

## The modulation of large airway smooth muscle phenotype and effects of epidermal growth factor receptor inhibition in the repeatedly allergen-challenged rat

S. Siddiqui, M. Novali, K. Tsuchiya, N. Hirota, B. J. Geller, T. K. McGovern, P. A. Risse, T. Jo, M. A. Zeroual, and J. G. Martin

Meakins-Christie Laboratories, Department of Medicine, McGill University and the Research Institute of the McGill University Health Center, Montreal, Quebec, Canada

Submitted 31 January 2012; accepted in final form 15 April 2013

**Siddiqui S, Novali M, Tsuchiya K, Hirota N, Geller BJ, McGovern TK, Risse PA, Jo T, Zeroual MA, Martin JG.** The modulation of large airway smooth muscle phenotype and effects of epidermal growth factor receptor inhibition in the repeatedly allergen-challenged rat. *Am J Physiol Lung Cell Mol Physiol* 304: L853–L862, 2013. First published April 19, 2013; doi:10.1152/ajplung.00047.2012.—Allergen challenges induce airway hyperresponsiveness (AHR) and increased airway smooth muscle (ASM) mass in the sensitized rat. Whether the remodeled ASM changes its phenotype is uncertain. We examined, in sensitized Brown Norway rats, the effects of multiple ovalbumin (Ova) challenges on ASM remodeling and phenotype and the role of the epidermal growth factor receptor (EGFR) in these processes. Rats were sensitized with Ova and challenged three times at 5-day intervals with phosphate-buffered saline or Ova and pretreated with the EGFR inhibitor AG-1478 (5 mg/kg) or its vehicle dimethyl sulfoxide. Ova challenges increased ASM mass in all-sized airways and in large airway mRNA expression of smooth muscle myosin heavy chain (sm-MHC), assessed by laser capture. Myosin light chain kinase and the fast myosin isoform SM-B mRNA expressions were not affected. Ova induced AHR to methacholine, and, based on the constant-phase model, this was largely attributable to the small airways and lung derecruitment at 48 h that recovered by 1 wk. The EGFR ligands amphiregulin and heparin-binding epidermal growth factor (HB-EGF) were increased in bronchoalveolar lavage fluid at 48 h after Ova exposure. AG-1478 inhibited AHR and prevented ASM growth. Epithelial gene expression of EGFR, HB-EGF, matrix metalloproteinase (MMP)-9, Gro- $\alpha$ , and transforming growth factor- $\beta$  was unaffected by Ova challenges. We conclude that EGFR drives remodeling of ASM, which results from repeated Ova challenge. Furthermore, the latter results in excessive small airway and, to a lesser degree, large airway narrowing to methacholine, and large airway gene expression of contractile protein is conserved.

Brown Norway rat; asthma; smooth muscle myosin heavy chain; airway hyperresponsiveness; remodeling; laser capture; amphiregulin; heparin-binding epidermal growth factor

AIRWAY REMODELING IS CONSIDERED a key feature of asthma along with airway hyperresponsiveness (AHR). The repeatedly allergen-challenged Brown Norway (BN) rat has been frequently used to investigate the mechanisms of airway smooth muscle (ASM) and epithelial remodeling that is substantially mediated by the epidermal growth factor receptor (EGFR) and its axis of ligands (21, 45, 48). An increased ASM mass is among the prominent remodeling changes. The mass of ASM may be increased potentially by hyperplasia (6, 12, 14, 32, 35, 45, 50), hypertrophy (2, 6, 35), migration of ASM or subepithelial myofibroblasts (2), recruitment of circulating fibrocytes (37), differentiation of local mesenchymal stem cells (42), and

epithelial-mesenchymal transition (51). The impact of the remodeling process on the function of remodeled ASM is uncertain. Hyperplasia of ASM is associated with loss of contractile proteins in vitro, although there are few studies addressing this issue in vivo (19, 23, 29, 50). ASM has been reported to be increased by hyperplasia after repeated allergen challenges in animal models (45) so that loss of contractile proteins might be expected. Some studies suggest possible downregulation of contractile proteins following allergen challenges in experimental models (19, 29), whereas studies of human asthmatic smooth muscle have failed to show such a downregulation of protein (23, 50). However, it is possible that ASM phenotype changes may only be seen in recently remodeled ASM, which may not have been the case in studies of human tissues.

Airway epithelium is also substantially remodeled in human asthma, and epithelial repair is dysregulated (9). When airway epithelium is injured in vitro, there is an upregulation of the EGFR and some of its ligands that drive proliferation and repair (13). This growth-promoting pathway also causes differentiation of epithelial cells into goblet cells (43). EGFR signaling dependent on amphiregulin, which is secreted by epithelial and mast cells, has recently been implicated in mucous cell metaplasia in nonallergic lung injury (28). A significant positive correlation between EGFR expression and hyperplastic goblet cells in asthmatic patients has been reported (44), suggesting pertinence for these mechanisms in vivo also. EGFR expression is induced in the epithelium of Ova-challenged F344 rats (43), and its expression has also been described to be increased in bronchial tissues of asthmatic patients (1, 33). Epithelial activation following allergen challenge may also have a role in ASM remodeling. In in vivo asthma models, inhibiting the EGFR leads to a decrease of ASM (21, 45, 48), and the epithelium is a likely source of the ligands responsible for EGFR activation.

Previous work from our laboratory demonstrated decreased mRNA and protein expression for several contractile proteins in the proximal airway tree at 48 h after multiple allergen challenges, concurrently with evidence of active proliferation of ASM (19). Smooth muscle total myosin heavy chain (sm-MHC) and the fast isoform SM-B, containing a seven-amino acid insert in the motor domain (22, 25), and myosin light chain kinase (MLCK) expression were assessed (19). However, a limitation of the studies was the fact that muscle proteins and their mRNA were assessed for whole airway tissue and were not assessed in muscle directly. Furthermore, the substrain of BN rat used did not develop AHR after allergen challenges. In a separate study, we demonstrated that AHR was a function of altered responses of the small airways; although the large airways contained substantially more smooth

Address for reprint requests and other correspondence: J. G. Martin, 3626 St. Urbain, Montreal, Quebec H2X 2P2, Canada (e-mail: james.martin@mcgill.ca).

