THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 48, pp. 33472–33482, November 28, 2008
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Acidic Mammalian Chitinase Is Secreted via an ADAM17/Epidermal Growth Factor Receptor-dependent Pathway and Stimulates Chemokine Production by Pulmonary Epithelial Cells*

Received for publication, July 21, 2008, and in revised form, September 15, 2008 Published, JBC Papers in Press, September 29, 2008, DOI 10.1074/jbc.M805574200

Dominik Hartl[1], Chuan Hua He[1], Barbara Koller, Carla A. Da Silva, Robert Homer, Chun G. Lee, and Jack A. Elias[2]
From the Section of Pulmonary and Critical Care Medicine, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06520-8057

Acidic mammalian chitinase (AMCase) is expressed in an exaggerated fashion in epithelial cells at sites of pulmonary T helper cell type 2 inflammation and plays important roles in the pathogenesis of anti-parasite and asthma-like responses. However, the mechanisms that control epithelial cell AMCase secretion and its effector responses have not been adequately defined. To address these issues, we used i*n vivo* and *in vitro* experimental systems to define the pathways of epithelial AMCase secretion and its epithelial regulatory effects. Here we demonstrate that, in murine T helper cell type 2 modeling systems, AMCase colocalizes with the epidermal growth factor receptor (EGFR) and ADAM17 (a membrane disintegrin and metallopeptidase 17) in lung epithelial cells. *In vitro* cotransfection experiments in A549 cells demonstrated that AMCase and EGFR physically interact with each other. Cotransfection of AMCase and EGFR also increased, whereas EGFR inhibition decreased AMCase secretion. Interestingly, AMCase secretion was not significantly altered by treatment with EGF but was significantly decreased when the upstream EGFR transactivator ADAM17 was inhibited. AMCase secretion was also decreased when the EGFR-downstream Ras was blocked. Transfected and recombinant AMCase induced epithelial cell production of CCL2, CCL17, and CXCL8. These studies demonstrate that lung epithelial cells secrete AMCase via an EGFR-dependent pathway that is activated by ADAM17 and mediates its effects via Ras. They also demonstrate that the AMCase that is secreted feeds back in an autocrine and/or paracrine fashion to stimulate pulmonary epithelial cell chemokine production.

Allergic asthma is characterized by an exaggerated T helper cell type 2 (Th2)[3] immune response with eosinophil-rich tissue and bronchoalveolar lavage inflammation, mucus metaplasia, airway remodeling, and airway hyperresponsiveness. Studies from our laboratory and others have demonstrated that the Th2-associated cytokine interleukin-13 (IL-13) is a central regulator of these responses (1–3). In accord with its importance, the mechanisms that IL-13 uses to induce these responses have been intensively investigated. These studies demonstrated that the effects of IL-13 are mediated by a variety of downstream mediators, including CC chemokines, TGF-β1, adenosine and adenosine receptors, vascular endothelial growth factor, IL-11, and the 18-glycosyl hydrolase protein, acidic mammalian chitinase (AMCase) (1, 4–6). The last is particularly intriguing, because these studies demonstrated that (*a*) AMCase is induced in epithelial cells and macrophages at sites of Th2 inflammation, (*b*) IL-13 is necessary and sufficient to stimulate AMCase production, (*c*) exaggerated AMCase expression can be readily appreciated in biopsies from patients with asthma, and (*d*) anti-AMCase-based interventions ameliorate Th2 inflammation and physiologic dysregulation in a chitin-free experimental system (6). These studies also demonstrated that epithelial cells store AMCase in intracellular granules and secrete the enzyme into their local microenvironment (7). However, the pathways regulating the secretion of AMCase and the effects that it has after secretion have not been adequately defined.

Epidermal growth factor receptor (EGFR) activation regulates epithelial cell activation, differentiation, proliferation, and survival (8–10). EGFR can be activated by a variety of cell surface-bound EGFR ligands like TGF-α and amphiregulin (11, 12). Most frequently, the availability of these ligands is regulated via ectodomain shedding and receptor transactivation by specific metalloproteinases (13, 14), such as the ADAM17 (a membrane disintegrin and metallopeptidase domain 17), also called TACE (tumor necrosis factor-α-converting enzyme) (14). Several lines of evidence have linked ADAM17/EGFR and asthma, including animal studies that demonstrated that many

* This work was supported, in whole or in part, by National Institutes of Health, NHLB, Grants R01-HL-081639 and P01-HL-056389 (to J. E.). This work was also supported by Deutsche Forschungsgemeinschaft Research Scholarship HA 5274/2-1 (to D. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed: Waldemar Von Zedtwitz Professor of Medicine, Chair, Dept. of Internal Medicine, Yale University School of Medicine, Chief, Beeson Service, Yale New Haven Hospital, 333 Cedar St., 1072 LMP, P.O. Box 208056, New Haven, CT 06520-8056. Tel.: 203-785-4119; Fax: 203-785-6954; E-mail: jack.elias@yale.edu.

[3] The abbreviations used are: Th2, T helper cell type 2; AMCase, acidic mammalian chitinase; EGFR, epidermal growth factor receptor; TGF-α, transforming growth factor-α; IL, interleukin; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline; WT, wild type; OVA, ovalbumin.

of the epithelial alterations at sites of Th2 inflammation can be ameliorated by EGFR inhibition (10) and human studies that documented the exaggerated EGFR activation in tissues from patients with asthma (16). A number of studies have also linked epithelial ADAM17 and EGFR signaling to IL-13. These studies demonstrated that IL-13 induces mucus metaplasia via an EGFR-dependent pathway (17), ADAM17 plays a critical role in the regulation of MUC5AC mucin expression in cultured human airway epithelial cells (18), and IL-13 induces the proliferation of normal human bronchial epithelial cells via ADAM17-induced ectodomain shedding of TGF-α (19).

Given the important roles of ADAM17 and EGFR signaling in IL-13-mediated activation of epithelial cells and allergic asthma, we hypothesized that a ADAM17/EGFR pathway contributes to the secretion of AMCase by lung epithelial cells. We also hypothesized that secreted AMCase feeds back to regulate epithelial cell function. To test these hypotheses, studies were undertaken to define the interactions of AMCase and EGFR and the role of the EGFR pathway in epithelial AMCase secretion. The effector responses of secreted AMCase were also evaluated. These studies demonstrate that respiratory epithelial cells secrete AMCase via a ADAM17/EGFR/Ras-dependent pathway. They also highlight the ability of AMCase to stimulate epithelial chemokine elaboration.

EXPERIMENTAL PROCEDURES

Reagents—Cell culture media and fetal bovine serum were purchased from Invitrogen. Restriction endonucleases and other DNA-modifying enzymes were obtained from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by IDT, Inc. (Coralville, IA) or the Yale University Keck facility center. cDNAs were purchased from Clontech. PCR kits for gene amplification or cloning were obtained from Stratagene (La Jolla, CA). The immunoprecipitation kit was from Millipore (Billerica, MA). Staurosporine, the PI3 kinase inhibitor wortmannin, and the Ras inhibitor manumycin A (a farnesyltransferase inhibitor of Ras) were from Sigma. The pharmacological inhibitors PD153035 (a kinase inhibitor that specifically blocks the ATP binding site and inactivates EGFR signaling) and AG1478 (a small molecule EGFR tyrosine kinase inhibitor) were purchased from BIOSOURCE (Camarillo, CA). The TAPI-1 and −2 (TNFα-processing inhibitor-1/2, ADAM17-specific inhibitors) were from Peptides International (Louisville, KY). The ERK inhibitor PD98059 was bought from Calbiochem. The primary anti-human polycloncal and the rabbit anti-mouse monocloncal AMCase antibodies were generated using phage display technology and were provided by Medimmune (Gaithersburg, MD). Recombinant human AMCase was from Medimmune (Gaithersburg, MD). Mouse monoclonal antibody reactive to β-tubulin was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary APC-labeled antibodies were from BD Biosciences. A primary anti-EGFR was from Abgent (San Diego, CA). Anti-human ADAM17 monoclonal antibody was purchased from R&D Systems (Minneapolis, MN) and employed as reported previously (20). Secondary Pe-Cy7- and APC-Cy7-labeled antibodies were from Santa Cruz Biotechnology. An anti-mouse CD31 fluorescein isothiocyanate antibody and the corresponding isotype control were from BD Biosciences. Anti-mouse pancytokeratin PE antibody was from Abcam. Anti-mouse CD45-PcP was from BD Biosciences.

