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ADAM33 Is Not Essential for Growth and Development and Does Not Modulate Allergic Asthma in Mice

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A disintegrin and metalloprotease 33 (ADAM33) is a transmembrane protease and integrin ligand that has been identified as an asthma susceptibility gene product. To determine whether ADAM33 plays important roles in mammalian development and the modulation of allergic airway dysfunction, we generated ADAM33-null mice by gene targeting. ADAM33-null mice were born at expected Mendelian ratios, and both male and females developed normally and were fertile. No anatomical or histological abnormalities were detected in any tissues. In an animal model of allergic asthma, ADAM33-null mice showed normal allergen-induced airway hyperreactivity, immunoglobulin E production, mucus metaplasia, and airway inflammation. Our results demonstrate that ADAM33 is not essential for growth or reproduction in the mouse and does not modulate baseline or allergen-induced airway responsiveness.

A disintegrin and metalloproteases (ADAMs) are a large family transmembrane proteases that are conserved from *Caenorhabditis elegans* to human. The family is defined by the presence of a metalloprotease domain, several epidermal growth factor (EGF)-like repeats, a disintegrin domain, and a transmembrane domain (6). Knockouts and induced and spontaneous mutations of ADAM family members have demonstrated a wide variety of functions for these proteins in development and disease. About half of the ADAMs contain a catalytic-site consensus motif for metalloprotease activity. Several of them have been shown to play a crucial role in protein ectodomain shedding. The best-characterized ADAM sheddase is ADAM17, which exhibits proteolytic activity towards tumor necrosis factor alpha, transforming growth factor α, HB-EGF (heparin-binding EGF-like growth factor), and other substrates. ADAM17 deficiency causes perinatal lethality in mice and induces a phenotype resembling that of mice homozygous for knockout of EGF receptor, transforming growth factor α, or HB-EGF (20, 32). ADAM10 knockout mice die during embryogenesis due to multiple defects in the central nervous system, somites, and cardiovascular system (17). ADAM12-deficient mice have reduced adipocytes and a defect in myogenesis (23), which may be due to processing of insulin binding growth factors 3 and 5 by ADAM12. ADAM19-deficient mice have defects in cardiovascular morphogenesis (24, 42). Less is known about the functions of most ADAMs that lack a metalloprotease catalytic site. However, fertilin α (ADAM1) and fertilin β (ADAM2), which are the two subunits of the heterodimeric sperm protein fertilin, play important roles in fertilization (7, 8, 11). Male mice deficient for ADAM3 are also infertile (36). ADAM23 is crucial for brain development (26). ADAM11-null mice have defects in learning and motor coordination (38). ADAM22-deficient mice display ataxia and die before weaning (35).

ADAM33, one of the more recently described family members, contains both a functional protease and an effective integrin binding domain. ADAM33 belongs to the ADAM12, -13, and -19 subfamily (16). The recombinant ADAM33 protein has been shown to have a functional metalloprotease domain (15). The ADAM33 catalytic domain has been crystallized (30). The catalytic site has the typical matrix metalloproteinase Zn^{2+} binding site. The structure of the entrance of the substrate pocket is unique to ADAM33, which could be the target of specific inhibitors. The ADAM33 disintegrin domain can interact with the integrin α9β1 (10). ADAM33 appears to be widely expressed (16, 41), but no studies to date have directly examined its functions in vivo.

On the basis of a genome-wide linkage analysis and both family-based and case control association studies, polymorphisms in the ADAM33 gene have been implicated in human asthma (39). Expression of ADAM33 in airway smooth muscle has led to the hypothesis that this protein plays a role in modulating the development or contraction of airway smooth muscle and thus contributes to the development of asthma. However, thus far, no functionally important disease-causing sequence variants have been identified in ADAM33, and some follow-up genetic association studies using the same polymorphisms evaluated in initial reports have been negative (5, 27, 34). We therefore decided to examine possible roles for ADAM33 in mammalian development and in models of asthma by generating mice homozygous for a null mutation of ADAM33. These mice are born and develop normally, are fertile, and have no evidence of morphological abnormalities in any organs up to 8 months of age. The mice also have normal airway narrowing in response to pharmacological bronchoconstriction and have normal morphological and functional responses to allergen sensitization and challenge. These mice should be a useful tool to probe potential roles for ADAM33 in other murine models of disease.