Table 1. *Primer sequences*

| Primer                | Sequence                 |
|-----------------------|--------------------------|
| S9 forward            | AGGATTCTTGGAGAGAAGGCTGC  |
| S9 reverse            | CTTCTGAGAGTCCAGGCGAACAA  |
| MHC forward           | CCAATCCTGGAGGCTTTCC      |
| MHC reverse           | CTCTCGTCCCTAGCATGTC      |
| MLCK forward          | ACATCCGTCAGGAGATCAGC     |
| MLCK reverse          | CACCTCCGCTCTGTTAGCTC     |
| Vimentin forward      | AACACTCCTGATTAAGACGGTTG  |
| Vimentin reverse      | TCATCGTGGTGTGAGAAAT      |
| CD90 forward          | CCACAAGCTCCAATAAACTATCAA |
| CD90 reverse          | AGCAGCCAGGAAGTGTTTTG     |
| TGF- $\beta$ forward  | CCTGGAAGGGCTCAACAC       |
| TGF- $\beta$ reverse  | CAGTTCTTCTCTGTGGAGCTGA   |
| Eotaxin forward       | CACGGCCACTTCCTTCAC       |
| Eotaxin reverse       | TGGGATCTTCTTACTGGTCA     |
| RANTES forward        | CTCACCGTCATCCTCGTTC      |
| RANTES reverse        | GAGTGGTGTCCGAGCCATA      |
| CLCA3 forward         | TCTCCTCAGGAATGCACTA      |
| CLCA3 reverse         | GGAGATTAACCTCTCTGCTCTCC  |
| MMP-9 forward         | CCTCTGCATGAAGACGACATAA   |
| MMP-9 reverse         | GGTCAGGTTTAGAGCCACGA     |
| GRO- $\alpha$ forward | CACACTCCAACAGAGACCA      |
| GRO- $\alpha$ reverse | TGACAGCGCAGCTCATTG       |
| IL-13 forward         | GGCCCTCAGGGAGCTTAT       |
| IL-13 reverse         | GCTGTTGCACAGGGAAGTCT     |
| EGFR forward          | TCCCTTTGGAGAACCTGCAGATCA |
| EGFR reverse          | GTGCTAAATCGCAGACACCGAT   |
| SMB forward           | CAAGGCCATCTTTTGCTCTAC    |
| SMB reverse           | CTCTCGTCCCTAGCATGTC      |
| Caldesmon forward     | GCTGGAGCAATATACCAATGC    |
| Caldesmon reverse     | TGATATTGCGGACACCTTCC     |
| p70S6 kinase forward  | AGACCTTTGGCTCGGAAG       |
| p70S6 kinase reverse  | GACTCACATCCTCTTCAGATTGC  |

MHC, myosin heavy chain; MLCK, myosin light chain kinase; CD90, cluster of differentiation 90; TGF- $\beta$ , transforming growth factor- $\beta$ ; RANTES, regulated on activation, normal T cell expressed and secreted; MMP-9, matrix metalloproteinase-9; EGFR, epidermal growth factor receptor; p70S6 kinase, p70 ribosomal S6 kinase.

muscle, they did not appear to be hyperresponsive to methacholine (MCh) (38). Either the enhancement of the ASM force-generating capacity by virtue of its increase in mass is not a determinant of large airway narrowing or a concomitant reduction in force production, presumably resulting from dedifferentiation, had occurred.

The aim of the current study was to examine the effects of repeated allergen challenge on ASM remodeling leading to

changes in mass and ASM phenotype. We reasoned that an increase in ASM in the large airways would likely be associated with changes in ASM phenotype, especially if the concurrent AHR is predominantly a small airway phenomenon. Additionally, we were interested to examine the effects of inhibition of the EGFR, the major pathway for ASM remodeling, on AHR and ASM growth and phenotype. Finally, we examined the airway epithelium for its expression of EGFR ligands and proinflammatory molecules with the potential to cause remodeling.

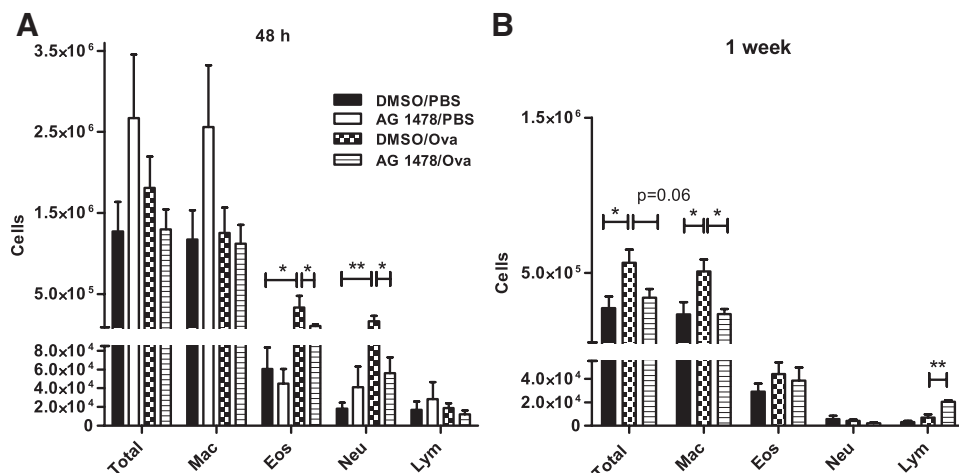
## MATERIALS AND METHODS

**Protocol for sensitization and challenge.** Male BN rats (SsN substrain) (Harlan/UK) were sensitized with 1 mg ovalbumin (Ova) along with 100 mg aluminum hydroxide dissolved in a 1-ml volume of phosphate-buffered saline (PBS) administered subcutaneously in the scruff of the neck (45). Immediately thereafter, the rats were intraperitoneally administered  $2 \times 10^9$  *Bordetella pertussis* heat-killed bacteria (provided by T. Issekutz, Dalhousie University, Halifax, NS, Canada). Animals under light anesthesia were endotracheally intubated and were administered aerosol challenges while continuing to breathe spontaneously. The tip of the endotracheal tube was placed in a small Plexiglas box into which aerosol was introduced from a Hudson nebulizer at 8 l/min for 5 min, with an output of 0.15 ml/min. Rats were challenged a total of three times with 5% Ova, or its control (PBS), at 5-day intervals (45) and studied 48 h after the third and final challenge. The first two study groups were 1) dimethyl sulfoxide (DMSO)-treated, PBS-challenged and 2) DMSO-treated, Ova-challenged animals. The protocols for this study were approved by the Animal Care Committee of McGill University (Montréal, QC, Canada).

**Administration of the EGFR inhibitor AG-1478.** Fourteen days after Ova sensitization as described above, two groups of rats were administered 5 mg/kg AG-1478 (Calbiochem, San Diego, CA) in DMSO, in a volume of 700  $\mu$ l, whereas the other two groups of animals received the vehicle intraperitoneally 1 h before each Ova or PBS challenge. The other two study groups were 3) AG-1478-treated, PBS-challenged and 4) AG-1478-treated, Ova-challenged animals.

**Airway responses to MCh challenge.** Airway responses to MCh (Sigma-Aldrich, St. Louis, MO) were assessed with the constant-phase model using a small animal ventilator and commercial software (Flexivent, Scireq, Montréal, QC) at 48 h after three Ova challenges (38). Animals were sedated and anesthetized with xylazine (10 mg/kg) and pentobarbital (35 mg/kg), respectively, and paralysis was induced with 1 mg/kg pancuronium bromide intraperitoneally (Sandoz Canada, Boucherville, QC, Canada). The animals received a booster at

Fig. 1. Bronchoalveolar lavage fluid inflammatory cells. A: at 48 h, there is a significant increase in the number of eosinophils (Eos,  $*P < 0.05$ ) and neutrophils (Neu,  $*P < 0.01$ ) with ovalbumin (Ova) challenges in the dimethyl sulfoxide (DMSO)/Ova group, and there is an inhibition of eosinophils and neutrophils with AG-1478 (AG) administration (AG-1478/Ova) ( $*P < 0.05$ ). In B at 1 wk, there is a significant increase in total inflammatory cells ( $*P < 0.05$ ) and macrophages (Mac,  $*P < 0.05$ ) and a borderline inhibition of total inflammatory cells ( $P = 0.0566$ ), an inhibition of macrophages ( $*P < 0.05$ ), and an increase in lymphocytes (Lym) with AG administration ( $**P < 0.01$ ). A one-way ANOVA and a Fisher's least-significant difference (LSD) post hoc test were performed ( $n = 11$ –17/group).