Flow Cytometry—Whole lung cell suspensions were obtained by a modified method according to Rice *et al.* (48). In brief, lung tissue was digested using dispase (5 mg/ml; Stem Cell Technologies), collagenase (0.04%; Sigma), and 100 units/ml DNase (Sigma). Whole lung suspension cells underwent several centrifugations (10 min, 300–1000 g) and hemolysis (precooled hemolysis solution containing 11 mM KHCO3, 152 mM NH_4Cl; washing for 5 min, 400 g at 4 °C), were then strained through progressively smaller cell strainers (100–20 μM) and nylon gauze, and were finally resuspended in fluorescence-activated cell sorting buffer (PBS, 2% bovine serum albumin, 2% fetal calf serum) supplemented with 10 units/ml DNase I. Macrophages were depleted from the lung cell suspensions by repeated means of adhesion to plastic plates at 37 °C. For cell surface staining, cells were treated for 30 min at 4 °C with appropriate combinations of specific antibodies for surface staining of CD45, CD31, EGFR, or ADAM17. The corresponding isotype controls or secondary antibodies only were stained in parallel. For intracellular staining, cells were fixed with 0.5 ml of ice-cold 2% paraformaldehyde and permeabilized using 0.5% saponin (Sigma) prior to antibody staining with anti-AMCase or anti-pancytokeratin or the respective isotype controls. Lung epithelial cells were characterized according to the following gating algorithm. Within digested and strained red cell and macrophage-depleted whole lung cell suspensions, nondebris cells were gated, and $CD45^+$ cells were further excluded. Within $CD45^-$ negative lung cells, $CD31^+$ endothelial cells were excluded. Within the $CD45^-CD31^-$ lung cell population, cells positive for intracellular pancytokeratin were considered as epithelial cells according to a modified method, as described previously (21, 22). For co-expression studies of intracellular proteins (AMCase) with extracellular receptors (ADAM17 and EGFR), lung cells were first surface-stained with anti-EGFR or anti-ADAM17, were permeabilized, and then underwent staining for intracellular AMCase. Saturating concentrations of the antibodies were used, as determined by titration experiments prior to the study. At least 10,000 cells/sample were analyzed. All antibodies and fluorescence-activated cell sorting reagents were from BD Biosciences except when otherwise indicated. Isotype controls were subtracted from the respective specific antibody expression, and the results are reported as mean fluorescence intensity. Calculations were performed with Cell Quest analysis software (BD Biosciences). All experiments were performed in triplicate.

IL-13-overexpressing Mice—C57BL/6 wild type (WT) were obtained from the Jackson Laboratories (Bar Harbor, ME). CC10-rtTA-IL-13 transgenic mice were generated in our laboratory (23), bred onto a C57BL/6 background, and used in these studies. These mice utilize the Clara cell 10-kDa protein (CC10) promoter and the reverse tetracycline transactivator (rtTA) to target IL-13 to the lung in a doxycycline-inducible manner. These animals have very low to undetectable levels of IL-13 in their lungs at base line and 1.9–2.1 ng/ml quantities after transgene induction by adding doxycycline for 4 weeks to the animals' drinking water. When CC10-IL-13 mice were being

evaluated, Tg (−) littermate animals were used as controls. These studies were approved by the Yale University School of Medicine Institutional Animal Care and Use Committee.

OVA Sensitization and Challenge—OVA sensitization and challenge were accomplished using a modification of the protocols previously described by our laboratory (23). In brief, 6–8-week-old WT mice were received injections containing 20 μg of chicken OVA (Sigma) complexed to alum (Resorptar, Indergen, New York, NY) or alum alone. This process was repeated 5 days later. After an additional 7 days, the animals received three aerosol challenges (40 min a day, 3 days) with 1% OVA (w/v) in endotoxin-free PBS or PBS alone. The aerosol was generated in a NE-U07 ultrasonic nebulizer (Omron Health Care, Vernon Hills, IL). The mice were sacrificed 24, 48, or 72 h after aerosol exposure.

Expression of Recombinant AMCase and EGFR—Human AMCase full-length cDNA plasmid constructed in expression vector pcDNA3.1 was obtained from MedImmune (Gaithersburg, MD). Human AMCase cDNA was amplified using PCR with the following primers: hAMCase-L-upper, 5′-ATG GAG GCC GAA TTC ATG GTT TCT ACT CCT GAG AAC-3′; hAMCase-L-lower, 5′-ATC TGC AGA ATT CCA CAT TGC CCA GTT GCA GCA ATC-3′. The wild-type human full-length EGFR cloned into the retroviral LXSN vector was a gift from David Stern (Yale University, New Haven, CT). The plasmid pCMV-β-Gal (American Type Culture Collection, Manassas, VA) was used to monitor transfection efficiency. A549 cells were incubated for 6 h with vector DNA mixtures that did not contain pcDNA 3.1 or contained AMCase or EGFR inserts. After incubation, the cells were washed and incubated for an additional 18 or 42 h in complete medium for immunoprecipitation assay and in Opti-MEM medium for bioactivity assay. Under these conditions, the transfection efficiency was found to be greater than 90% as determined by a β-galactosidase assay. The samples were centrifuged at 14,000 rpm in an Eppendorff microcentrifuge at 4 °C for 5 min, and the clear supernatants were saved. The presence of AMCase was verified by Western blotting. The activity of expressed AMCase was verified by an enzymatic assay as described below in detail.

Protein Extraction and Western Blot Analysis—Whole lung and cell monolayer lysates were evaluated by Western blotting. Lung lysates were prepared using lysis buffer as previously described. The cell monolayers were washed twice with ice-cold PBS containing 1 mM sodium orthovanadate and 1 mM sodium fluoride and lysed with lysis buffer (15 mM Hepes, pH 7.9, 10% glycerol, 0.5% Nonidet P-40, 250 mM NaCl, 0.1 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 10 mM dithiothreitol, and one tablet of complete miniprotease inhibitor mixture/10 ml of lysis buffer). The lysates were then clarified by centrifugation at 10,000 × *g* for 15 min, and supernatant protein concentrations were determined with a Bio-Rad assay kit. The samples were then mixed with an equal volume of 2× SDS-PAGE sample buffer (100 mM Tris-Cl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol) and heated in a boiling water bath, and equal amounts were loaded onto 12% SDS-polyacrylamide gels (Bio-Rad) and transferred to Immune-Blot polyvinylidene difluoride membrane (Bio-Rad). After transfer, the membranes were blocked for 1 h in nonfat dried milk, rinsed, incubated with the appropriate primary antibodies for 1.5 h at room temperature or overnight at 4 °C, washed, incubated with secondary antibody (diluted 1:1000–1:2000) for 1.5 h at room temperature, and washed in 0.05% Tween 20. Immunoreactive proteins were visualized using 20× LumiGLO Reagent and 20× peroxide according to the manufacturer's instructions (Cell Signaling Technology Inc., Beverly, MA). The membranes were exposed to BioMax MR film (Eastman Kodak Co.).

