Transgenic overexpression of $\alpha_7$ integrin in smooth muscle attenuates allergen-induced airway inflammation in a murine model of asthma

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Abstract
Asthma is a chronic inflammatory disorder of the lower airways characterized by modulation of airway smooth muscle (ASM) function. Infiltration of smooth muscle by inflammatory mediators is partially regulated by transmembrane integrins and the major smooth muscle laminin receptor $\alpha_7\beta_1$ integrin plays a critical role in the maintenance of ASM phenotype. The goal of the current study was to investigate the role of $\alpha_7$ integrin in asthma using smooth muscle-specific $\alpha_7$ integrin transgenic mice (TgSM-Itg$\alpha_7$) using both acute and chronic OVA sensitization and challenge protocols that mimic mild to severe asthmatic phenotypes. Transgenic over-expression of the $\alpha_7$ integrin in smooth muscle resulted in a significant decrease in airway resistance relative to controls, reduced the total number of inflammatory cells and substantially inhibited the production of crucial Th2 and Th17 cytokines in airways. This was accompanied by decreased secretion of various inflammatory chemokines such as eotaxin/CCL11, KC/CXCL3, MCP-1/CCL2, and MIP-1$\beta$/CCL4. Additionally, $\alpha_7$ integrin overexpression significantly decreased ERK1/2 phosphorylation in the lungs of TgSM-Itg$\alpha_7$ mice and affected proliferative, contractile, and inflammatory downstream effectors of ERK1/2 that drive smooth muscle phenotype in the lung. Taken together, these results support the hypothesis that enhanced expression of $\alpha_7$ integrin in vivo inhibits allergic inflammation and airway resistance. Moreover, we identify ERK1/2 as a potential target by which $\alpha_7$ integrin signals to regulate airway inflammation. We conclude that identification of therapeutics targeting an increase in smooth muscle $\alpha_7$ integrin expression could serve as a potential novel treatment for asthma.

Keywords
$\alpha_7\beta_1$ integrin, asthma, inflammation, ovalbumin mouse model, transgenic mice
1 | INTRODUCTION

Asthma is a chronic inflammatory syndrome of the airway, characterized by extensive smooth muscle cell proliferation, increased cytokine and chemokine production, airway hyperresponsiveness (AHR), and remodeling. Inflammation and structural remodeling represent “variable” and “persistent” airway components that affect AHR. The shortening velocity and generation of force in airway smooth muscle (ASM) has been implicated as a causative factor of AHR due to an increased response to inflammation, which contributes to enhanced airflow occlusion. Moreover, ASM cells secrete cytokines, chemokines, and growth factors that participate in the recruitment and activation of immune cells that further exacerbate AHR. The capacity of ASM cells to synthesize and secrete these inflammatory mediators is partly regulated by phenotype plasticity, a phenomenon defined as the reversible switching between a contractile and proliferative phenotype. Plasticity encompasses functional modifications including proliferation, contraction, and secretion yet its underlying regulatory mechanisms remain largely unknown. Together, inflammation-induced plasticity can exacerbate AHR and promote airflow occlusion.

Integrins are heterodimeric transmembrane receptors that link the extracellular matrix (ECM) to the cytoskeleton and play an essential role in airway remodeling. They consist of two distinct alpha (α) and beta (β) subunits. The α7 integrin is a laminin receptor that plays an important role in smooth muscle differentiation and contractile phenotype that is dysregulated in lung biopsies from asthmatic patients. A previous study showed that α7β1 promotes ASM cell survival by forming connections with laminin, suggesting that α7 integrin may modulate ASM phenotype. Additionally, in vitro studies have shown that the laminin-binding α7 integrin is required for both a mature contractile phenotype and ASM cell proliferation. Thus, at least in vitro, α7 integrin and laminin-211 interactions are key components of the ECM that affect ASM cell functions highly relevant to asthma. Extracellular matrix encapsulates ASM cells and can activate a wide range of intracellular pathways that regulate cell cycle, intracellular cytoskeleton organization, and movement of new receptors to ASM cell membranes. A previous study showed that α7 integrin-null mice exhibit increased vascular smooth muscle cell proliferation that is linked to mitogen-activated protein kinase (MAPK) signaling. In another study, inhibition of the α7 integrin-laminin signaling axis resulted in decreased α-smooth muscle actin (SMA) expression without reverting ASM cells to a proliferative phenotype. MAPKs integrate signals from diverse stimuli and directly regulate cellular proliferation, differentiation, and inflammatory response. Extracellular signaling-regulated kinase (ERK) MAPK is increased during proliferation and has been shown to modulate ASM cell phenotype in a rat model of chronic asthma. These observations underscore potentially larger roles for integrins as regulators of ASM cell phenotype and how variation in expression can modulate intracellular signaling to influence cellular function in asthma.

In this study, we demonstrate that expression of the α7 integrin subunit was significantly decreased in the airways of mice sensitized and challenged with ovalbumin (OVA). Therefore, we hypothesized that overexpression of α7 integrin in vivo inhibits allergic inflammation and AHR. To test our hypothesis, we analyzed the role of α7 integrin in asthma pathogenesis using smooth muscle-specific α7 integrin transgenic mice (TgSM-Itgα7).

2 | METHODS

2.1 | Animals

Initial OVA sensitization and challenge studies were performed in male and female wild-type BALB/c mice at 6–10 weeks of age. All subsequent experiments were performed using 8-10-week-old female transgenic α7 integrin (TgSM-Itgα7) mice and littermate controls with a mixed FVB×C57B10 genetic background. Mice were bred and housed in a pathogen-free environment at the University of Nevada, Reno (UNR) laboratory animal facility. All experiments and protocols were approved by the UNR Institutional Animal Care and Use Committee (IACUC). TgSM-Itgα7 mice were generated at the Nevada Transgenic Center at UNR as described below.