~45 min. The animals were placed on a heating pad, and their temperature was monitored to ensure that it remained close to 36°C. A positive end-expiratory pressure was maintained between 2 and 2.5 cmH<sub>2</sub>O, and the animals were ventilated at a tidal volume of 8 ml/kg and a breathing frequency of 90 breaths/min. We allowed 10–15 min after administration of anesthetic agents before intubating the animal and another ~3–5 min after placing the animal on the ventilator. The animals received a deep inflation to 25 cmH<sub>2</sub>O before the recordings were started. The constant-phase model was used to partition mechanical responses of the lungs to aerosolized MCh by fitting the data to an equation that has the following four parameters: Newtonian “airway” resistance ( $R_N$ ), airway inertance, dominated by the mass of gas in the central airways, and resistive and elastic impedances of tissues accounted for by G and H, respectively. G (tissue damping) is closely associated to peripheral airway and tissue resistance and reflects energy dissipation in the peripheral airways and lung tissues, whereas H is tissue elastance and reflects energy storage in the tissues (10). The perturbation used was the Quick Prime-3, a 3-s-long broadband (multifrequency) forced oscillation waveform with a frequency range of 1 to 20.5 Hz. The constant-phase parameter readings were assessed every 15 s, and the nebulization was synchronized to inspiration at 100% nebulization for a 10-s nebulization at any given dose. For each given MCh dose, we took the highest value for the constant-phase parameters that met a minimum requirement of a coefficient of determination of 0.75.

**Bronchoalveolar lavage fluid and assessment of inflammation.** Bronchoalveolar lavage (BAL) was performed by instilling PBS (5 × 5 ml aliquots) following the assessment of airway responses. Cells were pelleted upon centrifugation from all the aliquots and resuspended in PBS to perform cell counts. Cytospins were then prepared (Shandon Cytospin 4 cytocentrifuge; Thermo Scientific, Waltham, MA) and were subsequently stained with the Diff-Quik Stain Set (Dade Behring, Newark, DE). Differential cell counts were based on a cell count of 300.

**Bronchoalveolar lavage fluid assessment for EGFR ligands amphiregulin and heparin-binding epidermal growth factor by ELISA.** The volume recovered from the first 5 ml of bronchoalveolar lavage fluid (BALF) were stored in 1% bovine serum albumin at –80°C for further analysis. Amphiregulin levels were assayed in the BALF using the human amphiregulin kit (R&D Systems, Minneapolis, MN) as described previously (39). The lower limit of detection for amphiregulin using this kit is 15.6 pg/ml. Similarly, heparin-binding epidermal growth factor (HB-EGF) was assayed using the duo set Human HB-EGF kit (R&D Systems) where the lower limit of detection for HB-EGF is 31.25 pg/ml.

**Histology and morphometry.** For the assessment of remodeling, the left lung lobes were fixed overnight in formalin at a pressure of 25 cmH<sub>2</sub>O and were paraffin-embedded. Histological slides were prepared from 5-μm midsagittal and parahilar sections to sample medium and small airways and large airways, respectively. Tissue sections were stained for smooth muscle-specific  $\alpha$ -actin ( $\alpha$ -SMA), and the area of  $\alpha$ -SMA immunoreactivity was quantified as previously described (34, 45). Isotype controls were performed to confirm the specificity of  $\alpha$ -SMA staining. Airways  $\geq 1$  mm basement membrane perimeter ( $P_{BM}$ ) were considered to be medium and/or large airways and  $<1$  mm  $P_{BM}$  as small airways (34). Airways whose ratio of maximal to minimal internal diameter was equal to or greater than two were not included in the analyses.

In brief, sections were stained with a mouse monoclonal antibody to  $\alpha$ -SMA (clone 1A4; Sigma-Aldrich) as well as a biotinylated horse anti-mouse IgG, rat adsorbed (Vector Laboratories, Burlington, ON, Canada). The signal was developed with Vector Red. The area of ASM was traced using a camera lucida side-arm microscope attachment and was digitized with commercial software (SigmaScan Pro 5, Ashburn, VA). The area of ASM was normalized for airway size by dividing by the square of the  $P_{BM}$ . The number of bronchial blood vessels was also assessed and was similarly normalized for airway size.

**Laser capture microdissection for epithelial and ASM cells.** The right middle lobe was inflated with 50% optimal cutting temperature compound (OCT) and fixed with OCT before being snap-frozen in liquid nitrogen for subsequent laser capture microdissection (LCM; Arcturus Veritas). Seven-micrometer-thick sections were prepared in a cryostat (–20°C) in a RNase-free environment. The sections were stained with hematoxylin and eosin following the manufacturer’s protocol. LCM was performed using macro caps to select for epithelial cells. High-sensitivity caps were used to select for ASM cells. Once the cells were captured within 1–1.5 h after the staining, 4  $\mu$ l of Side-step lysis buffer (Stratagene, La Jolla, CA) was used to extract mRNA from the cells. The cells were placed in 500- $\mu$ l tubes and were vortexed for 1 min for lysis, and the samples were then stored at –80°C until reverse transcription was performed.

**Reverse transcription and RT-PCR.** The Agilent/Stratagene kit for reverse transcription (Santa Clara, CA) was used for the laser-captured cells. Epithelial cells were subsequently assessed for EGFR, HB-EGF, amphiregulin, transforming growth factor (TGF)- $\beta$ 1, IL-13, eotaxin [CC chemokine ligand (CCL)-11], regulated on activation, normal T cell expressed and secreted (RANTES, CCL-5), matrix