Immunoprecipitation—A549 cells (2×10^6/10-cm diameter dish) were transfected with 24 μg of DNA with 60 μl of Lipofectamine 2000. 48 h later, the cell monolayers were washed twice with ice-cold PBS containing 1 mM sodium orthovanadate and 1 mM sodium fluoride and lysed with lysis buffer mentioned above. The lysate was clarified by centrifugation for 10 min at 4 °C. AMCase and associated proteins were immunoprecipitated with rabbit anti-AMCase polyclonal antibody or anti-EGFR (Invitrogen), respectively, using the Catch and Release version 2.0 reversible immunoprecipitation system (Millipore). The precipitate was transferred to a polyvinylidene difluoride membrane and probed with monoclonal antibody against EGFR or AMCase followed by chemiluminescence detection. Parallel protein immunoblots of each sample were performed to confirm the expected expression of EGFR or AMCase constructs in the cells.

Immunofluorescence Staining and Confocal Microscopy—Immunohistochemistry of AMCase was performed as described by our laboratory previously (7), using a polyclonal rabbit anti-human AMCase or monocloncal rabbit anti-mouse antibody developed by MedImmune (Gaithersburg, MD) (171.204), with specificity for AMCase. The antibody was applied to the lung sections at a 1:100 dilution. To verify the specificity of the reactions, the rabbit antibody was incubated with AMCase-specific peptide (amino acids 428–446) at a 1:1 ratio for 2 h before being applied to the tissues. The mouse monoclonal antibody specificity was determined by ELISA against other murine chitinase family members (chitotriosidase, Ym1, Ym2, and YKL-40) and by comparison of the staining pattern with the rabbit antibody as described previously by our group (7). In all cases, BD Pharmingen™ Retrievagen A (pH 6) antigen retrieval solution was used for 20 min. The avidin/biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA) was selected to treat the section for two-color staining of lung tissue. For two-color fluorescence, secondary reagents included anti-mouse or anti-rabbit, either biotinylated or directly conjugated with Alexa-488 (Molecular Probes) or anti-rabbit Cy3 (Sigma). Tissues were mounted using VECTASHIELD mounting medium for fluorescence (Vector Laboratories, Inc., Burlingame, CA). For combined AMCase-EGFR staining, streptavidin Alexa-488 (green dye) was combined with Cy3 (red dye). Confocal microscopy was performed with a Zeiss LSM 510 META system with an optical thickness of either 5.0 or 1.0 μm.

Chitinase Bioactivity—The chitinase bioactivity in cell culture supernatants and cell lysates was determined using a fluorogenic substrate as described previously (24). Briefly, 50 μl of each sample was mixed with 30 μl of citrate/phosphate buffer

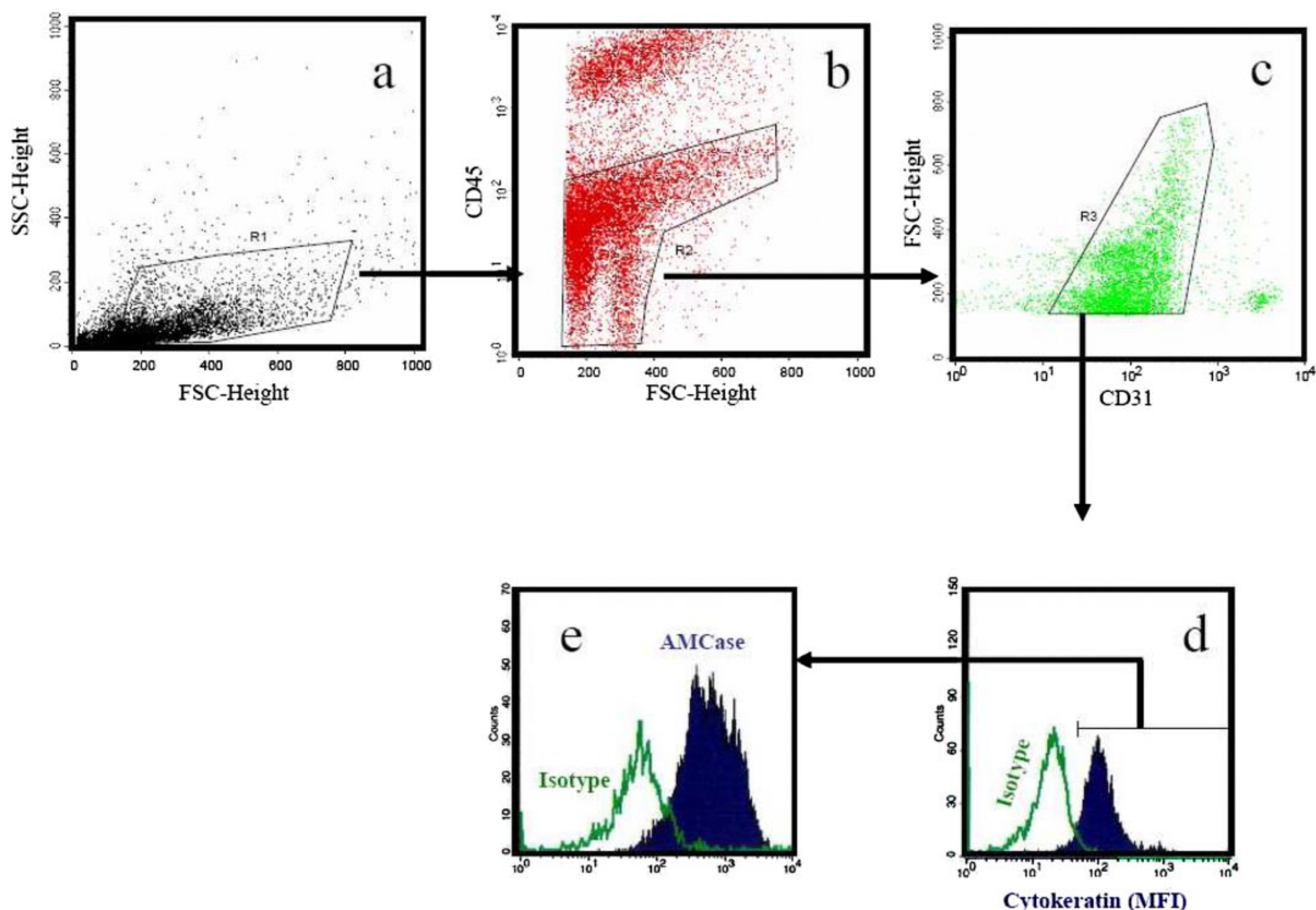

FIGURE 1. ***In vivo* analysis of lung epithelial cells.** Lung epithelial cells were characterized according to the following gating algorithm. Within digested/strained and red cell and macrophage-depleted whole lung cell suspensions (for details, see "Experimental Procedures"), nondebris cells were gated (*a*), and hematopoietic ($CD45^+$) cells were further excluded (*b*). Within $CD45^-$ negative lung cells, $CD31^+$ endothelial cells were excluded (*c*). Within the $CD45^-CD31^-$ lung cell population, cells positive for intracellular pancytokeratin (*d*) were considered as epithelial cells according to a modified method, as described previously (21, 22). Intracellular AMCase expression was robustly detectable in pancytokeratin$^+$ cells (*e*).