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MATERIALS AND METHODS

Targeting vector construction and generation of ADAM33-deficient mice.

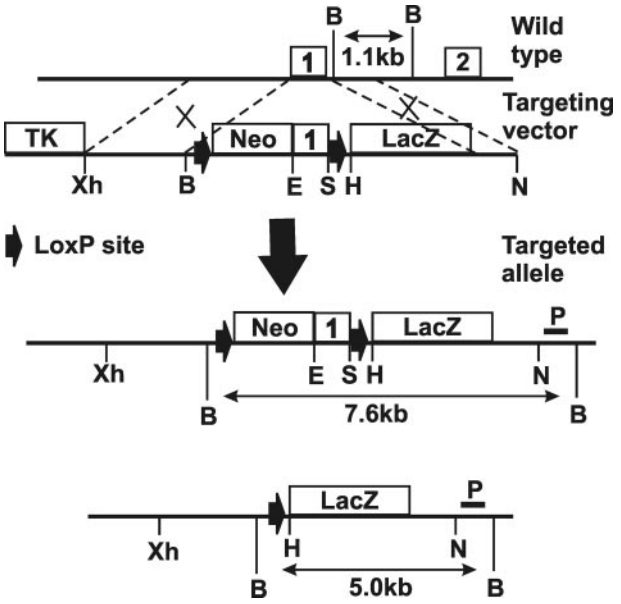

FIG. 1. Mouse ADAM33 locus, targeting vector, floxed allele, and knockout allele. The upper line shows a map of the ADAM33 locus with the first two exons defined by white boxes. The BamHI (B), XhoI (Xh), EcoRI (E), SalI (S), HindIII (H), and Not (N) restriction sites used for generation and analysis of the construct are indicated. Probe P was used for Southern blot analysis. The diagnostic BamHI restriction fragments (1.1 kb, 7.6 kb, and 5.0 kb) are shown with lines with double arrowheads. TK, thymidine kinase.

ADAM33 genomic DNA was amplified by three PCRs from mouse 129/SVJae genomic DNA. A 3.4-kb left targeting arm was obtained with two primers (5′-GCAATCTCGAGGTAATTCAAGCACAATATATACACAT and 5′-GCGGATCCTAGTGACATCTGGATTCTCCTTTCTAC) containing the XhoI and BamHI restriction sites, respectively. A 676-bp PCR product including the first exon and 325 bp of the 5′ nontranscript region was amplified with primers (5′-GCGAATTCAATCGATGTAGAAAGGAGAATCCAGATGTCACTA and 5′-GCGTCGACCTATTTGCTTTGCTAAGTAAGTGTGG). A 1.0-kb right targeting arm was obtained with primers (5′-GCAGATCTTTTAACCTCTCAGGAAGGCTTCTCTGTCCT and 5′-GCGCGGCCGCACTAGTATTTTTAGACTGGGTAGTAG) containing the BglII and NotI restriction sites. The cDNA encoding LacZ was cut from the pSV-β-galactosidase vector (Promega, Madison, WI) by HindIII and BamHI and ligated 5′ of the right arm. These three DNA fragments (left arm, right arm with LacZ cDNA, and exon 1-containing DNA) were subcloned into the pJB1 vector (21) as shown in Fig. 1. The targeting construct was linearized with NotI and electroporated into RF8 embryonic stem (ES) cells (provided by Robert V. Farese from Gladstone Institute of Cardiovascular Disease [29]). Genomic DNA isolated from 500 G418-resistant ES cell colonies was analyzed by Southern blotting. Two correctly targeted ES cell clones were identified and injected into blastocysts to generate chimeras and gave rise to two independent lines. Chimeras were mated to C57BL/6 females, and the resulting heterozygous animals were designed as ADAM33 Fl/+, since the neo and first exon are flanked by two LoxP sites. ADAM33 Fl/+ mice were subsequently mated to Tie-2 Cre females (provided by R. Wang from UCSF). The expression of Cre DNA recombinase in the female germ line mediated DNA recombination between the two LoxP sites (22). The neomycin resistance gene and the exon 1 for ADAM33 were removed from genomic DNA to generate ADAM33-heterozygous mice (ADAM33$^{+/-}$). These ADAM33-heterozygotes were mated with 129/SVJae wild-type mice for five generations. ADAM33 ±/129SVJae were bred to generate wild-type, ADAM33$^{+/-}$, and ADAM33$^{-/-}$ animals for subsequent analyses.