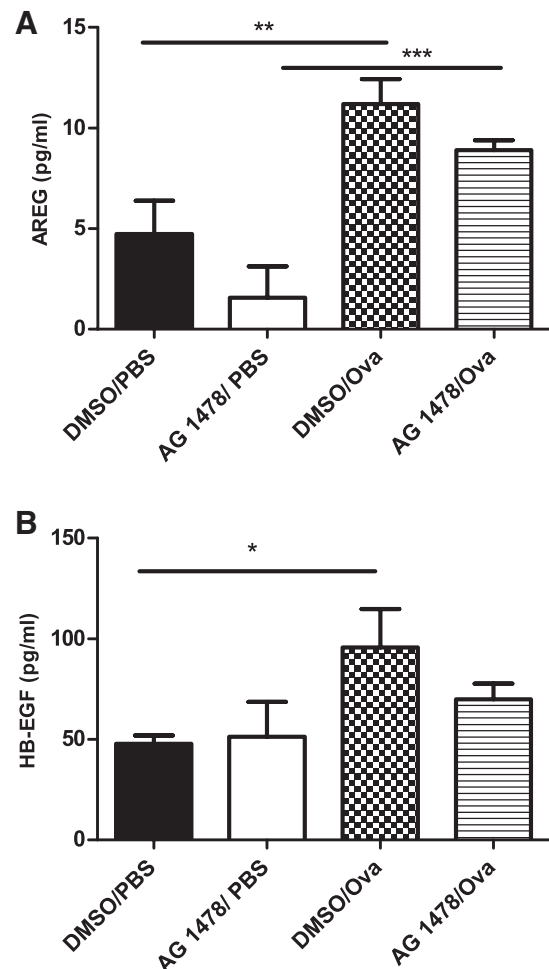


Fig. 2. ELISA for amphiregulin (AREG) and heparin-binding epidermal growth factor (HB-EGF) in the bronchoalveolar lavage fluid. A: at 48 h, there is a significant increase in the amphiregulin concentration in the DMSO/Ova animals compared with DMSO/PBS animals (\*\* $P < 0.01$ ) and in the AG-1478/Ova compared with the AG-1478/PBS animals (\*\*\* $P < 0.001$ ). B: at 48 h, there is a significant increase in the HB-EGF concentration in the DMSO/Ova animals compared with DMSO/PBS (\* $P < 0.05$ ). There were no observable differences between AG/PBS and DMSO/PBS. A one-way ANOVA and a Fisher’s LSD post hoc test were performed ( $n = 5$ –7/group).

metalloproteinase (MMP)-9, and the neutrophilic chemoattractant Gro- $\alpha$ . ASM cells were assessed for SM contractile proteins, including myosin heavy chain (MHC), MLCK, SM-B, caldesmon, in addition to vimentin (a marker for fibroblasts), p70 ribosomal S6 kinase (p70S6 kinase), a marker for hypertrophy, the EGFR ligand HB-EGF, and the mesenchymal stem cell marker cluster of differentiation 90 (CD90; Thy-1). The primer sequences were designed to be intron-spanning so as to avoid genomic amplification and were done so on the Roche website ([https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct\\_030000](https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=uplct_030000)). The primer sequences are described in Table 1. All genes of interest were normalized to S9 as a housekeeping gene.

**Statistical analysis.** Data are presented as means  $\pm$  SE. The statistical analysis was performed using GraphPad Prism, version 5 (GraphPad Software, San Diego, CA). For AHR data, repeated-measures ANOVA followed by Fisher's least-significant difference (LSD) test was performed. For BALF inflammatory cell count, ELISA, vessel assessment, ASM mass, and all gene expression data, a one-way ANOVA was performed followed by the Fisher's LSD test. For the gene expression data, the raw  $C_T$  values were log-transformed. A  $P$  value of  $\leq 0.05$  was considered significant.

## RESULTS

**Bronchoalveolar lavage fluid.** The eosinophils ( $P < 0.05$ ) and neutrophils ( $P < 0.01$ ) of the BALF cells were significantly increased in the Ova-challenged compared with the PBS-challenged animals. AG-1478-treated, Ova-challenged animals had a substantially lower number of eosinophils and neutrophils compared with Ova-challenged animals ( $P < 0.05$ ) (Fig. 1A). At 1 wk (Fig. 1B), there was a significant increase in the total number of inflammatory cells ( $P < 0.05$ ) and macrophages ( $P < 0.05$ ) in the Ova-challenged animals compared with PBS-challenged animals. At 1 wk, AG-1478-treated, Ova-challenged animals had a borderline reduction in the number of total inflammatory cells ( $P = 0.06$ ) and a marked decrease in macrophages ( $P < 0.05$ ) compared with the Ova-challenged animals. However, BALF lymphocytes were fur-

ther increased in the AG-1478-treated Ova-challenged animals compared with the Ova-challenged animals ( $P < 0.01$ ).

**Amphiregulin and HB-EGF protein assessment in the BALF.** At 48 h after challenges, the BALF concentration of amphiregulin was significantly higher in the Ova-challenged group compared with the PBS-challenged animals ( $P < 0.01$ , Fig. 2A). Similarly, the AG-1478-treated, Ova-challenged animals had a significantly higher amphiregulin concentration compared with the AG-1478-treated, PBS-challenged animals ( $P < 0.001$ , Fig. 2A). The Ova-challenged group also had a significantly higher HB-EGF concentration compared with the PBS-challenged animals ( $P < 0.05$ , Fig. 2B).

**Allergen-induced AHR to MCh; total respiratory system AHR and constant-phase parameters.** The baseline values of the constant-phase parameters, measured after saline aerosolization, were greater in the Ova-challenged animals compared with PBS-challenged animals, and these increases were prevented by AG-1478 treatment (Fig. 3A). The increase in responsiveness to MCh following repeated Ova challenges was predominantly attributable to changes in G and H, whereas there were fewer significant changes in  $R_N$  among treatment groups (Fig. 3, A-C). AG-1478 prevented the increase in airway responses as reflected by G and H. AG-1478-treated, Ova-challenged animals had higher values of  $R_N$ , G, and H compared with PBS-challenged, AG-1478-treated animals. AG-1478 treatment itself significantly reduced airway responses to MCh in repeatedly saline-challenged animals in  $R_N$  (Fig. 3A). At 1 wk, there were no significant differences between the groups for the constant-phase parameters (data not shown).

**Morphometric assessment of ASM mass.** There was a significant increase in the mass of ASM in the Ova-challenged animals in the large ( $P < 0.05$ ) and small ( $P < 0.05$ ) airways. The increased mass in both the large and small airways was prevented by AG-1478 administration ( $P < 0.001$  and  $P < 0.05$ , respectively, Fig. 4, A and B). At 1 wk, there was a trend for an increased ASM mass to persist in the medium/larger