2.2 | Generation of TgSM-Itgα7 transgenic mice

The smooth muscle myosin heavy chain (smMHC) promoter that drives rat α7-integrin (rITGA7) transgene expression was cloned using standard methodologies. Briefly, the rITGA7 cDNA was amplified from the rITGA7-PCRscript plasmid with Pfu polymerase (Promega, Madison, WI) following standard procedures using primers 1 and 2 (Table S1). A partial digest of rITGA7 with SmaI and full digest with SalI were performed. Standard procedures were followed to ligate the rITGA7 fragment into the SalI/Smal digested pGEX-4 T3 smMHC promoter plasmid (donated by Joe Miano, University of Rochester) using T4 DNA ligase (Promega). The smMHC- rITGA7 SV40 promoter was then purified away from the pGEX vector backbone for mouse transgene integration using Swal/AatII digestion. The smMHC-rITGA7 transgenic mouse
was generated by the UNR Transgenic Mouse Core following standard procedures and screened by PCR using primers 3 and 4 (Table S1). Positives mouse lines were assessed for elevated α7-integrin expression in the aorta by western blotting and RT-qPCR as previously described. The transgenic TgSM-Itgα7 mice were fertile and exhibited a normal phenotype.

### 2.3 Real time quantitative-PCR (RT-qPCR)

Total RNA was isolated using TRIzol™ reagent (Invitrogen™) according to the manufacturer’s protocol. RNA was purified and resuspended in ddH2O supplemented with rRNasin RNase Inhibitor (1 μL/1 μL ddH2O) and RQ1 RNase-Free DNase (0.5 μL/1 μL ddH2O) (Promega). Synthesis of cDNA from 500 ng total RNA was performed using SuperScript IV Reverse Transcriptase according to the manufacturer’s protocol (Invitrogen™). For studies in BALB/c mice, RT-qPCR was performed using a StepOne Plus PCR System (Applied Biosystems™). All reactions were performed in triplicate in 25 μL total volume containing a 1X concentration of SYBR Green PCR Master Mix according to the manufacturer’s protocols using primer sequences previously reported and summarized in Table S2. For transgenic animal studies, RT-qPCR was performed on a 7900 HT Fast Real-time PCR system (Applied Biosystems™) using TaqMan® Gene expression assays with ThermoFisher™ Fast Universal PCR Master Mix (2X), no AmpErase™ UNG. Table S2 summarizes the TaqMan® assays and primers used for these studies.

### 2.4 Western blot

Proteins were extracted from tissue using a lysis buffer containing 60 mM Tris–HCL, 2% SDS, 10% glycerol with protease and phosphatase inhibitors (1 μM leupeptin, 1 mM EGTA, 1 mM Na2EDTA, 1 mM AEBSF, 5 mM NaF, and 1 mM Na3VO4). Samples were sonicated for 5 s using a probe sonicator and centrifuged at 14,000 rpm for 25 min at 4°C. Total protein concentration for all extracts was determined by the bicinchoninic acid (BCA) method using bovine serum albumin as the standard. Total proteins (10 μg/well for α7 integrin and 50 μg/well for the remaining blots) were separated on 4%–12% Bis-Tris Plus gels (Life Technologies™) and transferred to nitrocellulose membrane at 19 V for 2 h. To characterize α7 integrin expression, membranes were probed with an anti-α7B integrin antibody previously described (1:5000; from D. Burkin) followed by the secondary goat anti-rabbit 800 (1:25,000) from Rockland Immunochemicals (Gilbertsville, PA).

Membranes were scanned and then stained with the reagent 700 total protein stain (Lior Biosciences) following the manufacturer’s recommended protocol. The α7 integrin protein expression was normalized to total protein. To analyze the ERK1/2 activity, blots were probed with anti-phospho-ERK1/2 (Cat. 9101S, 1:1000, Cell Signaling Technologies) and anti-total ERK1/2 (Cat. 4696S, 1:1000, Cell Signaling Technologies). Protein expression was assessed using anti-α-SMA (Cat. A2647, 1:1000, Sigma), anti-proliferating cell nuclear antigen (PCNA, Cat. 13110S, 1:1000, Cell Signaling Technologies) and antiglyceraldehyde-3-phosphate dehydrogenase (GAPDH, Cat. A303-878A, 1:1000, Thermo Fisher Scientific) for normalization. Anti-cyclooxygenase (COX-2, Cat. sc-1745, 1:1000, Santa Cruz Biotechnology) and anti-COX-1 (Cat. sc-1752, 1:1000, Santa Cruz) were used, with COX-2 normalized to COX-1 expression. IRDye 680RD and 800CW secondary antibodies (Lior Biosciences) were diluted 1:25,000. Imaging and analysis were performed using the LI-COR Odyssey Infrared Imaging System 9120 (Lior Biosciences).

### 2.5 OVA-sensitization and challenge

Acute and chronic OVA protocols were performed as previously described using 8-10-week-old female mice. For the acute protocol, mice were sensitized with an intraperitoneal (i.p.) injection of 50 μg chicken-egg OVA-Grade V (Sigma) on days 1, 7, and 14. OVA was prepared in 1 mg aluminum hydroxide adjuvant (Alum, Thermo Pierce Scientific). Intranasal (i.n.) OVA-challenge was performed on days 21, 22, and 23 with 100 μg OVA in 50 μL of saline. To induce a chronic inflammatory response, mice were sensitized with two i.p. injections of 10 μg OVA on days 1 and 7 followed by 5 weeks of OVA challenge.

