] While no animal model encompasses all features of severe asthma, in this study, we recapitulated the high Th17 and low Th2 neutrophilic inflammatory response associated with severe asthma patients in a mouse model in which sensitization of mice with LPS/OVA induced a dominant neutrophil infiltration into the lung and bronchoalveolar space. Even though ORMDL3 has been linked to severe asthma,[57,58] we observed that overexpression of *Ormdl3* in transgenic mice challenged with LPS/OVA reduced neutrophil accumulation in BALF that correlated with decreased levels of Th17-associated cytokine Il-17A. Moreover, consistent with the function of ORMDL3 as a negative regulator of SPT,[19,21] we found that *Ormdl3* overexpression reduced lung and circulation levels of DHS, the direct product of SPT activity.[38] However, consistent with previous studies,[17,39] the most prominent effect on sphingolipids was the reduction of S1P levels in circulation. Thus, our study revealed a new connection between functions of ORMDL3, circulating levels of S1P, and neutrophilia.

Neutrophilic inflammation induced by pro-inflammatory stimuli including LPS is often associated with IL-17, which stimulates granulocyte colony stimulating factor (G-CSF/CSF-3) production, leading to increased granulopoiesis in the bone marrow.[45] IL-17 also induces epithelial cells to release chemokines/cytokines that attract neutrophils to the site of lung inflammation.[59] Subsequently, neutrophils transmigrate into tissues, rapidly undergo apoptosis, and are cleared by tissue-resident phagocytic macrophages and dendritic cells,

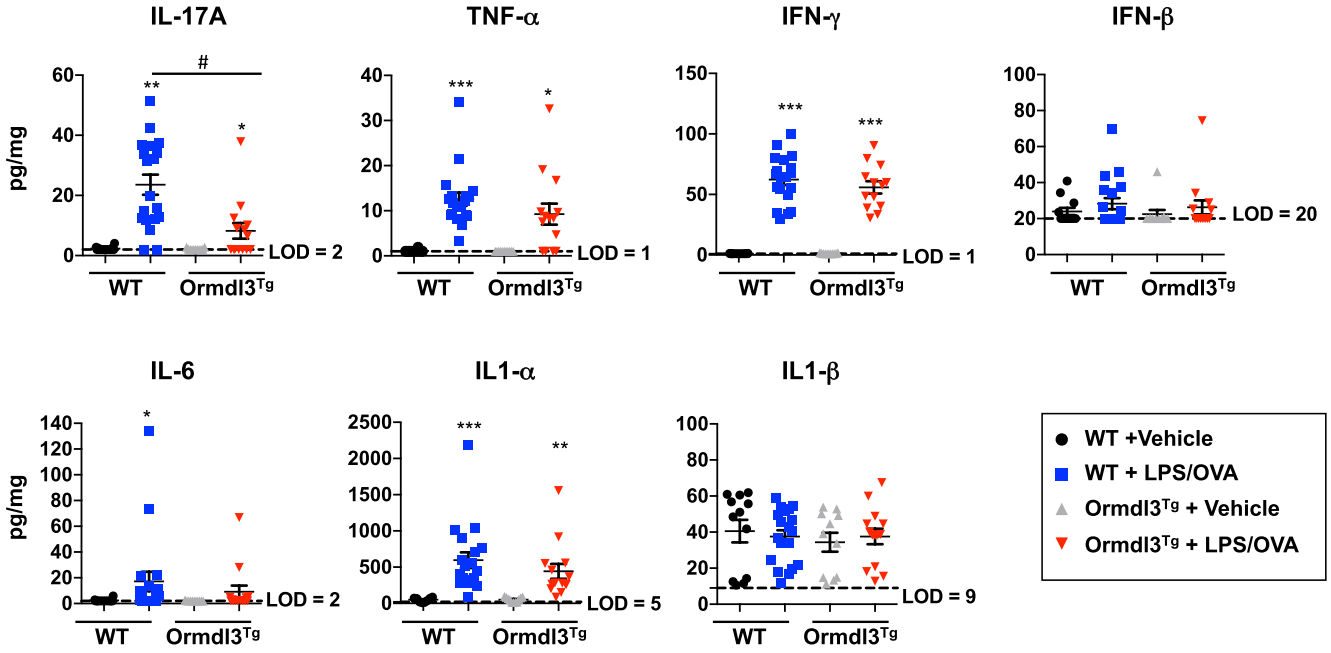

FIGURE 4 *Ormdl3* overexpression reduces lung IL-17A. Pro-inflammatory cytokines were measured in the lungs from wild type (WT) and global *Ormdl3* transgenic ($Ormdl3^{Tg}$) mice after challenge with LPS/OVA or vehicle. Data are means ± SEMs. $n = 12$, $n = 19$, $n = 11$, and $n = 14$ for WT treated with vehicle, WT treated with LPS/OVA, TG treated with vehicle, and TG treated with LPS/OVA, respectively. $^{*}p < .05$ compared to vehicle-treated mice, $^{\#}p < .05$ WT challenged with LPS/OVA compared to $Ormdl3^{Tg}$ mice challenged with LPS/OVA.