(0.1 and 0.2 M, respectively), pH 5.2, and 20 μl of 0.5 mg/ml substrate 4-methylumbelliferyl-D-*N*,*N'*-diacetylchitobioside (Sigma) at a final concentration of 0.17 mM. The samples were incubated at 37 °C for varying amounts of time, and the reactions were stopped by adding 1 ml of stop solution (0.3 M glycine/NaOH buffer, pH 10.6). The fluorescence intensity of released 4-methylumbelliferone was measured with a fluorometer (excitation 350 nm and emission 450 nm). A standard curve was generated using serially diluted 4-methylumbelliferone (Sigma). Chitinase extracted from *Serratia marcescens* (Sigma) was used as a positive control.

Cytokine and Chemokine ELISA—Immunosandwich enzyme-linked immunosorbent assay kits for IL-6, MCP-1/CCL2, IL-8/CXCL8, and TARC/CCL17 were used according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Statistics—All data were initially checked for normal/parametric distribution (Kolmogorov-Smirnov test). If parametric distribution was found, analysis of variance was applied to screen for differences among at least three groups. To compare two individual groups, Student's *t* test was applied. If nonparametric distribution was found, the Kruskal-Wallis test was applied to screen for differences among at least three groups, followed by the Mann-Whitney *U* test (Wilcoxon rank sum test) to compare two individual groups. Statistical analyses were performed using Prism 4.0 (Graph Pad Software) and STATA version 8.2 for Windows (STATA Corp.) according to the approach described in Ref. 25.

RESULTS

AMCase, EGFR, and ADAM17 Are Induced and Co-localize in Vivo at Sites of Th2 and Th2-Cytokine-mediated Airway Inflammation—To characterize and quantify intracellular AMCase expression in lung epithelial cells *in vivo*, we established a flow cytometric epithelial evaluation method based on light scatter parameters, negative expression of CD45, negative expression of CD31, and positive expression of cytokeratin (Fig. 1). Using this method, we successfully compared the levels of intracellular AMCase in lung epithelial cells from control mice, mice sensitized and challenged with OVA, and IL-13-overexpressing transgenic animals. These studies demonstrated a significant increase in intracellular AMCase in both of the modeling systems (Fig. 2, *a–d*). The increase in intracellular AMCase in these Th2 inflammation/asthma models was paralleled by an

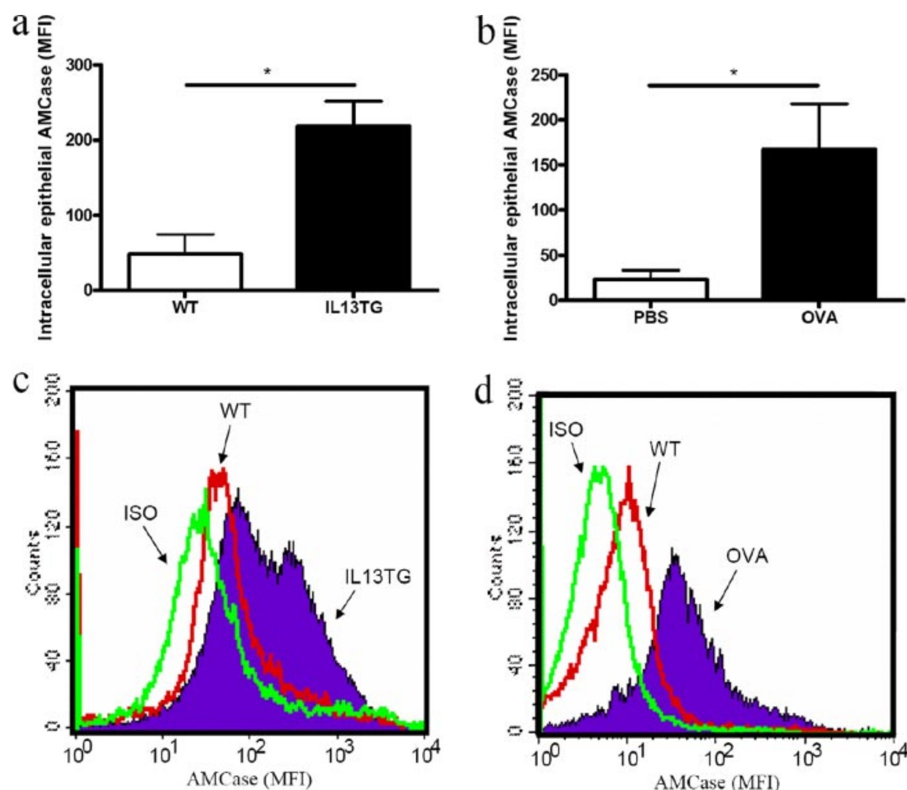

FIGURE 2. **Th2 airway inflammation increases AMCase expression in lung epithelial cells.** Intracellular AMCase was stained in permeabilized $CD45^-CD31^-cytokeratin^+$ lung epithelial cells in WT and IL-13-overexpressing transgenic mice (*IL 13 TG*) (*a* and *c*), PBS-sensitized and challenged mice (*PBS*), and OVA-sensitized and challenged mice (*OVA*) (*b*). *Bars*, means ± S.D. (*, $p < 0.05$; Student's *t* test). *c*, a representative histogram of intracellular AMCase expression in $CD45^-CD31^-cytokeratin^+$ cells. *Green line*, isotype (*ISO*) control; *red line*, AMCase expression in $CD45^-CD31^-cytokeratin^+$ cells from WT mice; *filled blue area*, AMCase expression in $CD45^-CD31^-cytokeratin^+$ cells from IL-13 Tg mice. *d*, a representative histogram of intracellular AMCase expression in $CD45^-CD31^-cytokeratin^+$ cells. *Green line*, isotype control; *red line*, AMCase expression in $CD45^-CD31^-cytokeratin^+$ cells from WT mice; *filled blue area*, AMCase expression in $CD45^-CD31^-cytokeratin^+$ cells from OVA-sensitized and challenged (*OVA*) mice. *MFI*, mean fluorescence intensity.

increase of total EGFR in lung lysates (Fig. 3*a*) as well as specific EGFR surface expression on lung epithelial cells as analyzed by immunohistochemistry (Fig. 3*b*) and flow cytometry (Fig. 3, *c* and *d*). Using confocal microscopy, AMCase and EGFR were found to co-localize in lung epithelial cells of lungs of IL-13 transgenic and OVA-sensitized and -challenged mice (Fig. 3*e*) (data not shown).

Similar to EGFR, our studies demonstrated that ADAM17 surface expression on lung epithelial cells was up-regulated in IL-13 transgenic mice (Fig. 3, *f* and *g*) and OVA-sensitized and challenged mice (Fig. 3*f*). In OVA-sensitized and saline-challenged or saline-sensitized and OVA-challenged mice, no induction of AMCase, EGFR, or ADAM17 was observed (data not shown). Further analyses using flow cytometry showed co-expression of AMCase, EGFR, and ADAM17 on lung epithelial cells in OVA-sensitized and -challenged and IL-13 transgenic mice on a single cell level (Fig. 4) (data not shown). When viewed in combination, these data demonstrate that AMCase, EGFR, and ADAM17 are concomitantly up-regulated in lung epithelial cells at sites of Th2/allergic inflammation *in vivo*, where they co-localize with each other.

EGFR Physically Interacts with AMCase—To determine if AMCase and EGFR physically interact with one another, lung epithelial cells (A549) were transfected with vectors driving the expression of full-length cDNA encoding these moieties, alone and in combination. The interaction of AMCase and EGFR was then evaluated using combined immunoprecipitation and immunoblotting protocols. These studies demonstrated that these molecules bind to each other in these cells, since antibodies against one consistently precipitated the other (Fig. 5).