Chronic-OVA mice were challenged three times (every other day) a week for 4 weeks and four times on week 5. Control mice (Alum) received adjuvant or saline for sensitization and challenge, respectively.

### 2.6 Invasive measurement of airway resistance and compliance

Airway function was analyzed 24 h after the last OVA challenge. Mice were anesthetized by an i.p. injection of 0.1 ml/10 g animal weight of a 10 mg/mL ketamine:1 mg/mL xylazine mix followed by an additional dose of 0.1 ml/10 g ketamine (10 mg/mL). Mice were then tracheostomized and subjected to mechanical ventilation. Airway resistance (cm H2O/mL/s) and dynamic lung compliance (mL/cm H2O) were measured in response to increasing
concentration of aerosolized methacholine (MCh) (0–100 mg/mL in PBS) using FinePoint software (Buxco Research Systems).

2.7 | Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was collected as previously described. Briefly, lungs were lavaged eight times with 500 μL of sterile saline-EDTA solution (2.6 mM EDTA and 0.9% sterile saline) through the tracheostomy tube. Cell pellets from all lavages were collected by centrifugation at 300 g for 5 min at 4°C, pooled and hemolyzed with a 150 mM potassium chloride solution, and resuspended in 500 μL BAL lavage solution. Total cell counts were obtained by Coulter counting (Beckman Coulter Z series). Supernatants (BAL fluid) from the first three lavages were combined and subjected to Luminex analysis.

2.8 | Luminex multiplex assays

The Luminex multiplex assay was processed at Eve Technologies Corp using 50 μL BAL fluid per sample with the Mouse Cytokine Array/Chemokine Array 32-Plex Discovery Assay® (MD31). The assay was analyzed on the Bio-Plex™ system (Bio-Rad Laboratories, Inc.) using a Milliplex Mouse Cytokine/Chemokine kit (Millipore) according to the manufacturer’s instructions. The 32-plex consisted of 21 cytokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 [p40 and p70], IL-13, IL-15, IL-17, IFNγ, G-CSF, GM-CSF, M-CSF, LIF and TNFα), ten chemokines (eotaxin/CCL11, IP-10/CXCL10, KC/CXCL2, LIX/CXCL6, MCP1/CCL2, MIG/CXCL9, MIP-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL2 and RANTES/CCL5), and vascular endothelial growth factor (VEGF). The sensitivities of these markers ranged from 0.1–33.3 pg/mL.

2.9 | Statistical analysis

Data was analyzed using unpaired t-test for two groups comparison or one-way analysis of variance (ANOVA) when comparing three or more groups with one independent variable. Two-way ANOVA was used when comparing multiple groups with two-independent variables. ANOVA was followed by Dunnett’s Post-hoc multiple comparison test. p values < 0.05 were considered statistically significant. The statistical analysis was performed using GraphPad Prism (GraphPad Software Inc.).

3 | RESULTS

3.1 | ITGA7 expression is decreased in a mouse model of acute allergic inflammation

We initially analyzed the expression of ITGA7 in response to acute allergic inflammation in vivo using male and female BALB/c mice sensitized and challenged with OVA. These animals were compared to control mice that received adjuvant or saline for sensitizations and challenges, respectively. While there are many animal models established to study asthma pathogenesis, we chose OVA as a well-established model of allergic lung inflammation that has proven useful in furthering understanding of the molecular and cellular changes that occur in the lung. Our results show that the transcript levels of ITGA7 were significantly decreased in female OVA-sensitized mice compared to the control animals (Figure 1A). In contrast, no effect of OVA on ITGA7 expression was seen in male animals (Figure 1B). These results indicate that targeting α7 integrin in subsequent experiments is a viable approach and that female animals are likely to provide more robust results than male animals.

Figure 1 Effect of ovalbumin (OVA) sensitization and challenge on ITGA7 mRNA expression. Expression of ITGA7 in tracheal airway smooth muscle (ASM) was analyzed by RT-qPCR in (A) female and (B) male mice acutely sensitized and challenged with OVA. Data were normalized to GAPDH mRNA, n = 5–6 ± SEM, *p < 0.05 significant difference from female Alum control mice via unpaired t-test.
3.2 Characterization of smooth muscle-specific α7 integrin transgenic mice (TgSM-Itgα7)

To determine if increased α7 integrin expression modulates the asthmatic phenotype in an animal model of asthma, we generated a smooth muscle-specific α7 integrin transgenic mouse (TgSM-Itgα7) for further study. We used a construct in which the smMHC promoter drives the expression of the rat α7-integrin (ITGA7) gene (Figure 2A). We assessed ITGA7 and α7 integrin expression in the aorta, trachea, and kidney (control) in transgenic animals relative to non-transgenic littermates. Transcript and protein expressions were determined by RT-qPCR, using rat-specific α7 integrin probe-primer mix, and western blotting, respectively. The rat α7 integrin mRNA levels were significantly enhanced in the aorta of TgSM-Itgα7 mice relative to the trachea and kidney tissues of the same group, as well as to the WT mice (Figure 2B). This was accompanied by a significant increase of α7 integrin protein expression in the aorta of transgenic mice versus WT controls (Figure 2C,D). However, the protein levels of α7 integrin in the trachea and kidney remain unchanged in TgSM-Itgα7 animals relative to respective WT controls (Figure 2C,D). These findings indicate that the rat α7 integrin transgene is specifically overexpressed in the aortic vascular smooth muscle of transgenic mice.