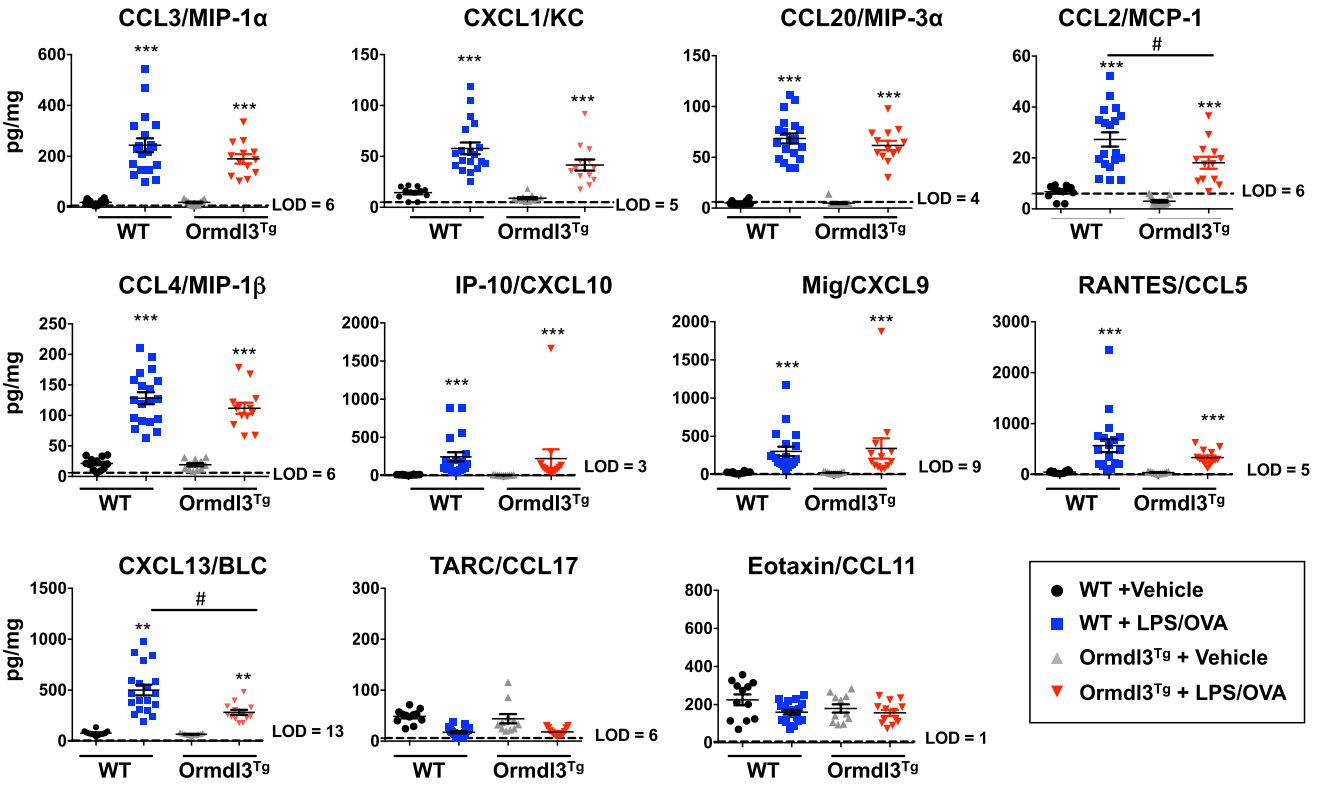

FIGURE 5 Effect of *Ormdl3* overexpression on lung chemokines. Chemokines were measured in the lungs of wild type (WT) and global *Ormdl3* transgenic ($Ormdl3^{Tg}$) mice after challenge with LPS/OVA or vehicle. $n = 12$, $n = 19$, $n = 11$, and $n = 14$ for WT treated with vehicle, WT treated with LPS/OVA, $Ormdl3^{Tg}$ treated with vehicle, and $Ormdl3^{Tg}$ treated with LPS/OVA, respectively. $^{*}p < .05$ compared to WT vehicle, $^{\#}p < .05$ WT challenged with LPS/OVA compared to $Ormdl3^{Tg}$ mice challenged with LPS/OVA.