EGFR Modulates AMCase Secretion—Studies were next undertaken to define the intracellular pathway(s) that epithelial cells use to secrete AMCase. In these experiments, A549 cells were transfected with recombinant human full-length AMCase. This resulted in the secretion of bioactive AMCase into the cell supernatant that could be appreciated after 24 h and was most prominent after 48 h of incubation (Fig. 6*a*). The co-transfection of these cells with EGFR enhanced AMCase activity in a dose-dependent manner (Fig. 6*a*). In contrast, transfection of EGFR without AMCase had no effect on supernatant chitinase activity (data not shown). Based on these findings, we hypothesized that EGFR signaling is required for the secretion of AMCase. To test this hypothesis, we evaluated the effects of exogenous EGF and small molecule EGFR kinase inhibitors on AMCase secretion in AMCase/EGFR-co-transfected A549 cells. The former demonstrated that exogenous EGF up to 5 ng/ml had no effect on AMCase secretion by these cells, whereas very high concentrations of EGF (>200 ng/ml) only caused slight but not statistically significant increases in AMCase elaboration (Fig. 6*b*) (data not shown). However, pharmacologic blockade of the ATP binding site of EGFR, which inactivates its downstream signaling, caused a significant decrease in the levels of supernatant AMCase (Fig. 6*b*). Importantly, the levels of intracellular AMCase were significantly increased in lysates from these EGFR inhibitor-treated A549 cells (Fig. 6*c*). This demonstrates that AMCase is produced but cannot leave the cell without EGFR signaling activity. In combination, these studies demonstrate that ligand-independent EGFR signaling plays a critical role in epithelial cell AMCase secretion.

ADAM17 and Ras Regulate AMCase Secretion—Because EGFR regulation of AMCase secretion was largely ligand-independent, we hypothesized that constitutive EGFR activation via protease-mediated liberation of membrane-bound ligands (11) plays a major role in this process. Since ADAM17 is a major

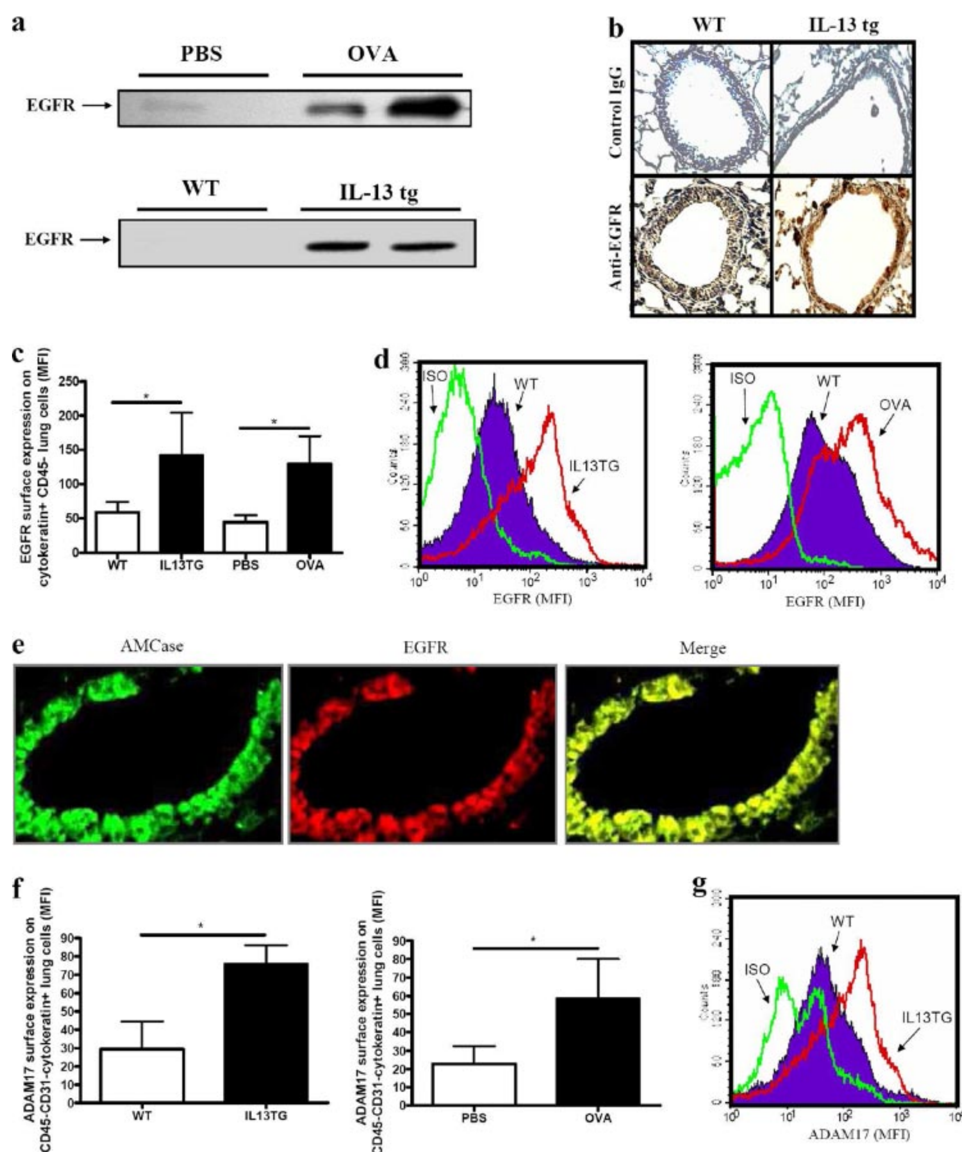

FIGURE 3. **Th2 airway inflammation increases EGFR and ADAM17 expression in lung epithelial cells.** *a*, immunoblotting of EGFR expression in whole lung cell lysates in PBS-sensitized and challenged (*PBS*) and OVA-sensitized and challenged mice (*OVA*) (*top*). WT and IL-13-overexpressing transgenic mice (*IL-13 tg*) are seen in the *lower panel*. *b*, EGFR expression in lung epithelial cells using immunohistochemistry in WT and IL-13-overexpressing transgenic mice. In the *upper panel* an isotype control IgG antibody was used instead of the specific anti-EGFR antibody. *c*, EGFR surface expression on CD45$^-$CD31$^-$cytokeratin$^+$ cells in WT and IL-13-overexpressing transgenic mice, PBS-sensitized and -challenged mice, and OVA-sensitized and -challenged mice. *Bars*, means ± S.D. (*, $p < 0.05$; Student's *t* test). *d*, representative histogram of EGFR surface expression on CD45$^-$CD31$^-$cytokeratin$^+$ cells. The *left panel* shows EGFR expression in lungs from IL-13-overexpressing transgenic mice, and the *right panel* shows expression in lungs from OVA-sensitized and -challenged (*OVA*) mice. *Green line*, isotype (*ISO*) control; *filled blue area*, EGFR expression on CD45$^-$CD31$^-$cytokeratin$^+$ cells of WT mice; *red line*, EGFR expression on CD45$^-$CD31$^-$cytokeratin$^+$ cells from IL-13-overexpressing transgenic mice. *e*, AMCase (Alexa-488, *green dye*) and EGFR (Cy3, *red dye*) were localized in tissues from IL-13-overexpressing transgenic mice. Confocal microscopy was performed with a Zeiss LSM 510 META system. Co-localization of AMCase and EGFR after merging was observed as a *yellow color* with confocal microscopy. *f*, ADAM17 surface expression was evaluated on CD45$^-$CD31$^-$cytokeratin$^+$ lung epithelial cells from WT and IL-13-overexpressing transgenic (*left*) mice, PBS-sensitized and challenged mice, and OVA-sensitized and challenged mice (*right*) ($p < 0.05$; Student's *t* test). *g*, representative histogram of ADAM17 expression on CD45$^-$CD31$^-$cytokeratin$^+$ cells. *Green line*, isotype control. The *filled blue area* and the *red line* illustrate the ADAM17 expression on CD45$^-$CD31$^-$cytokeratin$^+$ cells from WT and IL-13-overexpressing transgenic mice, respectively. *MFI*, mean fluorescence intensity.