3.3 α7 integrin expression decreases inflammatory cell infiltration in response to acute OVA sensitization and challenge

We used both acute and chronic OVA sensitization and challenge protocols that mimic mild to severe asthmatic phenotypes in transgenic animals. To determine the role of α7 integrin expression in allergic inflammation, we compared the total cell counts from BAL fluid in mice subjected to acute OVA with those from mice sensitized and challenged with the chronic OVA treatment. Acutely sensitized WT-OVA mice showed significantly higher total cell counts than Alum controls and a substantial increase in total cell counts compared to the TgSM-Itgα7-acute-OVA mice (Figure 3A). Surprisingly, total cell counts in TgSM-Itgα7-chronic-Alum mice were reduced and the total cell counts in WT-chronic-OVA mice were not significantly different from their respective control, WT-chronic-alum controls (Figure 3B). Despite the lack of significant statistical changes between TgSM-Itgα7-acute-OVA mice and WT-acute-OVA mice, there was a substantial (43%) decrease in total BAL cell counts between the two groups. Further, we noted that the TgSM-Itgα7-OVA group had fewer replicates than all other groups, which may have affected the statistical significance between this group and the WT-OVA mice. These findings indicate that α7 integrin expression decreases the total inflammatory cell counts in mice subjected to the acute OVA protocol, suggesting inflammatory cells may be reduced to a lesser extent in the chronic OVA model.

3.4 Increased α7 integrin expression decreases airway pulmonary resistance in response to chronic inflammation

The role of α7 integrin on AHR was assessed in response to increasing doses of aerosolized MCh (0–100 mg/mL) in both acute and chronic OVA models. Airway resistance and compliance were utilized as a measure of airway hyperreactivity. As expected, WT-OVA mice displayed a significantly higher pulmonary resistance relative to WT and TgSM-Itgα7 unchallenged animals, in response to both acute (Figure 4A) and chronic (Figure 4C) OVA exposures. This was paralleled with significantly decreased pulmonary resistance in TgSM-Itgα7-OVA compared to the WT-OVA mice following the chronic protocol (Figure 4C) but was not observed in acutely sensitized animals. Surprisingly, no change in airway compliance was observed between all four groups (Figure 4B,D), indicating that elasticity of the lung is not compromised. Overall, these data indicate that α7 integrin is more effective in reducing pulmonary resistance in response to chronic sensitization and challenge protocol with OVA.

3.5 Effect of OVA sensitization and challenge on cytokine and chemokine secretion in FVB×C57B10 mice

BALB/c is the most widely used mouse strain to model allergic inflammation in mice, as these mice develop a strong Th2-biased inflammatory response that contributes to AHR and remodeling. Other strains, including C57BL/6, FVB, and A/J have also been successfully utilized to study asthma. Despite this, the hallmarks of an antigen-induced allergic response in FVB×C57B10 mice are not well characterized. Thus, we determined the effect of acute and chronic OVA sensitization and challenge on cytokine and chemokine production in FVB×C57BL10 mice by assaying an array of 31 inflammatory mediators. These results demonstrated that acute OVA mice displayed a higher BAL concentration of key inflammatory cytokines (Table 1) and chemokines (Table 2) compared to chronic OVA mice. For instance, secretion of major Th2 (IL-4 and IL-5) and Th17 (IL-17A) cytokines was significantly increased in acute OVA mice compared to Alum
Targeted smooth muscle over-expression of α7 integrin in vivo. (A) Construct used for pronuclear injections. (B) RT-qPCR analysis of the rat ITGA7 transcript levels in the aorta, trachea, and kidney normalized to 18S rRNA. (C and D) Western analysis of α7 integrin protein expression in aorta, trachea, and kidney normalized to total protein. N = 4–8 ± SEM, *p < 0.05 and **p < 0.01 significant difference between the α7 integrin expression levels in the TgSM-Itgα7 mice’ aorta and the other groups, via one-way ANOVA followed by Dunnett’s multiple comparison test. TPS (Total Protein Stain), M (Marker), WT (wild type), Tg (Transgenic), and α7 int. (alpha 7 integrin)
controls. These cytokines were undetectable in all chronic OVA mice (Table 1). Levels of leukemia inhibitory factor (LIF), macrophage colony-stimulating factor (M-CSF), and granulocyte colony-stimulating factor (G-CSF) were also substantially increased in acute OVA mice relative to chronic OVA (Table 1). The secretion levels of eotaxin/CCL11, keratinocyte-derived chemokine (KC)/CXCL3, and monocyte chemoattractant protein-1 (MCP-1)/CCL2 was significantly higher in acute OVA mice compared to alum controls and chronic OVA mice (Table 2). In contrast, none of the chemokines assayed were significantly elevated in response to chronic inflammation (Table 2). Overall, these results suggest that acute OVA exposure was more effective than chronic OVA at inducing an inflammatory response in FVB x C57BL10 mice. Therefore, we chose to analyze the role of α7 integrin on inflammatory mediator production solely in response to the acute OVA protocol.

### 3.6 | α7 integrin regulates the secretion of key inflammatory cytokines in mice sensitized and challenged to acute OVA

Allergic inflammation and asthma are often associated with increased production of Th2 and Th17 cytokines. Despite associations between α7β1 integrin and asthma, the effect of α7 integrin on cytokine secretion is not well characterized. After establishing the baseline inflammatory response in the FVB x C57BL10 mouse strain, Luminex assays were used to investigate the role of α7 integrin on cytokine secretion in response to OVA-induced inflammation. We show that levels of IL-4, M-CSF, G-CSF, LIF, and IL-17A cytokines were significantly inhibited in acute OVA TgSM-Itgα7 mice relative to WT acute OVA animals (Figure 5A-E). Furthermore, we noted an increase of IL-1α in TgSM-Itgα7-OVA relative to WT-OVA (Figure 5F). No change in cytokine production was observed between WT and transgenic Alum controls. These findings indicate that α7 integrin regulates key Th2 and Th17 cytokine secretion and restores the production of IL-1α.