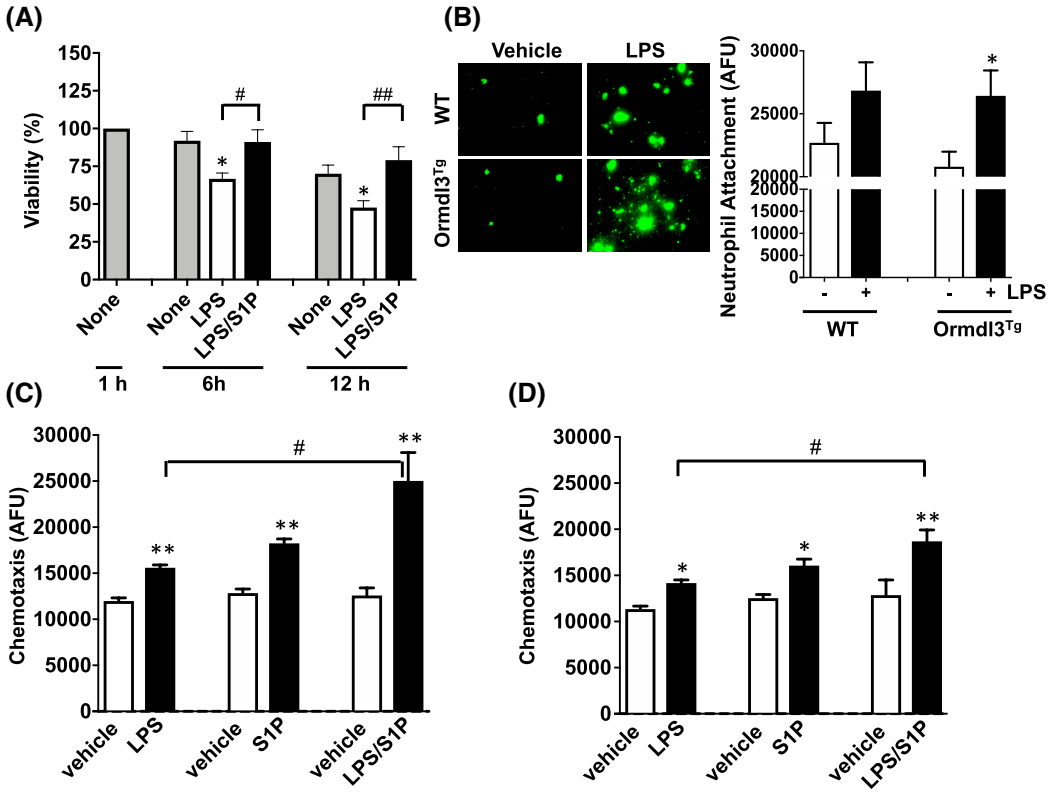

FIGURE 6 Effect of S1P on survival, attachment and migration of murine neutrophils. (A) Murine neutrophils were treated without (None) or with LPS (200 ng/mL) in the presence or absence of S1P as indicated. After 1, 6, or 12 h, cell viability was measured by CCK-8 assay, and absorbance was normalized to the values at 1 h. $n = 10$ per group. $^{*}p < .05$, $^{**}p < .01$ compared to the corresponding untreated cells at 6 or 12 h (None) by one-way analysis of variance test followed by Dunnett's multiple comparisons test. $^{\#}p < .05$; $^{\#\#}p < .01$ compared to LPS by unpaired two-tailed *t*-test. (B) Primary lung epithelial cells were incubated for 20 h with vehicle or LPS (200 ng/mL). After extensive washing, purified labeled neutrophils from wild type (WT) and *Ormdl3* transgenic (Ormdl3^{Tg}) mice were added for 4 h. Adhesion was assessed by fluorescence. Representative fluorescent images (left) and quantification of fluorescence (right). $n = 12, 15$ for each group. $^{*}p < .05$ compared to appropriate untreated cells by unpaired two-tailed *t*-test. (C,D) Isolated neutrophils from WT mice (C) and Ormdl3^{Tg} mice (D) were added to transwell inserts and allowed to migrate toward fMLP with LPS (200 ng/mL), S1P (100 nM), or both present in the lower well for 1 h. Migration of neutrophils in the lower chambers was assessed by calcein labeling of migrated neutrophils by fluorescence. Data expressed as arbitrary fluorescence units and are means ± SEM. $n = 7, 9$ for each group. Each sample represents an individual donor mouse. $^{*}p < .05$, $^{**}p < .005$ compared to appropriate vehicle control by unpaired two-tailed *t*-test. $^{\#}p < .005$ compared to LPS by one-way analysis of variance test followed by Dunnett's multiple comparisons test.