PCR and Southern blot analyses. Genomic DNA isolated from ES cells was digested with BamHI, separated through a 0.8% agarose gel with Tris-borate-EDTA buffer at pH 7.5, and transferred onto Zeta-Probe GT membrane (Bio-Rad, Hercules, CA). Probe P (Fig. 1), outside the targeting region, was used for genotyping. The probe was labeled with digoxigenin-dUTP with a digoxigenin DNA-labeling kit (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instructions. Hybridization of BamHI-cut DNA with the 0.6-kb P probe resulted in a band of 1.1 kb for the wild-type allele, whereas the correctly targeted mutant allele resulted in a band of 7.6 kb (Fig. 1). The P probe fragment was obtained by PCR within ADAM33 intron 1 using primers PF1 (5′-TGCCTCCTTCTTCCCAGCCCCTCTG) and PR1 (5′-GTAGCACTTCCAGGATTTGGTATTC).

Standard PCR was also used to test genotype from mouse tail DNA. PCR primers F1 (5′-TCAACACGAAAGCAATGTCC) and R2 (5′-GTTGTAAAACGACGGGATCG) were used to generate a 630-bp fragment detect the ADAM33-null allele. PCR primers F1 and R1 (5′-TGGCGTTATCTGCAACAATC) detect the wild-type allele as a 440-bp fragment.

RT-PCR. Total RNA was extracted from freshly isolated mouse tissue using TRIzol solution (Invitrogen, Carlsbad, CA) according to the company's recommendations. Single-stranded cDNA was transcribed from RNA using a Superscript cDNA synthesis system and random hexamers (Invitrogen, Carlsbad, CA). PCR was performed with the ADAM33-specific primers mADAM33-RTF (5′-CCACTACAGGCCAGATGGGCATC) and mADAM33-RTR (5′-GAGAATCTGGTCCACGCAAT) to show that the transcription of ADAM33 was disrupted in ADAM33 knockout mice. Beta actin primers (5′-GGTACCACCATGTACCCAGG and 5′-ACATCTGCTGGAAGGTGGAC) were used as a positive control for reverse transcription-PCR (RT-PCR).

Histology analyses. For histology, mouse tissues were fixed in 10% formalin for 48 h and embedded in paraffin. Sections (5 mm thick) were stained with hematoxylin and eosin (H&E).

Sensitization and challenge. Six- to eight-week-old sex-matched (each group had five male and five female mice) ADAM33$^{-/-}$ and littermate control mice were sensitized on days 0, 7, and 14 by intraperitoneal injection of 50 μg ovalbumin (OVA) (Sigma-Aldrich) emulsified in 10 mg of aluminum potassium sulfate in a total volume of 200 μl. Control animals received an equal volume of aluminum potassium sulfate. Subsequently, lightly anesthetized mice (isoflurane inhalation) were intranasally challenged with ovalbumin (1 mg in 50 μl of saline) or with saline alone on days 21, 22, and 23.

OVA-specific IgE assay. Sera were obtained from blood collected by cardiac puncture from antigen- or vehicle-treated mice after airway responsiveness measurements. OVA-specific immunoglobulin E (IgE) levels were measured by enzyme-linked immunosorbent assay using microplates coated with OVA. Diluted serum samples were added to each well, and the bound IgE was detected with biotinylated anti-mouse IgE (R35-118; Pharmingen). Color development was achieved using streptavidin-conjugated horseradish peroxidase (Pharmingen) followed by addition of horseradish peroxidase substrate (TMB; BD Biosciences Pharmingen). Optical density readings of samples at 450 nm were obtained, and the results were expressed as optical density values.