regulator of EGFR activity by liberation of cell surface-bound EGFR ligands and subsequent transactivation of EGFR (14), studies were next undertaken to determine if inhibition of ADAM17 altered epithelial cell AMCase secretion. This was accomplished with the specific ADAM17 inhibitors TAPI-1 and TAPI-2. In these experiments, ADAM17 inhibition decreased AMCase secretion (Fig. 7). The effects of TAPI-1 an TAPI-2 were comparable with those that were seen with small molecule kinase inhibitors that blocked the ATP binding site of EGFR (PD153035) and interfered with EGFR tyrosine kinases (AG1478) (Fig. 7). In accord with these findings, inhibition of Ras, an oncogene downstream of EGFR, also decreased AMCase secretion (Fig. 7*a*), In contrast, inhibition of ERK did not alter AMCase secretion (Fig. 7). To further define the specificity of this finding, we used different doses of these pharmacological inhibitors and compared AMCase secretion with IL-6 secretion by A549 cells (Fig. 7*b*). These studies demonstrated that manumycin A, a natural inhibitor of Ras farnesyltransferase, inhibited AMCase secretion in a dose-dependent manner, whereas ERK inhibition had no effect at all doses tested (Fig. 7*b*, *top*). In contrast to AMCase secretion, IL-6 secretion was not affected by Ras inhibition (Fig. 7*b*, *bottom*). When viewed in combination, these studies demonstrate that lung epithelial cell AMCase secretion is selectively regulated via a pathway involving ADAM17, EGFR, and Ras.

AMCase Stimulates Epithelial Chemokine Production—Previous studies from our laboratory demonstrated that the *in vivo* inhibition of AMCase decreased aeroallergen-induced inflammatory cell accumulation in the airway (6). This prompted us to determine if AMCase stimulated epithelial cell chemokine production. To address this issue we compared the levels of MCP-1/CCL2, TARC/CCL17, and IL-8/CXCL8 in supernatants from A549 cells transfected with AMCase and control constructs and A549 cells incubated with varying concentrations of exogenous recombinant AMCase or vehicle control. In both experimental systems, AMCase stimulated epithelial cell production of these cytokines (Fig. 8, *a–d*). This stimulation was most prominent after 48 h of A549 cell-AMCase incubation. (Fig. 8 and data not shown). These studies demonstrate that the AMCase that is produced by epithelial cells feeds back in an auto-

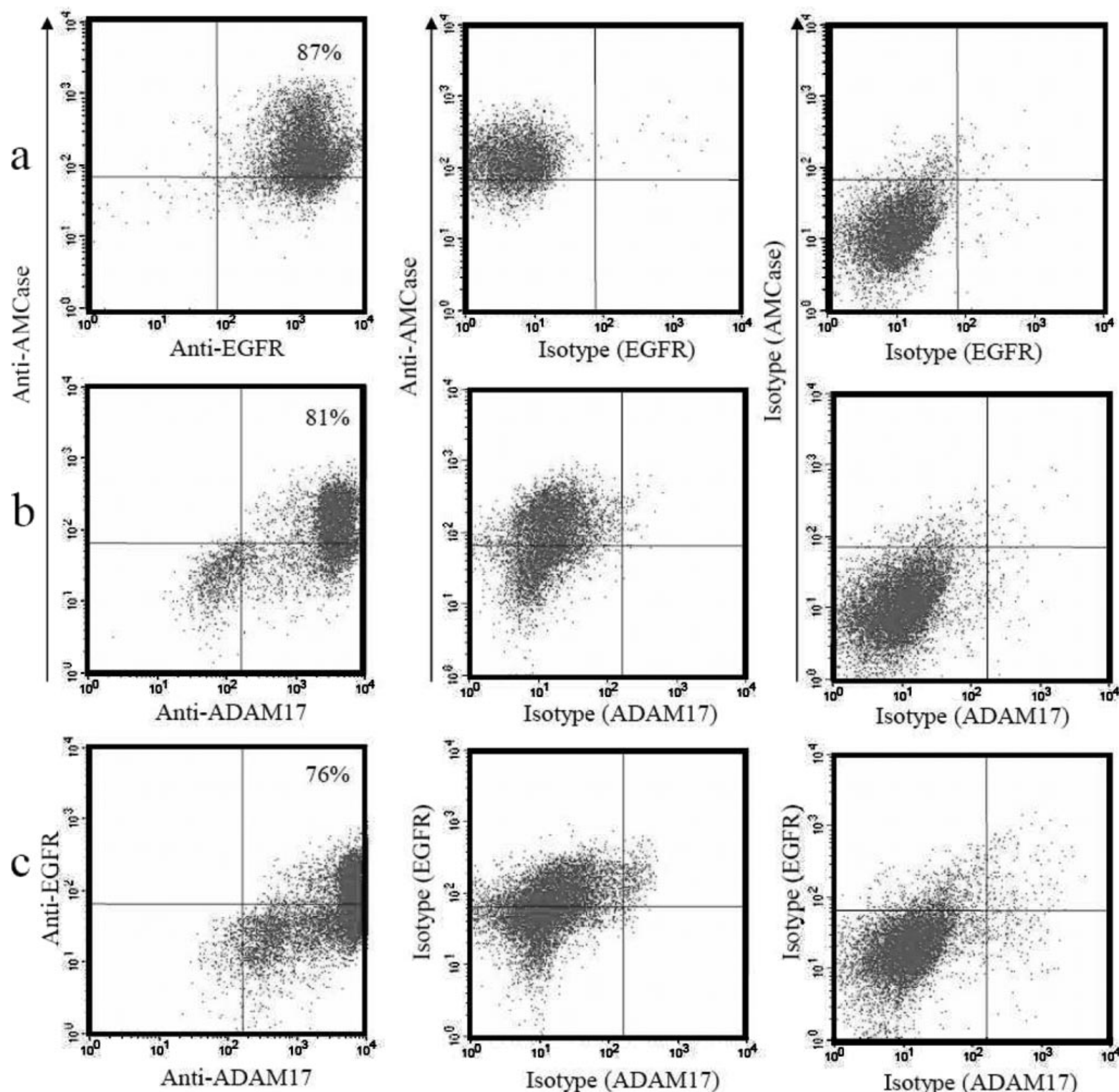

FIGURE 4. **AMCase, EGFR, and ADAM17 colocalize in lung epithelial cells.** AMCase, EGFR, and ADAM17 expression were quantified within $CD45^{-}CD31^{-}cytokeratin^{+}$ cells in IL-13-overexpressing transgenic mice. *a* and *b* show AMCase coexpression with EGFR (*a*) and ADAM17 (*b*), and *c* shows coexpression of EGFR with ADAM17. The respective isotype controls are depicted.

crine and/or paracrine manner to stimulate epithelial cell chemokine production.

DISCUSSION

AMCase is induced during and plays an important role in anti-parasite and Th2 responses (6, 26). Surprisingly, the molecular mechanisms controlling AMCase elaboration and its effector responses in these settings have not been adequately defined. To address these issues, we defined the mechanisms that regulate epithelial AMCase secretion and the epithelial regulatory effects that it has once it is elaborated. These studies demonstrate that AMCase physically interacts with EGFR and co-localizes with EGFR and ADAM17 and that epithelial AMCase secretion is regulated by an ADAM17/EGFR/Ras-dependent pathway. They also demonstrate that the AMCase that is produced feeds back in an autocrine and/or paracrine manner to induce epithelial chemokine elaboration.