### 3.7 | The α7 integrin inhibits levels of inflammatory chemokines in mice sensitized and challenged to acute OVA

Subsequently, the contribution of the α7 integrin on chemokine secretion was evaluated. We first focused on chemokines that were dysregulated in response to OVA challenge in WT mice as reported in Table 2. We found that eotaxin/CCL11 and KC/CXCL3 levels were significantly decreased in TgSM-Itgα7 OVA mice compared to WT OVA animals (Figure 6A,B). Furthermore, we noted that MCP-1/CCL2 and MIP-1β/CCL4 chemokines were only present in the BALF of WT-OVA mice, and not detectable (N.D.) in alum controls and in TgSM-Itgα7 OVA mice (Figure 6C,D). These results suggest that α7 integrin inhibits the secretion of distinct set of chemokines following acute OVA sensitization and challenge.

### 3.8 | MAPK activity is decreased by α7 integrin in mice independent of OVA sensitization and challenge

A previous study showed that α7 integrin-null mice exhibit increased vascular smooth muscle cell proliferation that is linked to MAPK signaling. Thus, to better understand the broader role of α7 integrin in smooth phenotype plasticity, we focused on canonical MAPK signaling through ERK1/2. Protein phosphorylation and expression in lung tissue of control and OVA-challenged mice was assayed via immunoblot. We found significantly decreased ERK1/2 phosphorylation in the lungs of TgSM-Itgα7 mice compared to WT animals (Figure 7A), irrespective of Alum or OVA. These results indicate that increased α7 integrin...
expression decreases endogenous levels of ERK1/2 phosphorylation in TgSM-Itgα7 animals and that these reduced levels of ERK1/2 activity persist during allergic inflammation. We also assayed proliferative, contractile, and inflammatory downstream effectors of ERK1/2 that drive smooth muscle phenotype in the lung. We found that the proliferative marker PCNA (Figure 7B), while increased in WT-OVA mice as expected, remained unchanged in TgSM-Itgα7 mice. We also measured α-SMA (Figure 7C) as a marker of contractile smooth muscle and found expression was significantly increased in TgSM-Itgα7-OVA animals, which was unexpected given the reduction in pulmonary resistance shown in earlier experiments. We further found that a reduction in ERK activity correlated with a 23.2% decrease in COX-2 expression in TgSM-Itgα7 OVA mice (Figure 7D). While these results were not significant, they support the overall reduction in inflammatory mediators we observed in TgSM-Itgα7-OVA mice. Together, these data suggest a role for α7 integrin in regulating specific components of the cellular contractile

**FIGURE 4** Effect of α7 integrin expression on airway hyperresponsiveness. Mice were subjected to acute (A and B) or chronic (C and D) OVA protocols. Airway resistance (A and C) and dynamic lung compliance (B and D) were measured in response to increasing concentration of aerosolized methacholine (0–100 mg/mL in PBS) in tracheostomized and mechanically ventilated mice. Data were expressed as mean ± SEM, n = 5–9, *p < 0.05, Alum vs OVA-challenged mice and ## < 0.01 WT-OVA compared to TgSM-Itgα7- OVA using two-way ANOVA followed by Dunnett’s multiple comparison test.
apparatus and signaling pathways that are important to the phenotype of smooth muscle in the lung.

4 | DISCUSSION

Our results show that overexpression of α7 integrin in smooth muscle decreases airway resistance, inflammatory response, and ERK1/2 phosphorylation in a mouse model of allergic asthma. Allergic asthma is associated with leukocyte infiltration and the release of an array of cytokines and chemokines that modulate ASM phenotype. Thus, prolonged inflammation due to allergen exposure can lead to airway remodeling and promote declining lung function. In this study, we characterized the effect of smooth muscle-specific overexpression of α7 integrin on airway inflammation and resistance using acute and chronic mouse models of allergic asthma. The effect of α7 integrin downregulation in asthmatic smooth muscle in vitro has been demonstrated. However, to the best of our knowledge, this is the first study that uses an overexpression mouse model as an in vivo gain of function strategy targeting smooth muscle to analyze the contribution of the α7 integrin subunit on asthma pathogenesis. This focus on α7 integrin is further supported by recent studies demonstrating that α7 integrin expression

### TABLE 1
Effect of ovalbumin (OVA) sensitization and challenge on cytokine secretion in FVB×C57Bl10 mice

| Cytokines (pg/mL) | Acute Alum | Acute OVA | Chronic Alum | Chronic OVA |
|------------------|------------|-----------|--------------|-------------|
| IL-1α            | 25.07 ± 2.27 | 6.72 ± 1.49**** | 28.27 ± 2.90 | 19.30 ± 4.52 |
| IL-1β            | N.D.       | N.D.      | 1.37 ± 0.44  | 0.23 ± 0.08* |
| IL-2             | 1.43 ± 0.31 | 1.56 ± 0.13 | 2.85 ± 0.35  | 2.23 ± 0.72 |
| IL-4             | 0.03 ± 0.01 | 11.49 ± 3.92** | N.D.         | N.D.        |
| IL-5             | 0.14 ± 0.04 | 10.21 ± 6.10 | N.D.        | N.D.        |
| IL-6             | 4.32 ± 2.47 | 45.27 ± 24.47 | 5.06 ± 1.66  | 16.61 ± 10.31 |
| IL-7             | 0.32 ± 0.15 | 0.08 ± 0.05  | N.D.        | N.D.        |
| IL-9             | 2.12 ± 0.80 | 5.63 ± 1.52  | 5.83 ± 1.44  | 6.94 ± 2.70 |
| IL-10            | N.D.       | 0.42 ± 0.29  | N.D.        | N.D.        |
| IL-15            | N.D.       | N.D.       | 4.56 ± 0.40  | 4.69 ± 0.27 |
| IL-17A           | 0.08 ± 0.02 | 1.58 ± 0.33**** | N.D.        | N.D.        |
| G-CSF            | 1.18 ± 0.28 | 12.26 ± 4.29** | 1.76 ± 0.63  | 4.20 ± 1.76 |
| M-CSF            | 0.01 ± 0.01 | 1.44 ± 0.42** | N.D.        | N.D.        |
| LIF              | 0.10 ± 0.08 | 2.92 ± 0.75** | 0.66 ± 0.22  | 1.08 ± 0.39 |