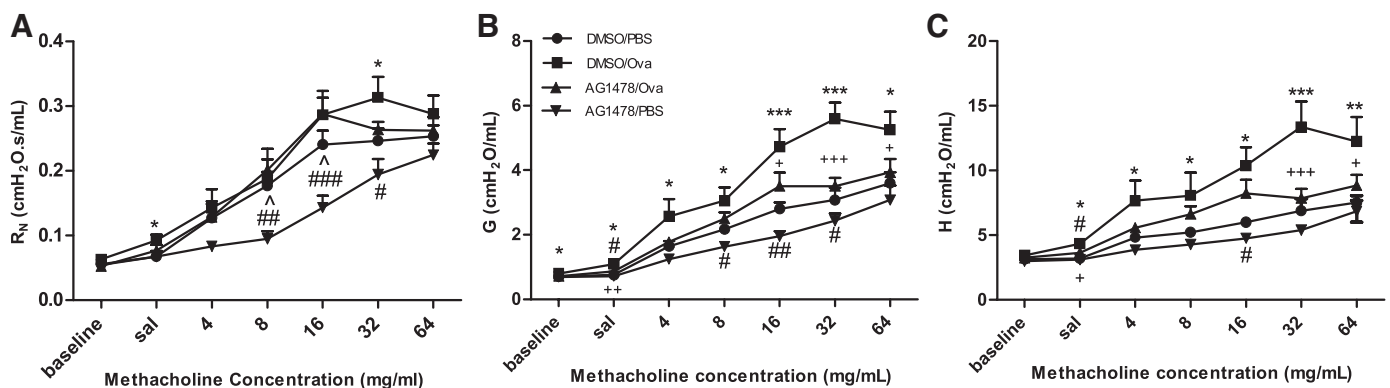


Fig. 3. Airway responses to methacholine challenge. Newtonian airway resistance ( $R_N$ , A), tissue damping (G, B), and tissue elastance (H, C) indicate that, at saline, DMSO/Ova was increased (\* $P < 0.05$ , \*\* $P < 0.05$ , \*\*\* $P < 0.001$ , respectively) and, for G and H, this increase was prevented in AG-1478/Ova (++ $P < 0.01$  and + $P < 0.05$ , respectively). For  $R_N$ , AG-1478/Ova was higher than AG-1478/PBS at 8, 16, and 32 mg/mL (## $P < 0.01$ , ### $P < 0.001$ , and # $P < 0.05$ ) and AG/PBS was lower than DMSO/PBS at 8 and 16 mg/mL (^ $P < 0.05$ ). At 32 mg/mL,  $R_N$  indicates that DMSO/Ova is higher than DMSO/PBS (\* $P < 0.05$ ) (G, B), where there is an increase in the DMSO/Ova compared with DMSO/PBS at baseline (\* $P < 0.05$ ) and 4, 8, 16, 32, and 64 mg/mL (\* $P < 0.05$ , \*\* $P < 0.05$ , \*\*\* $P < 0.001$ , \*\*\* $P < 0.001$ , and \* $P < 0.05$ , respectively) and prevented with AG administration, as observed in AG-1478/Ova in 16, 32, and 64 mg/mL (+ $P < 0.05$ , +++ $P < 0.001$ , and + $P < 0.05$ , respectively). For G, there is a significant increase in AG-1478/Ova compared with AG-1478/PBS for saline, 8, 16, and 32 mg/mL (# $P < 0.05$ , # $P < 0.05$ , ## $P < 0.01$ , and # $P < 0.05$ ) and the H parameter (C) where there is an increase in DMSO/Ova compared with DMSO/PBS at 4, 8, 16, 32, and 64 mg/mL (\* $P < 0.05$ , \* $P < 0.05$ , \* $P < 0.05$ , \*\*\* $P < 0.001$ , and \*\* $P < 0.01$ , respectively), and this increase is inhibited by AG-1478/Ova at 32 and 64 mg/mL (+++ $P < 0.001$  and + $P < 0.05$ ). AG-1478/Ova was higher than AG-1478/PBS at saline (# $P < 0.05$ ) and 16 mg/mL (# $P < 0.05$ ). Repeated-measures ANOVA with subsequent Fisher's LSD post hoc tests were performed ( $n = 12$ –15/group).

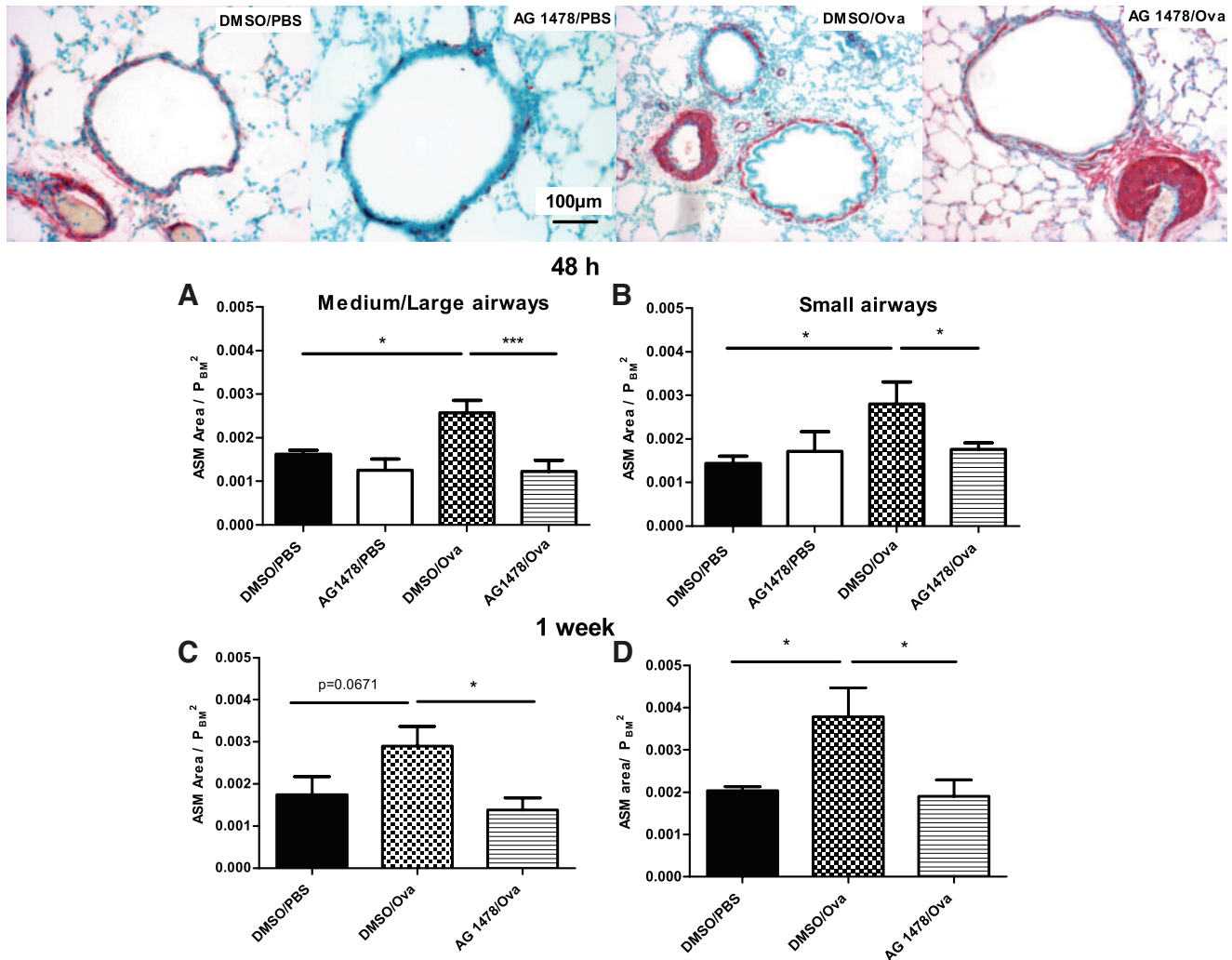


Fig. 4. Morphometric assessment of airway smooth muscle (ASM) mass. At 48 h, there is a significant increase in the ASM mass [assessed as sum of smooth muscle bundles normalized for basement membrane perimeter ( $P_{BM}^2$ )] in the medium/large airways (A) and the small airways (B) of the DMSO/Ova animals compared with DMSO/PBS ( $*P < 0.05$ ), and this increase is prevented by AG administration, as observed in the AG-1478/Ova group ( $***P < 0.001$  and  $*P < 0.05$ , respectively). There are no differences observed between AG-1478/PBS ( $n = 4$ ) and DMSO-PBS. At 1 wk, there is a marked reduction in the medium/large ASM mass in the AG-1478/Ova animals compared with the DMSO/Ova animals ( $*P < 0.05$ ) (C) while there is an increase in the small ASM mass by DMSO/Ova ( $*P < 0.05$ ) that is prevented by AG administration, as observed in AG-1478/Ova ( $*P < 0.05$ ) (D). There are no differences observed between AG-1478/PBS ( $n = 4$ ) and DMSO-PBS. A one-way ANOVA and a Fisher's LSD post hoc test were performed ( $n = 4-8$ /group).

airways ( $P = 0.0671$ ), whereas there was still a significantly increased ASM mass in the smaller airways ( $P < 0.05$ ) following Ova challenge. These increases were not seen in the animals that received AG-1478 ( $P < 0.05$ ) (Fig. 4, C and D).