EGFR has been strongly implicated in the pathogenesis of Th2 tissue inflammation via its ability to regulate goblet cell metaplasia, mucin synthesis, and epithelial cell proliferation, differentiation, and survival (12, 27–29). Recent studies demonstrated that EGFR signaling mediates allergen-induced chemokine (TARC/CCL17) release by lung epithelial cells (30). EGFR signaling can be triggered by EGF as well as TGF-α, heparin binding epidermal growth factor, and amphiregulin (31). These latter ligands are synthesized as

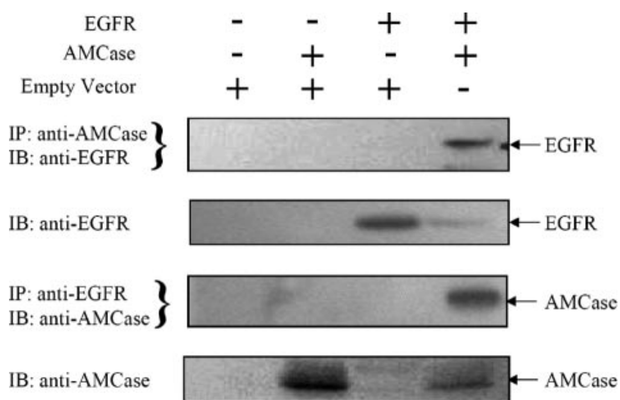

FIGURE 5. **Physical interaction of EGFR and AMCase.** A549 cells were transfected with empty vector, the indicated AMCase or EGFR expression plasmids, or both. In *rows 1* and *3* (from the *top*), cell lysates (200 μg of protein) were subjected to immunoprecipitation (*IP*) with anti-AMCase or anti-EGFR antibodies as noted, and the resulting immune complexes (total sample from a single precipitation) were size-fractionated on a denaturing 10% polyacrylamide gel. These proteins were transferred to polyvinylidene difluoride, and the EGFR or AMCase proteins were detected by Western blotting (*IB*) using the indicated antibody. In *rows 2* and *4*, cell lysates (50 μg) were size-fractionated on a denaturing 10% polyacrylamide gel, transferred to polyvinylidene difluoride, and evaluated via Western blotting using the noted antibodies.

transmembrane proforms and are processed to their mature, receptor-activating forms by ADAM proteases, such as ADAM17/TACE (14). ADAM17 acts as a sheddase where it processes and thereby regulates a variety of mediators and receptors, including EGFR transactivation (14), ectodomain shedding of TNF-α and TNFI and TNFII (TNF receptors I and II, respectively) (32), platelet glycoproteins (33), neurotrophin receptors (34), L-selectin (35), angiotensin-converting enzyme-2 (36), and fractalkine (37). ADAM17 activity in pulmonary epithelial cells has been reported to be induced by IL-13 (19, 30), and TACE/ADAM17 processing of EGFR ligands has been found to play a critical role in IL-13-induced airway responses and proliferation of epithelial cells (19, 30). Previous studies from our laboratory demonstrated that IL-13 stimulates epithelial AMCase production, that AMCase accumulates in the cytoplasm of distal airway epithelial cells in a distinctly granular manner, and that AMCase plays a critical role in the pathogenesis of IL-13-induced tissue alterations (6, 7). This caused us to hypothesize that EGFR and AMCase interact with each other and that ADAM17/EGFR-dependent pathways are involved in

FIGURE 6. **Functional interactions of EGFR and AMCase.** *a*, cultured A549 cells were transfected with empty vector, the indicated quantity of AMCase or EGFR expression plasmid, or both. The amount of EGFR that was transfected is expressed in relation to the amount of AMCase that was employed. EGFR and AMCase were used at 1:1 (*1*), 2:1 (*2*), and 3:1 (*3*) ratios. Chitinase activity was measured in supernatants from cells incubated for 24 and 48 h as noted. *Bars*, means ± S.D. (*, $p < 0.05$; **, $p < 0.01$; Student's *t* test). *b*, A549 cells were transfected with empty vector (*AMCase* −) or AMCase (*AMCase* +) and were treated with recombinant human EGF at the indicated concentrations or with the EGFR inhibitor PD153035. Supernatant chitinase activity was measured after 48 h in culture (**, $p < 0.05$, PD153035-treated *versus* -nontreated AMCase-transfected cells; Student's *t* test). *c*, A549 cells were transfected with empty vector or AMCase and were treated with human EGF at the indicated concentrations or with the EGFR inhibitor PD153035. Cell lysate chitinase activity was measured after 48 h of incubation. AMCase and β-actin were evaluated in cell lysates by Western blotting (*, $p < 0.05$; Student's *t* test).