Measurement of airway response to acetylcholine. Twenty-four h after the last challenge, mice were anesthetized with ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg). A tracheostomy was performed, and a tubing adaptor (20 gauge) was used to cannulate the trachea. The mice were then attached to a rodent ventilator and pulmonary mechanics analyzer (FlexiVent; SIRAQ Inc, Canada) and ventilated at a tidal volume of 9 ml/kg, a frequency of 150 breaths/minute, and 2 cm H_2O positive end-expiratory pressure. Mice were paralyzed with pancuronium (0.1 mg/kg intraperitoneally). A 27-gauge needle was placed in the tail vein, and measurements of airway mechanics were made continuously using the forced oscillation technique. Mice were given increasing doses of acetylcholine (0.03, 0.1, 0.3, 1, and 3 μg/g of body weight) administered through the tail vein to generate a concentration-response curve.

Assessment of pulmonary inflammation and mucus production. Lungs were subjected to lavage five times with 0.8 ml of phosphate-buffered saline (PBS). After centrifugation (1,000 rpm, 5 min), the cell pellet was resuspended in normal saline after lysis of red blood cells. Total cells were counted with a hemacytometer. Cytospin preparations were prepared and stained with a HEMA 3 stain set (Fisher), and bronchoalveolar lavage fluid cell differential percentages were determined based on light microscopic evaluation of >300 cells/slide.

After lavage, lungs were inflated with 10% buffered formalin to 25 cm H_2O of pressure and transferred into tubes containing 10% buffered formalin. Multiple paraffin-embedded 5-μm sections of the entire mouse lung were prepared and stained with H&E for regular morphology and with periodic acid-Schiff for evaluation of mucus production.

RESULTS

Generation of ADAM33 knockout mice. An ADAM33-targeting vector was designed to delete the whole exon 1 including the 5′ untranslated region and the first 33 codons (Fig. 1). Correctly targeted alleles were detected by Southern blotting