downregulating their production of IL-23, leading to reduction in IL-17 and inhibiting G-CSF production.[60] It is tempting to speculate that enhanced apoptosis of neutrophils due to reduction of S1P in *Ormdl3* transgenic mice might contribute to decreased IL-17A observed in these mice. In this regard, the IL-23/IL-17/G-CSF cytokine-controlled loop dependent on neutrophil trafficking to tissues was disturbed in S1P lyase-deficient mice that are unable to degrade S1P, resulting in elevated S1P levels.[61] Lower S1P levels in the *Ormdl3* transgenic mice may also affect this feedback loop via decreased activation of S1PR1 on T cells, as it was previously shown that S1P/S1PR1 signaling increases the number of Th-17 cells and their secretion of IL-17 to the same extent as IL-23.[62] It is also possible that S1P in circulation might affect other cells of the immune system that produce Th17 promoting cytokines. For example, binding of S1P to S1PR4 in dendritic cells controls their capacity to polarize CD4 T-helper cells toward the Th17 linage.[63]

No functional differences in neutrophils were detected in neutrophils from *Ormdl3* transgenic mice compared to WT, suggesting no inherent differences in neutrophil functions due to increases of their *Ormdl3*. It is more likely that the decreased circulating levels of S1P in *Ormdl3* transgenic mice affect neutrophil homeostasis directly due to decreased neutrophil S1PR signaling, which has been shown to be involved in their trafficking and survival.[52–56] Consistent with previous studies,[52,64] we have shown that S1P increased neutrophil migration and greatly enhanced the effects of LPS. In this respect, Toll-like receptor 4 (TLR4), the key receptor for LPS, is essential for S1P-dependent pulmonary inflammation in

mice.[55] Furthermore, in mice immunized with ovalbumin, FTY720 that targets S1PR1/3/4/5 abrogated neutrophil migration from inflamed tissues toward draining lymph nodes.[64] Interestingly, a meta-analysis of GWAS data revealed a novel association of a missense variant in *S1PR4* that supports the role of S1P signaling in neutrophil trafficking. Moreover, reduced numbers of tissue neutrophils in the liver and lungs of S1PR4-deficient mice further confirmed the association observed in humans and altered kinetics of neutrophil recruitment and resolution in response to tissue injury.[53] Together, these findings support the notion that the S1P/S1PR4 axis is a regulator of neutrophil motility, especially during inflammation.

Increased neutrophil lifespan due to local production of proinflammatory mediators is involved in lung functional impairment and injury during the establishment of asthma.[4] Previously, it was shown that S1P and its receptors are also involved in regulating neutrophil survival and resolution of inflammation in the pleural cavity and that inhibitors of S1P synthesis or its receptor facilitated the efferocytosis of apoptotic neutrophil.[56] Consistent with this study, we observed that stimulation with S1P markedly enhanced survival of neutrophils in vitro. Thus, ligation of S1PR also promotes the life span of mature neutrophils. This would explain the reduced peripheral neutrophil counts when S1PR signaling is abrogated owing to decrease of its ligands S1P and dihydro-S1P in the circulation. In this regard, neutrophilic asthma is resistant to mainstay glucocorticoid therapies as a result of increased migration of neutrophils from bone marrow to blood and importantly their enhanced survival as they are resistant to glucocorticosteroid-induced apoptosis.[4,65]

In summary, our data demonstrate that neutrophilic inflammation and IL-17A are reduced by overexpression of ORMDL3, which negatively regulates SPT, and predominantly decreases circulating levels of S1P and dihyro-S1P, the ligands of S1PRs. Taken together with previous studies, our data emphasize an important role for S1P/S1PR in regulating the outcome of neutrophilic inflammation and support the notion that targeting the S1P-S1PR axis might be a useful approach for treatment of patients with severe asthma associated with neutrophil inflammation and high IL-17 levels.

AUTHOR CONTRIBUTIONS

Briana N. James contributed to the design of the study, writing of the manuscript first draft, analysis of the data, administered LPS/OVA challenges, and measured cytokines and chemokines; Rebecca K. Martin and Anuj Tharakan performed LPS/OVA challenges; Rebecca K. Martin also carried out FACS and supervised the study; Cynthia Weigel purified epithelial cells and neutrophils and carried out neutrophil experiments and staining; Ryan D. R. Brown performed Western blots, Christopher D. Green and Elisa N. D. Palladino performed neutrophil experiments and staining; Richard L. Proia generated the Ormdl3 transgenic mice. Sheldon Milstien contributed to the analysis of data and writing of the manuscript; Sarah Spiegel supervised the study, contributed to the conception and design of the study, analysis of data, and writing of the manuscript. All authors reviewed the results and approved the manuscript.

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DISCLOSURES

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

All data needed to support the findings of this study are available in the Methods and the Results sections of the paper.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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