Note: Data are expressed as mean ± SEM, n = 4–9, *p < 0.05, **p < 0.001, and ****p < 0.00001 versus Alum control by one-way ANOVA followed by Tukey's multiple comparison test.

### TABLE 2
Effect of ovalbumin (OVA) sensitization and challenge on chemokines secretion in FVB×C57BL10 mice

| Chemokines (pg/mL) | Acute Alum | Acute OVA | Chronic Alum | Chronic OVA |
|--------------------|------------|-----------|--------------|-------------|
| Eotaxin/CCL11      | 2.83 ± 0.72 | 18.54 ± 4.28** | 7.84 ± 1.55 | 8.14 ± 2.38 |
| IP-10/CXCL10       | 2.52 ± 0.60 | 32.33 ± 16.51 | 4.35 ± 0.47 | 10.15 ± 4.97 |
| KC/CXCL3           | 3.85 ± 1.50 | 17.23 ± 2.45*** | 3.85 ± 1.13 | 5.85 ± 1.83 |
| LIX/CXCL6          | N.D.       | N.D.      | N.D.       | N.D.        |
| MCP-1/CCL2         | N.D.       | 7.67 ± 4.51 | 1.94 ± 0.33 | 2.91 ± 0.70 |
| MIG/CXCL9          | 2.38 ± 1.10 | 14.46 ± 5.61 | 3.28 ± 1.10 | 12.54 ± 4.99 |
| MIP-1α/CCL3        | 3.91 ± 1.36 | 53.07 ± 25.53 | 9.95 ± 2.00 | 18.13 ± 1.83 |
| MIP-1β/CCL4        | N.D.       | 19.72 ± 14.16 | N.D.        | N.D.        |
| MIP-2/CXCL2        | 15.48 ± 4.51 | 16.00 ± 1.48 | 33.08 ± 9.55 | 29.38 ± 6.66 |
| RANTES/CCL5        | 0.19 ± 0.05 | 0.92 ± 0.21  | 1.03 ± 0.21  | 1.83 ± 0.60 |

Note: Data are expressed as mean ± SEM, n = 4–9, **p < 0.001 and ***p < 0.0001 versus Alum control by one-way ANOVA followed by Tukey's multiple comparison test.

Abbreviation: N.D., not detectable.
FIGURE 5  Effect of α7 integrin expression on the secretion of cytokines in acute OVA mouse model of asthma. (A) IL-4, (B) M-CSF, (C) G-CSF, (D) LIF, (E) IL-17A, and (F) IL-1alpha cytokine levels in BAL fluid of mice exposed to acute OVA protocol were analyzed via Luminex assays. Data were expressed as mean ± SEM, n = 5–9, *p < 0.05, **p < 0.01 ***p < 0.001 ****p < 0.0001 WT-OVA group relative to alum controls and TgSM-Itga7-OVA animals, one-way ANOVA followed by Dunnett’s multiple comparison test.
was dysregulated in asthmatic patients. Given that the α7 integrin is coupled to the β1 integrin, we did conduct initial studies to determine whether ITGB1 expression was affected by OVA in our animal model and found that changes in ITGA7 were greater than that seen with ITGB1 expression (data not shown). Since the β1 integrin can heterodimerize with multiple α integrins, it was not surprising that expression levels of ITGA7 and ITGB1 did not change to the same extent. In addition, it is the α integrin subunit that binds the ECM to direct β integrin signaling, suggesting that the α integrin may be the more critical target in directing functions. The importance of α7 integrin over β1 integrin has also been supported by previous studies in ASM cells further strengthening this approach.

While animal models have proved invaluable in studying mechanisms of asthma, we appreciate that the OVA model does not cause disease in humans and that many of the available models also do not fully recapitulate human disease. However, despite the limitations of the OVA model, it has proven useful in furthering our understanding of the pathophysiology of the lung relevant to allergic airway disease. Given the novelty of our hypothesis and the transgenic animals available to us for these experiments, we chose OVA as a well-established model of allergic lung inflammation for which our group has experience. These protocols elicit an inflammatory response characterized by IgE-dependent eosinophilia and inflammation in response to acute exposure, followed by thickening of the epithelium, and airway remodeling, in response to
chronic allergic exposure. We first assessed the role of α7 integrin on airway AHR in response to acute and chronic OVA exposures. We found that TgSM-Itgα7-OVA mice experienced a significant decrease in airway resistance relative to the WT-OVA group following the chronic protocol. These findings imply that α7 integrin expression reduces airway resistance. Despite changes in resistance with the chronic model, we did not find any apparent difference in airway compliance between TgSM-Itgα7-OVA and WT-OVA mice. We recognize that these results respond to just one model of allergic inflammation and that other models elicit different physiologically relevant responses, particularly relevant to ASM function.40–45 However, given the numerous variables using FVB x C57BL10 mice, the novel application of TgSM-Itgα7 animals in this study, and the predicted responses generated with the Alum controls, we conclude that α7 integrin has a larger role in attenuating inflammation. Results from total BAL cell counts showed that enhanced α7 integrin expression in smooth muscle reduced inflammatory cell infiltration in the acute model but no clear effect in response to chronic inflammation. These results may indicate that the α7 integrin function plays a more important role in the earlier stages of an immune response. To our surprise, no significant
differences in total BAL cell count or cytokine secretions were observed between WT-Alum and WT-OVA in the chronic model. Thus, these results further suggest that acute OVA exposure was more effective than chronic OVA at inducing an inflammatory response in FVBxC57BL10 mice.