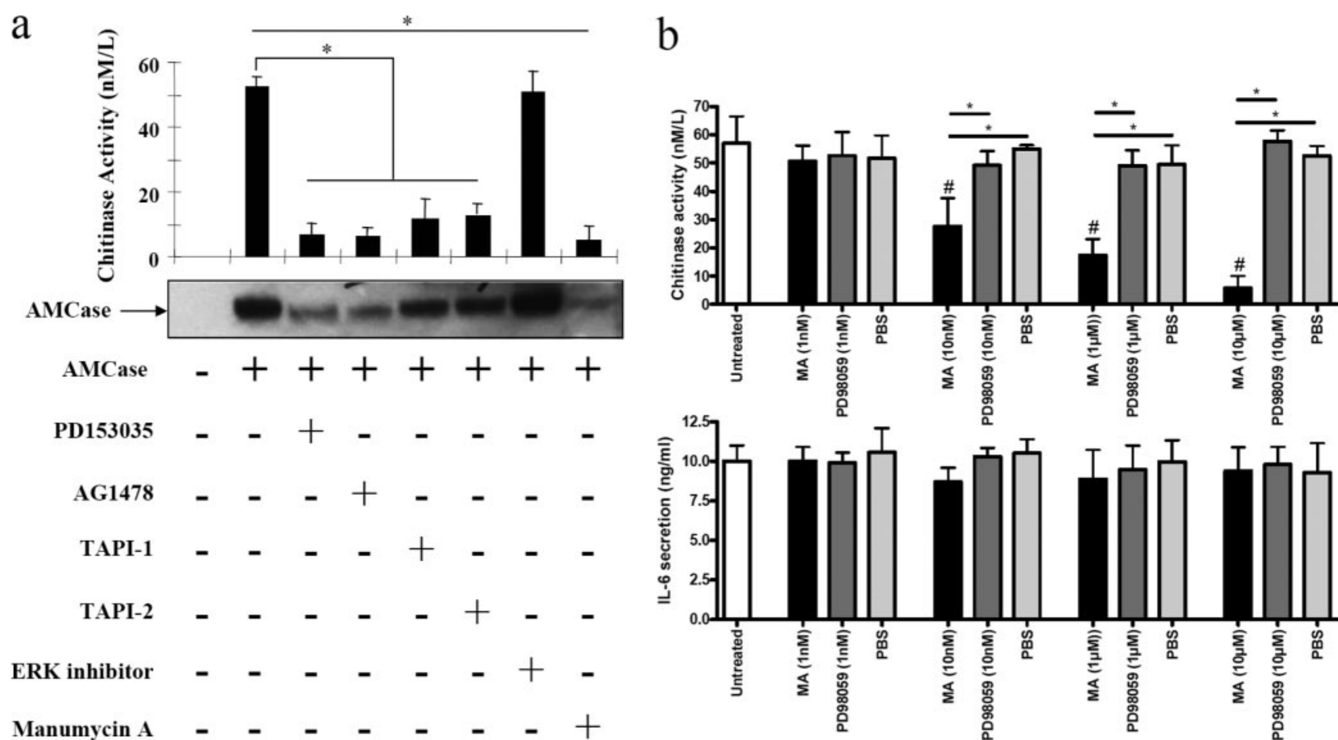

FIGURE 7. **ADAM17 and Ras modulate AMCase secretion.** *a*, A549 cells were transfected with empty vector (*AMCase* −) or AMCase (*AMCase* +) and were treated with the EGFR inhibitors PD153035 (1 μM) or AG1478 (1 μM), the ADAM17 inhibitors TAPI-1 (25 μM) and TAPI-2 (25 μM), the ERK inhibitor PD98059 (10 μM), or the Ras/Raf inhibitor manumycin A (1 μM). Supernatant chitinase activity was measured after 48 h of cell culture (*, $p < 0.05$ chitinase activity in supernatants of untreated *versus* inhibitor-treated AMCase-transfected A549 cells; Mann-Whitney *U* test). Supernatant AMCase was evaluated by Western blotting. *b*, A549 cells were transfected with AMCase and were treated with manumycin A (*MA*) (at the indicated doses; 1 nM to 10 μM), PD98059 (1 nM to 10 μM), or PBS. Supernatant chitinase activity (*top*) and IL-6 levels (*bottom*) were measured after 48 h of cell culture (*, $p < 0.05$, Mann-Whitney *U* test; #, $p < 0.05$, Mann-Whitney *U* test *versus* untreated).

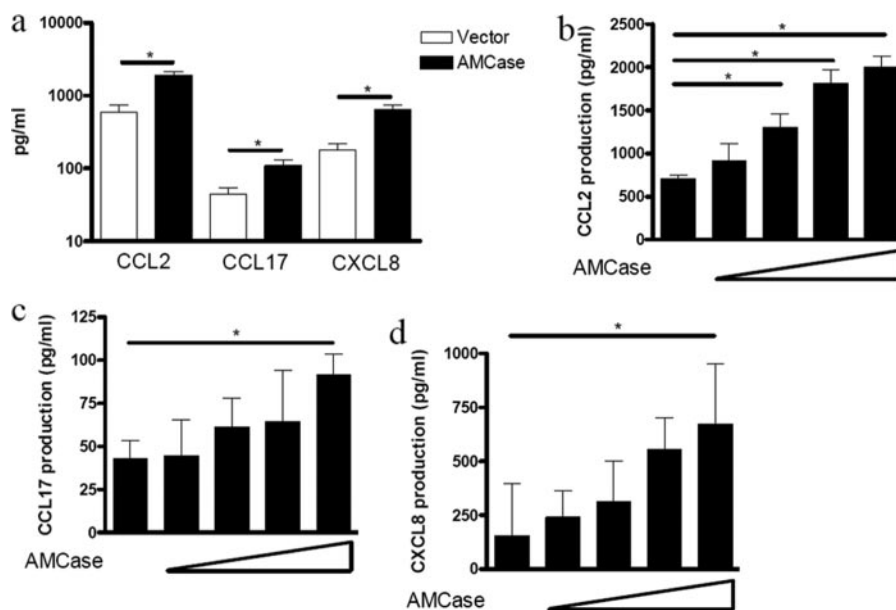

FIGURE 8. **AMCase induces chemokine production by lung epithelial cells.** *a*, CCL2/MCP-2, CCL17/TARC, or CXCL8/IL-8 levels were quantitated in supernatants of vector-transfected (*white bars*) or AMCase-transfected (*black bars*) A549 cells. *Bars*, means ± S.D. *, $p < 0.05$; Student's *t* test). *b–d*, CCL2/MCP-1 (*b*), CCL17/TARC (*c*), or CXCL8/IL-8 levels (*d*) were quantitated in supernatants of A549 cells treated for 48 h at 37 °C with increasing doses of AMCase (10 ng/ml, 100 ng/ml, 1 μg/ml, and 10 μg/ml). *, $p < 0.05$; Student's *t* test.

the secretion of AMCase by epithelial cells. Our data support this hypothesis in a number of ways. First, they demonstrate that the IL-13-induced increase in lung epithelial cell AMCase expression is paralleled by a similar increase of EGFR and ADAM17 expression and that all three proteins colocalize in lung epithelial cells *in vitro* and *in vivo*. They also demonstrate that AMCase and EGFR physically interact with each other. Last, they demonstrate that inhibition of EGFR phosphorylation, ADAM17 inhibition, or inhibition of the EGFR downstream signaling molecule Ras impair epithelial cell AMCase secretion, whereas inhibition of ERK had no effect. This effect was not due to general inhibition of the cell secretion apparatus, since secretion of IL-6 was unaffected by inhibition of this pathway. When viewed in combination, these data tempt us to speculate that the following scenario is taking place in allergic/Th2 inflammation. IL-13, while inducing AMCase production, concomitantly activates ADAM17 on pulmonary epithelial cells (13, 19). ADAM17 transactivates EGFR on epithelial cells, which then in turn triggers a downstream signaling

cascade involving Ras, thereby facilitating the trafficking and secretion of AMCase (11, 38–40). The involvement of Ras in AMCase secretion is in accord with its known roles in exocytosis and vesicular trafficking (41, 42). EGFR may also participate in the trafficking of intracellular AMCase, since EGFR is well known to be internalized and undergoes further endosomal processing and recycling (43–45). Our demonstration that AMCase and EGFR physically bind to each other supports this possibility. Further support for this hypothesis can be seen in recent data from our laboratory that demonstrate that the CAML (calcium modulator and cyclophilin ligand) protein, which is required for appropriate EGFR recycling (46), binds AMCase intracellularly.[4]

Studies from our laboratory and others have demonstrated that 18 glycosyl hydrolase proteins, including AMCase, BRP-39 (murine breast regression protein 39), and YKL-40 (the human homologue of BRP-39), are stimulated during parasite infections and Th2 inflammation (4, 6, 47). To understand the processes that are involved in these important responses, the biologic repertoires of these moieties have begun to be investigated. These studies demonstrated that AMCase is an important regulator of responses to chitin-containing pathogens and contributes to tissue inflammation in chitin-free situations (6, 26). The present studies add to our understanding of the biology of these responses by demonstrating that epithelial cell AMCase secretion is regulated by an EGFR/ADAM17/Ras-dependent mechanism and that AMCase feeds back in an autocrine and/or paracrine manner to induce epithelial chemokine production. The EGFR-related observations raise the intriguing possibility that many of the important roles of EGFR in Th2 responses are the result of EGFR regulation of AMCase secretion. The chemokine findings also provide an intriguing explanation for the decrease in bronchoalveolar lavage and tissue inflammation that was seen when free AMCase was neutralized in the ovalbumin sensitization and challenge asthma model (6). Last, an appropriate anti-parasite response needs to rapidly attack the pathogen and induce an inflammatory response that is powerful enough to control the invading agent. The present studies and studies from others (26) suggest that AMCase contributes to both of these responses, with the chitinolytic activity of AMCase mediating anti-parasitic effects and the ability of AMCase to stimulate epithelial cell chemokines (15) contributing to the induction of tissue inflammation. Further experimentation will be required to evaluate the validity of this mechanistic construct.

In summary, these studies provide novel insights into the secretion and effector functions of AMCase. They demonstrate that AMCase colocalizes and physically interacts with EGFR and that AMCase secretion is mediated via an ADAM17/EGFR/Ras-dependent pathway. They also demonstrate that the AMCase that is secreted stimulates epithelial chemokine production. These findings will help to clarify the utility of AMCase-based interventions in the treatment of allergic inflammation as well as other Th2-dominated disorders.

[4] C. W. He and J. A. Elias, manuscript in preparation.

Acknowledgment—We thank Medimmune (Gaithersburg, MD) for antibodies and reagents.

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