**Assessment of bronchial vessels.** There was no significant increase in the number of bronchial vessels in the large airways at 48 h after multiple allergen challenges. However, the number of blood vessels associated with the peripheral airways was significantly higher in the AG-1478-treated, Ova-challenged animals than in either the PBS-challenged or the Ova-challenged animals ( $P < 0.05$ ). We also assessed the area of vascular smooth muscle but found no significant differences in the vascular smooth muscle areas in the Ova-challenged animals compared with controls for either the large or small airways nor the Ova-challenged animals vs. the AG-1478-treated, Ova-challenged animals (data not shown).

**Gene expression in laser-captured ASM cells.** We assessed the expression of a number of genes involved in ASM con-

traction by LCM and subsequent quantitative RT-PCR. There was a significant increase in the expression of sm-MHC that was not present in the AG-1478-treated, Ova-challenged animals ( $P < 0.05$ , Fig. 5A). There was no difference in total MLCK and in the fast myosin isoform SM-B among the groups (Fig. 5, B and C, respectively). None of the samples in any of the groups expressed caldesmon or p70S6 kinase at a detectable level. Additionally, there were no differences in HB-EGF expression or in vimentin (data not shown). However, CD90, a marker highly expressed in mesenchymal stem cells, was significantly decreased in the Ova-challenged animals ( $P < 0.05$ ), and this decrease was prevented by AG-1478 ( $P < 0.05$ ) (Fig. 5D). All RT-PCRs were normalized to the expression of S9, a housekeeping gene (Fig. 5).

**Gene expression in laser-captured epithelium.** We examined the expression of the eosinophil chemoattractants eotaxin and RANTES and found no differences among the three study groups: PBS-challenged, Ova-challenged, and AG-1478-treated, Ova-chal-

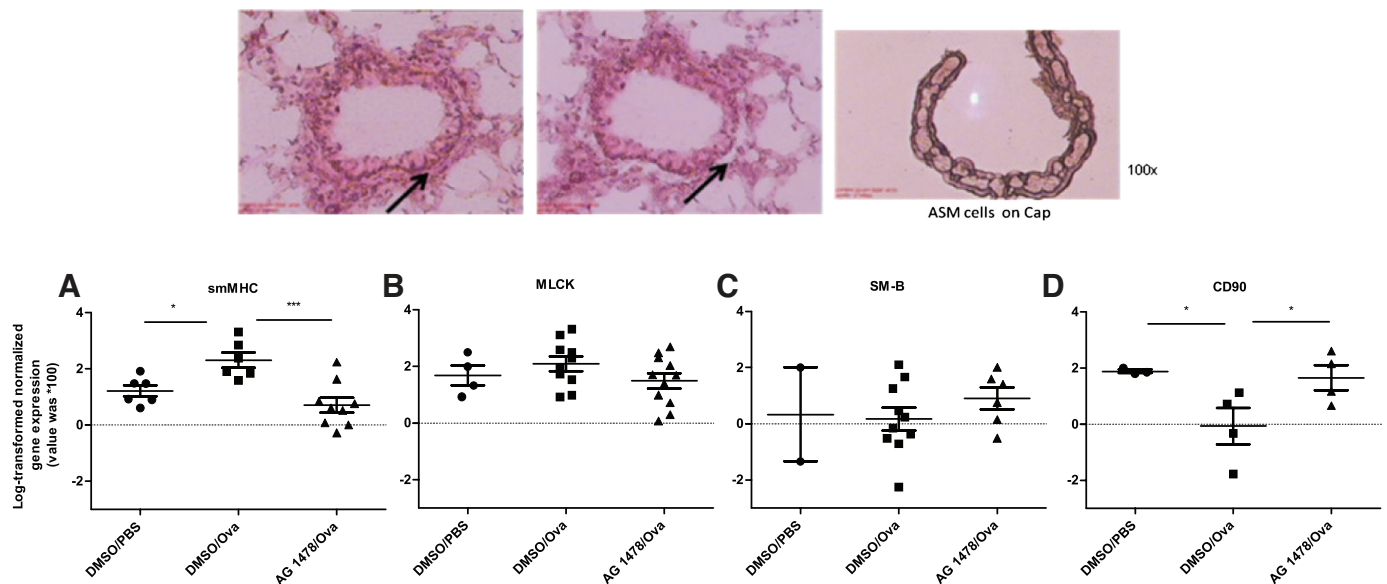


Fig. 5. mRNA gene expression from laser-captured ASM cells. A: smooth muscle myosin heavy chain (sm-MHC) expression, which is upregulated in the Ova-challenged animals compared with controls ( $*P < 0.05$ ,  $n = 6-9/\text{group}$ ) and prevented by AG administration ( $***P < 0.001$ ). There are no differences in myosin light chain (MLCK) ( $n = 4-11/\text{group}$ ) (B) and SM-B ( $n = 2-10/\text{group}$ ) (C) gene expressions. In D, cluster of differentiation 90 (CD90) ( $n = 3-4/\text{group}$ ), there was a decrease in the DMSO/Ova group compared with DMSO/PBS ( $*P < 0.05$ ) that recovered with AG-1478/Ova ( $*P < 0.05$ ). A one-way ANOVA and a Fisher's LSD post hoc test were performed.

lenged animals. We also examined CLCA3, a gene closely associated with mucus production (53). Other genes tested included EGFR (Fig. 6A), TGF- $\beta$ 1 (Fig. 6B), MMP-9 (data not shown), Gro- $\alpha$  (data not shown), and IL-13 (data not shown). There were no significant differences detected among the groups for any of these aforementioned genes.

## DISCUSSION

Repeated Ova challenges of the sensitized BN rat led to AHR to inhaled aerosols of MCh. Analysis of mechanics indicated that AHR, in the Ova-challenged animals, was largely attributable to responses within the peripheral airways. Remodeling of ASM affected both large and small airways, as reflected in an increase in ASM mass. There was also an increase in the expression at the mRNA level of one of the important contractile proteins, MHC, in the ASM in the large airways, suggesting a possible element of hypertrophy. AHR, the increase in ASM mass, and the increase in MHC expression were all attenuated by inhibiting the EGFR. BAL leukocytosis was also reduced by inhibiting the EGFR, suggesting a role for this receptor in the inflammatory process. There was an increase in protein levels of the EGFR ligands amphiregulin and HB-EGF in the BALF in the Ova-challenged animals compared with the respective controls. Changes in airway epithelial phenotype were not detected by analysis of some candidate chemokines, cytokines, and growth factors. Furthermore, the changes in AHR were no longer significant at 1 wk after allergen challenges despite the persistent increase in ASM mass, in particular in the small airways, and an increase in inflammatory cells. Both the peripheral ASM and overall burden of inflammatory cells in the BALF were reduced by EGFR inhibition. No differences were observed in the contractile protein gene expression at 1 wk.