Allergic asthma is also accompanied by the release of an array of cytokines and chemokines by T helper (Th) cells. Our results show that α7 integrin expression inhibited the production of Th2 cytokines (IL-4 and LIF) and members of the colony-stimulating factors family (M-CSF and G-CSF) cytokines. Th2 cells produce major cytokines, such as IL-4, that participate in allergic asthma. IL-4 is a predominant signature of eosinophilic asthma, and it modulates the differentiation of naïve T cells (Th0) into Th2 cells and IgE production by B cells while inhibiting macrophage activation.46–48 LIF is a principal inflammatory mediator that belong to the IL-6 family. LIF is involved in the differentiation of various cell types, including stem cells, and it plays a role in cytokine production and airway inflammation.49 M-CSF and G-CSF are members of colony-stimulating factors (CSFs), which consists of the macrophage CSF (M-CSF), granulocyte-macrophage CSF (GM-CSF), and granulocyte CSF (G-CSF). M-CSF promote the growth and differentiation of monocytes, and its production levels is enhanced in the airways of asthmatic patients.50 G-CSF stimulates the proliferation and differentiation of granulocytes hematopoietic progenitor cells. It is involved in the development and function of neutrophils as well as cytokine production.51–53 Overall, these findings suggest that α7 integrin plays an important role in the production of Th2 and CSF cytokines relevant to asthma pathophysiology.

We additionally found that α7 integrin expression decreased the secretion of IL-17A, a Th17-derived cytokine. Th17 cells are highly associated with neutrophilic inflammation and severe forms asthma, they produce mainly IL-17A and IL-17F cytokines. IL-17A and IL-17F secretion levels are increased in asthmatic patients’ septum and linked to the disease severity.54,55 Despite the high sequence homology and sharing the same receptor, these two cytokines are thought to function via the distinct signaling mechanism. For instance, IL-17A was involved in the OVA-induced neutrophilic asthma, whereas IL-17 F was associated with Aspergillus oryzae-induced neutrophilia.56 In the present study, we noted that IL-17A cytokine was secreted in the BALF of mice that underwent the acute OVA protocol but was not detectable in the chronic OVA model. Prior work has shown that a subset (Th2/Th17⁺) of Th2 cells were able to secrete IL-17A cytokine in addition to Th2 cytokines such as IL-4. The Th2/Th17⁺ frequency was correlated with increased levels of IL-17 in the BAL and to asthma severity.57 Further, the role of IL-17 in allergic asthma has also been demonstrated. For example, neutralization of IL-17A expression increased AHR and airway eosinophilic infiltration, and knockout of IL-17 receptor A (IL-17 RA) decreased allergic airway inflammation.58,59 Thus, our finding of IL-17A cytokine increased production in a Th2-bias acute OVA model, may suggest that this cytokine is produced by Th2/Th17⁺ cells in response to eosinophilic inflammation.

IL-1α is a member of IL-1 family cytokines with pro-inflammatory properties, and is it upregulated in asthmatic patients’ biofluids, including BAL, blood, and sputum.60 Surprisingly, our results showed that IL-1α was downregulated in OVA-exposed mice. This was additionally correlated with the increase of major Th2 and Th17 cytokines. These findings may be attributed to the imbalance between various cytokine classes during asthma pathogenesis. Overall, we found that overexpression of α7 integrin in the OVA mouse model of allergic inflammation restored IL-1α secretion while decreasing IL-4, M-CSF, G-CSF, LIF, and IL-17A cytokines, indicating that α7 integrin restores the cytokine secretion imbalance that accompanies allergic asthma.

Although cytokines have been shown to play a crucial role in asthma pathogenesis, chemokines are also essential contributors to this process. Chemokines are polypeptide chemoattractant cytokines that modulate leukocytes migration and activation.61 They are classified into four groups based on the first two cysteine residue positions in the conserved region; however, the two major groups are alpha (CXC) and beta (CC) chemokines. Alpha (CXC) chemokines are principal neutrophil chemoattractants and activators, while the beta (CC) chemokines induce adhesion in nearly all inflammatory cells except neutrophils.62 Our results indicated significantly decreased levels of eotaxin/CCL11 and KC/CXCL3 in TgSM-Itgα7 OVA mice relative to WT OVA animals. Eotaxin/CCL11 controls the recruitment and activation of inflammatory leukocytes, specifically eosinophils.60 KC/CXCL3 is a neutrophilic chemoattractants. In our hands, the secretion of various chemokines including Eotaxin/CCL11, KC/CXCL3, MCP-1/CCL2 and MIP-1β/CCL4 were substantially increased in the BAL of WT OVA mice. This agrees with previous studies showing upregulated profiles of these chemokines in the biofluids and biopsied of asthma patients at various stages of disease progression.60,63 Taken together, these observations highlight a crucial role of these chemokines in asthma pathogenesis. The over-expression of α7 integrin abrogated the secretion levels of key cytokines and chemokines associated with eosinophilia, neutrophilia, and asthma severity, indicating that α7 integrin regulates a broad range of inflammatory processes at various stages of asthma pathogenesis.
Airway remodeling is an important feature of asthma that contributes to ASM-mediated bronchoconstriction and AHR.\textsuperscript{64,65} It is characterized by structural changes that correlate with increased ASM mass, mucus cell hyperplasia, basal membrane thickening, and ECM.\textsuperscript{64,66–68} Asthmatic BAL fluid contains increased levels of fibronectin, laminin and hyaluronate, reflecting increased ECM turnover.\textsuperscript{65,69} A previous study has shown that α7 integrin knockout mice displayed vascular smooth muscle hyperplasia and hypertrophy,\textsuperscript{70} suggesting that α7 integrin over expression may inhibit airway remodeling and ECM deposition. Studies examining αβ integrin receptors have shown that laminin binding to α7 integrin is required for the contractile phenotype of ASM while the β1 integrins mediate ASM cell proliferation likely through α5 integrin.\textsuperscript{17} In asthmatic ASM cells in culture, expression of both fibronectin and laminin binding integrins is elevated, indicating that composition of the ECM alters smooth muscle functions in diseased cells.\textsuperscript{71}

Translocation of phosphorylated ERK1/2 to the nucleus activates a range of transcription factors and downstream effectors that regulate inflammatory mediator gene expression.\textsuperscript{72,73} As expression shifts, secretion of cytokines and chemokines by smooth muscle cells can promote sustained inflammation by signaling in an autocrine fashion, providing feedback input that further modulates phenotype.\textsuperscript{74} Previous studies have demonstrated that ERK1/2 is a primary mediator of mitogen-induced ASM cell proliferation, growth, and secretion.\textsuperscript{75–80} ERK1/2 activation is increased in asthmatic patients and in chronic asthmatic rats, and was shown to play a role in the modulation of asthmatic phenotype.\textsuperscript{19} ERK1/2 is also critical to Th2 differentiation and the regulation and production of numerous cytokines and chemokines in ASM cells.\textsuperscript{81,82} Another study has shown that inhibition of Ras, an upstream regulator of ERK1/2, in α7 integrin-deficient smooth muscle cells significantly reduced nuclear localization.\textsuperscript{83} We found that overexpression of α7 integrin decreased ERK1/2 phosphorylation in the lungs of transgenic mice (TgSM-Itgα7) mice independent of OVA exposure. These results corroborate previous studies that showed enhanced MAPK activity in vascular smooth muscle of α7 integrin-knockout mice.\textsuperscript{18} There are also numerous downstream effectors of ERK1/2 that interact to drive smooth muscle phenotype in animal models of asthma\textsuperscript{64–66} and we chose to focus on a selection of well-characterized proteins to assess the functional role of ERK1/2 in the lung. Overexpression of α7 integrin prevented a significant increase in PCNA expression in OVA-treated TgSM-Itgα7 mice compared to WT animals that correlated with reductions in ERK 1/2 phosphorylation, consistent with previous studies in vascular smooth muscle.\textsuperscript{18} In addition, prior studies of a suppressive role for prostaglandin E2 (PGE2) in animal models\textsuperscript{87} of airway inflammation are consistent with the trend we found in COX-2 expression, although we did not measure PGE2 in our studies. Moreover, our findings do support other studies in suggesting α7 integrin expression is inversely correlated with ERK1/2 activation and positively paralleled with α-SMA expression during allergic inflammation.\textsuperscript{15,18} Further investigation into the mechanisms underlying these alterations is required to better understand integrin-mediated smooth muscle phenotype and regulation of inflammatory mediator secretion.

### Conclusion/Future Implications

In summary, we have shown that α7 integrin participates in asthma pathogenesis. Targeted smooth muscle α7 integrin overexpression in preclinical OVA models of

![Summary of implicated roles for α7 integrin in airway inflammation and airway remodeling. Overexpression of α7 integrin in smooth muscle decreases ERK1/2 phosphorylation in the lung and increases α-SMA expression in mice during allergic inflammation. These shifts correlate with decreased secretion of inflammatory mediators in BAL of an OVA mouse model of allergic inflammation.](image-url)
asthma reduced BAL total cell counts, the secretion of critical cytokines and chemokines, and inhibited airway resistance in the chronic OVA model. The inhibition of ERK1/2 phosphorylation via MAPK signaling pathway may be one plausible mechanism by which α7 integrin modulates airway inflammation (Figure 8). Thus, α7 integrin expression can serve as a potential novel candidate for asthma-targeted therapies. Disentangling the regulatory mechanisms underlying α7 integrin-mediated airway inflammation has the potential to translate into valuable pharmaceutical therapies relevant to human asthma.

AUTHOR CONTRIBUTIONS
M.A.B and C.A.S conceived and designed experiments; R.D.W. and D.J.B generated the transgenic mouse and performed the initial characterization experiments; M.A.B and A.A. performed experiments; M.A.B, A.A, and K.H analyzed data and drafted the manuscript; J.M.E. performed experiments and analyzed data; M.A.B, A.A, K.H, J.M.E., and C.A.S interpreted results, M.A.B and K.H prepared figures. M.A.B, A.A., K.H., J.M.E., R.D.W., D.J.B, and C.A.S edited and approved the final manuscript.

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CONFLICT OF INTERESTS
The authors have no competing or financial interests to declare.

DATA AVAILABILITY STATEMENT
The data that support this study’s findings are available from the corresponding author upon reasonable request.

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