Repeated allergen challenges in the sensitized BN rat have been shown previously to result in AHR (24, 38). The mech-

anisms of AHR have not been definitively elucidated although it seems likely that there are different stimulus-dependent pathways that may lead to the same excessive narrowing of the airways. ASM remodeling has been proposed as a likely cause of AHR (20), but direct proof of a cause and effect relationship is lacking. We have shown recently that the site of the altered mechanics accounting for AHR is located in the periphery of the lung based on the evaluation of respiratory mechanics using the constant-phase model (38). Our present data are in concordance with these earlier findings in that our present data indicate that the airway responsiveness is predominantly a small airways phenomenon. In the current study, with a larger sample size, we do observe an increase in responsiveness after Ova challenges measured with the  $R_N$  parameter. Increases in G and H after repeated Ova challenge implicate the peripheral airway compartment. Whether there are changes in the properties of the airways facilitating constriction or simply lung derecruitment from airway closure is uncertain, but the latter has been suggested (26). The administration of AG-1478 to the Ova-challenged animals prevented the increases in responsiveness. However, AG-1478 also reduced the airway responses in PBS-challenged animals, but this effect was manifest only in large, conducting airways as observed in the values of  $R_N$  at submaximal concentrations of MCh. This was an unexpected finding, and the mechanism of the effect will require further study.

In the current study, repeated allergen challenges led to increased ASM growth in all-sized airways. Concurrent EGFR inhibition reduced allergen-induced ASM growth as reported earlier (45). We did not detect an increase in either blood vessel density or in vascular smooth muscle area in the Ova-challenged animals compared with controls. Unexpectedly, EGFR inhibition led to a greater number of bronchial blood vessels compared with both the PBS and Ova-challenged animals. One week after allergen challenges, interestingly, AHR was no

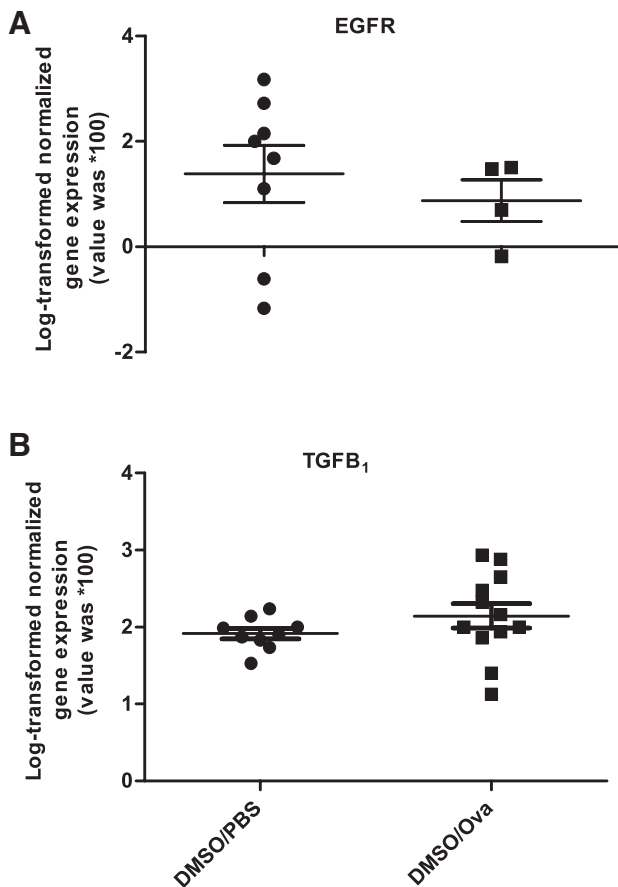


Fig. 6. mRNA gene expression from laser-captured epithelial cells. There are no differences in the expressions of epithelial growth factor receptor (EGFR) ( $n = 3-8$ /group, AG1478/Ova not shown) (A) and transforming growth factor (TGF)- $\beta$  (B) among the DMSO/PBS and DMSO/Ova groups ( $n = 4-12$ /group, AG1478/Ova not shown). A one-way ANOVA and a Fisher's LSD post hoc test were performed.

longer present, whereas total inflammatory cells and macrophages were increased, and the increase in ASM mass was maintained, although only statistically significantly in the peripheral lung. These data support earlier reports suggesting a lack of a clear association between the two asthmatic features (38). A more rapid rate of resolution of ASM remodeling after allergen challenges was found in a previous study (19), but these differences may relate to the source of the BN rats (United Kingdom vs. the United States). The U.S. BN rats also fail to develop AHR (19). We have previously observed differences in the degree of vascular remodeling and inflammation and of expression in Th2 cytokines between U.S. and UK BN rats (15, 47).

Previous studies of allergen-challenged BN rats have shown that increased ASM mass is attributable to hyperplasia (31, 34, 45). Based on cell culture models, we would have expected intrinsic changes associated with proliferative and secretory characteristics. Indeed, we reasoned that a possible explanation for the failure of the increase in ASM in the large airways to cause AHR was a reduction in contractile protein expression. To address such an alteration in ASM phenotype, we examined the expression of proteins in the contractile pathway, sm-MHC, its isoform SM-B, and MLCK. Only sm-MHC was significantly upregulated at the mRNA level in large airway muscle.

No alterations in SM-B or MLCK were detected. Although at odds with *in vitro* findings in ASM undergoing proliferation, our findings are not inconsistent with reports on human ASM harvested from asthmatics. Some investigators have failed to find changes in mRNA expression for contractile proteins in ASM from asthmatic subjects (50). Others have reported alterations in contractile protein expression related to clinical outcomes; sm-MHC, sm- $\alpha$ -actin, and desmin expression levels have been shown to correlate with MCh responsiveness in asthmatic subjects (23, 40). Airway responsiveness to MCh has been reported to be inversely related to the expression of sm- $\alpha$ -actin, desmin, and elastin (40). In addition, forced expiratory volume in 1 s (%predicted) was positively related and deep inspiration-induced bronchodilation was inversely related to desmin, MLCK, and calponin expression (40).

The observed increase in total sm-MHC mRNA after repeated allergen challenges suggests that an element of hypertrophy was triggered. Hypertrophy of ASM is described in some asthmatic subjects (6) and may be mediated by cytokines such as TGF- $\beta$  (8). EGFR inhibition prevented the increase in sm-MHC, linking the effects of the receptor to both hyperplasia and hypertrophy. We did not, however, observe differences in the gene expression of total MLCK. In the current study, none of the laser-captured smooth muscle samples had a detectable gene expression of p70S6 kinase, which has been reported to be implicated in hypertrophy (5). Our results are in accordance with the results of the analysis of human asthmatic bronchoscopic biopsies using the LCM technique to harvest mRNA from ASM where no increases in MLCK expression were detected (50). These findings are in contrast to findings of an upregulation of MLCK mRNA in mild asthmatic bronchial biopsies (23). Our data are suggestive of the possibility that the activity of MLCK may be modulated rather than the expression of MLCK itself.

We assessed CD90 expression, a marker that is highly expressed in mesenchymal stem cells and myofibroblasts. We anticipated that CD90 expression might be increased after allergen challenges if either stem cells or myofibroblasts were recruited as part of the remodeling process. Contrary to our expectations, we found a reduction in CD90 in the Ova-challenged animals and a similar expression of CD90 in the ASM of AG-1478-treated, Ova-challenged animals and the PBS-challenged animals, arguing against either of these cell types as a source of increase in muscle mass. Expression of vimentin in the ASM, a differentiation marker for fibroblasts, was also not altered. The gene expression of EGFR ligand HB-EGF, which has been reported to be upregulated in the ASM in tissue from asthmatic human biopsies by *in situ* hybridization and by immunohistochemistry and in proliferating human ASM cells in culture by RT-PCR (11), was not significantly different among the groups. A recent study reports that two weeks of oral prednisolone ( $0.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ ) also induces changes in the gene expression of ASM in asthmatic patients (52). Two of the genes, family with sequence similarity 129, member A (FAM129A) and synaptopodin 2 (SYNPO2), correlated with lung function and responsiveness to MCh (52). This study confirms the plasticity of gene expression by ASM.

Since the epithelium is a source of EGFR ligands and the proforms of these molecules may be cleaved at the membrane by metalloproteinases such as MMP-9, we assessed the expres-

sion of HB-EGF, MMP-9, and the EGFR itself. However, there were no differences in the expression of EGFR in the epithelium among the groups. There is evidence for epithelial influence on ASM phenotype via a MMP-9-dependent mechanism (27). We thus assessed the gene expression of epithelial-derived MMP-9, but we observed no changes among the groups. TGF- $\beta$ 1 is a profibrotic cytokine and has also been implicated in cellular hypertrophy in vitro (8), and in vitro it has also been shown to induce ASM proliferation (3). Epithelial-derived TGF- $\beta$ 1 has been reported to be upregulated in the mouse after allergen challenge in vivo (16), but we found no differences among the groups, arguing that epithelial-derived TGF- $\beta$ 1 is not responsible for the increase in ASM mass, as had been previously proposed, or that we have sampled the epithelial cells too late in the remodeling process. We assessed IL-13 as well as a potential modulator of ASM phenotype as the cytokine most strongly associated with the development of AHR (49). Very few cell samples expressed IL-13; thus, it was not possible to assess its potential role. Gro- $\alpha$ , an important neutrophilia-promoting chemokine, was also not different among the groups. In an attempt to demonstrate epithelial cell metaplasia to goblet cells, we assessed CLCA3 (Gob5), commonly considered as a mucus gene (30, 53). Despite earlier data demonstrating goblet cell differentiation in this BN rat model (38), we did not find differences among the three study groups for CLCA3. These data support findings where CLCA3 was shown not to be important in mucus overproduction in an allergic mouse model (36).

Ova challenge induced eosinophilic and neutrophilic inflammation while EGFR inhibition decreased both eosinophilia and neutrophilia. We have previously shown that EGFR inhibition during the sensitization process inhibited the Ova-induced influx of CD4<sup>+</sup> T cells to airways (46). The current data show that, at 1 wk post-Ova challenge, there was a significant increase in lymphocytes with AG-1478 administration compared with the Ova-challenged animals even though there was an overall decrease in granulocytes and macrophages. It appears that AG-1478 administration at the time of Ova challenge has different effects to those administered during sensitization. Perhaps there is a rebound effect of the inhibition of the EGFR on lymphocyte trafficking once the drug effects are no longer present. Even though these results suggested to us the possibility of an effect on EGFR activation during allergen challenge on chemokine expression, we failed to detect changes in transcripts by laser capture in the epithelial-derived eosinophilic chemotactic factors, RANTES and eotaxin. We did not explore the other mediators implicated in cellular recruitment in vivo (18), including the 5-lipoxygenase product 5-oxo-6,8,11,14-eicosatetraenoic acid (41) and leukotriene B<sub>4</sub> (4).

A significant limitation of our studies is the timing of the assessment of mRNA for genes of interest. It is possible that these changes may have occurred much earlier following challenge and were restored to normal levels by the time we harvested tissue. Likewise, the expression of the cytokines and chemokines we assessed at the protein level in BALF may also have returned to usual levels.

The BN rat model has many features in common with human allergic asthma. Of particular interest to the current study is the rapid remodeling of the airways that occurs after as few as three challenges with allergen (45). ASM remodeling in human asthma has been described by many investigators, but there is

only one report to our knowledge showing active proliferation of ASM cells in excess of that seen in control tissues (11). ASM remodeling in human asthma is attributed also to hyperplasia for the most part (12, 14, 50), but it may occur early in disease and may not be easily detected unless tissues are sampled at a time when the triggers for hyperplasia are present. In the BN rat model, hyperplasia has been demonstrated (45). In the context of the current study, it is noteworthy that, in addition to the evidence of increased EGFR expression in human asthmatic tissues, the ligands HB-EGF and amphiregulin, which were elevated in the BALF of the rat, are increased in expression at the mRNA and protein level in tissue biopsies from severe asthmatics for HB-EGF (11) and in sputum from asthmatic subjects for amphiregulin (7, 17). This model therefore may have the potential to provide valuable insights into tissue remodeling in human asthma.

In summary, we have explored the relationships among ASM remodeling, AHR, and ASM gene expression. There is an increase in ASM in all-sized airways and an increase in sm-MHC in large airways. Small changes in the large airway responses to aerosolized MCh were observed and more substantial changes affecting the small airways, confirming that the enhanced responses in vivo involve the small airways mainly. These changes appear to be mediated via the EGFR. There was no evidence for a downregulation of contractile proteins in association with the remodeling of the ASM.

#### ACKNOWLEDGMENTS

We thank Drs. Barbara Tolloczko and Isao Ito for guidance on using the laser capture microdissection equipment. We acknowledge Dr. John MacSharry for helpful suggestions and Muhammad Abu Shadeque Mullah for a statistical consultation. We also thank Megha Sandhu, Zahra Talat, Cinzia L. Marchica, Helena Giménez Palau, Sarah McCuaig, Michael O'Sullivan, and Ludovic Gaut for excellent technical help.

#### GRANTS

This work was supported by a grant from the Canadian Institutes of Health Research (MOP-93747) and the J.T. Costello Memorial Research Fund.

#### DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

#### AUTHOR CONTRIBUTIONS

Author contributions: S.S. and J.G.M. conception and design of research; S.S., M.N., K.T., N.H., B.J.G., T.K.M., T.J., and M.A.Z. performed experiments; S.S., K.T., N.H., and P.-A.R. analyzed data; S.S., M.N., P.-A.R., and J.G.M. interpreted results of experiments; S.S. prepared figures; S.S. and J.G.M. drafted manuscript; S.S. and J.G.M. edited and revised manuscript; S.S., M.N., K.T., N.H., B.J.G., T.K.M., P.-A.R., T.J., M.A.Z., and J.G.M. approved final version of manuscript.

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