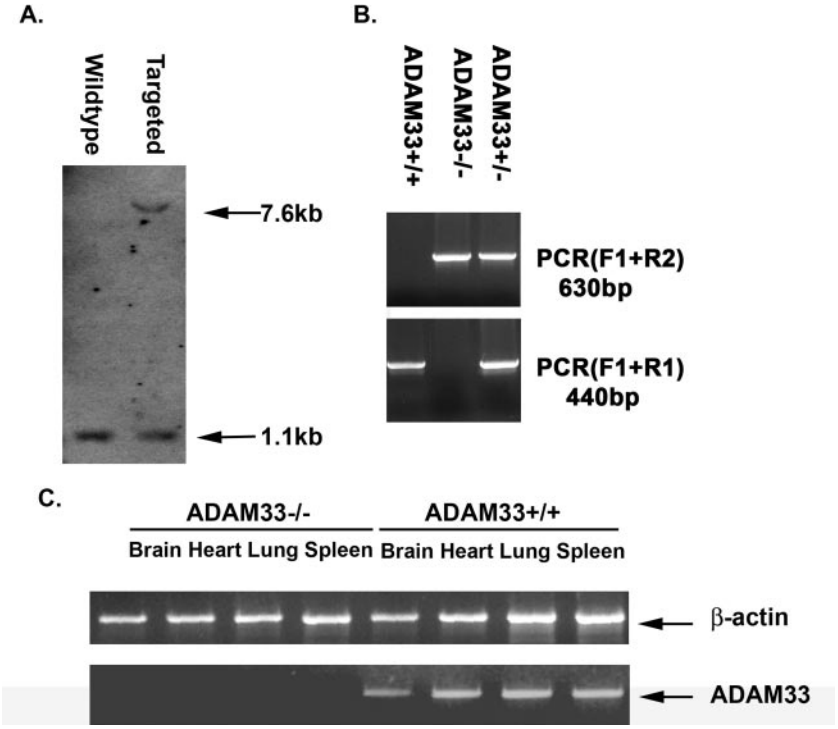

FIG. 2. (A) Southern blot analysis of wild-type and floxed allele heterozygous ES cell clones. DNA from ES clones was digested with BamHI, transferred onto a membrane, and hybridized with digoxigenin-labeled probe P. The predicted bands are seen for each genotype. (B) PCR analysis of tail DNA from ADAM33 wild-type ($ADAM33^{+/+}$), ADAM33-heterozygous ($ADAM33^{+/-}$), and ADAM33 knockout ($ADAM33^{-/-}$) mice. PCR with plasmids F1 and R2 [PCR(F1+R2)] generated the expected 630-bp product from the ADAM33 knockout allele. PCR with plasmids F1 and R2 generated the expected 440-bp product from the wild-type allele. (C) Analysis of ADAM33 RNA expression. RT-PCR amplification of ADAM33 from various organs isolated from wild-type and ADAM33 knockout mice. Amplification of actin was used as an internal positive control.

of ES cells (Fig. 2A). After confirmation of ADAM33 Fl/+ mice, we crossed them with Tie2-Cre female mice, which have Cre recombinase expression in oocytes (22). The generation of $ADAM33^{+/-}$ was confirmed by PCR (Fig. 2B).

LacZ cDNA was located behind the LoxP site (Fig. 1). We hoped that LacZ cDNA would be expressed under control of the ADAM33 promoter in $ADAM33^{+/-}$ mice. However, we could not detect any beta-galactosidase staining (data not shown), suggesting that LacZ either was not expressed from this construct or was expressed at a level too low to be detected by beta-galactosidase staining.

ADAM33 mRNA was also examined. RT-PCR analysis of RNA derived from mouse brain, spleen, lung, and heart showed that an expected 440-bp fragment was amplified from the cDNA of $ADAM33^{+/+}$ mice, but no amplification product was detectable in the cDNA of $ADAM33^{-/-}$ mice (Fig. 2C).

Analysis of offspring from heterozygous breeding pairs. Mice that carried one copy of the deleted gene were interbred to generate $ADAM33^{-/-}$, $ADAM33^{+/-}$, and $ADAM33^{+/+}$ mice. Out of 202 pups born, 57 were $ADAM33^{-/-}$, 87 were $ADAM33^{+/-}$, and 58 were $ADAM33^{+/+}$. This is close to the predicted Mendelian ratios of 1:2:1 expected for nondeleterious alleles. Thus, the ADAM33 knockout pups were no less viable than their wild-type littermates. The postnatal growth rates of $ADAM33^{-/-}$, $ADAM33^{+/-}$, and $ADAM33^{+/+}$ mice were indistinguishable over an 8-week period (data not shown). $ADAM33^{-/-}$ and $ADAM33^{+/-}$ mice did not exhibit gross morphological or behavioral abnormalities compared to wild-type mice. Histological examination showed that there were no apparent differences in lung, heart, liver, kidney, brain, spleen, stomach, uterus, testis, ovary, small intestine, spleen, or skin (data not shown).

To determine whether fertility was affected in $ADAM33^{-/-}$ mice, multiple matings were carried out. Each pair of $ADAM33^{+/+}$ and $ADAM33^{-/-}$ mice was housed separately and allowed to mate for 3 months. Similar numbers of pups were born, and pups were separated after weaning. There was no difference in the numbers of pups born or in the average number of pups per litter, suggesting that knockout ADAM33 does not affect the fertility of male or female mice (Table 1).

Loss of ADAM33 does not impair allergen-induced IgE production. ADAM33 knockout and appropriate control mice were sensitized and then challenged by intranasal administration of ovalbumin to produce an allergic response in the lung and airways. To determine whether loss of ADAM33 affected

TABLE 1. Offspring from multiple matings

Genotype	No. of pups			No. of litters	Avg litter size (no. of pups)
	Female	Male	Total		
+/+ × +/+	56	62	118	12	7.38 ± 1.02
−/− × −/−	62	66	128	13	7.53 ± 1.59

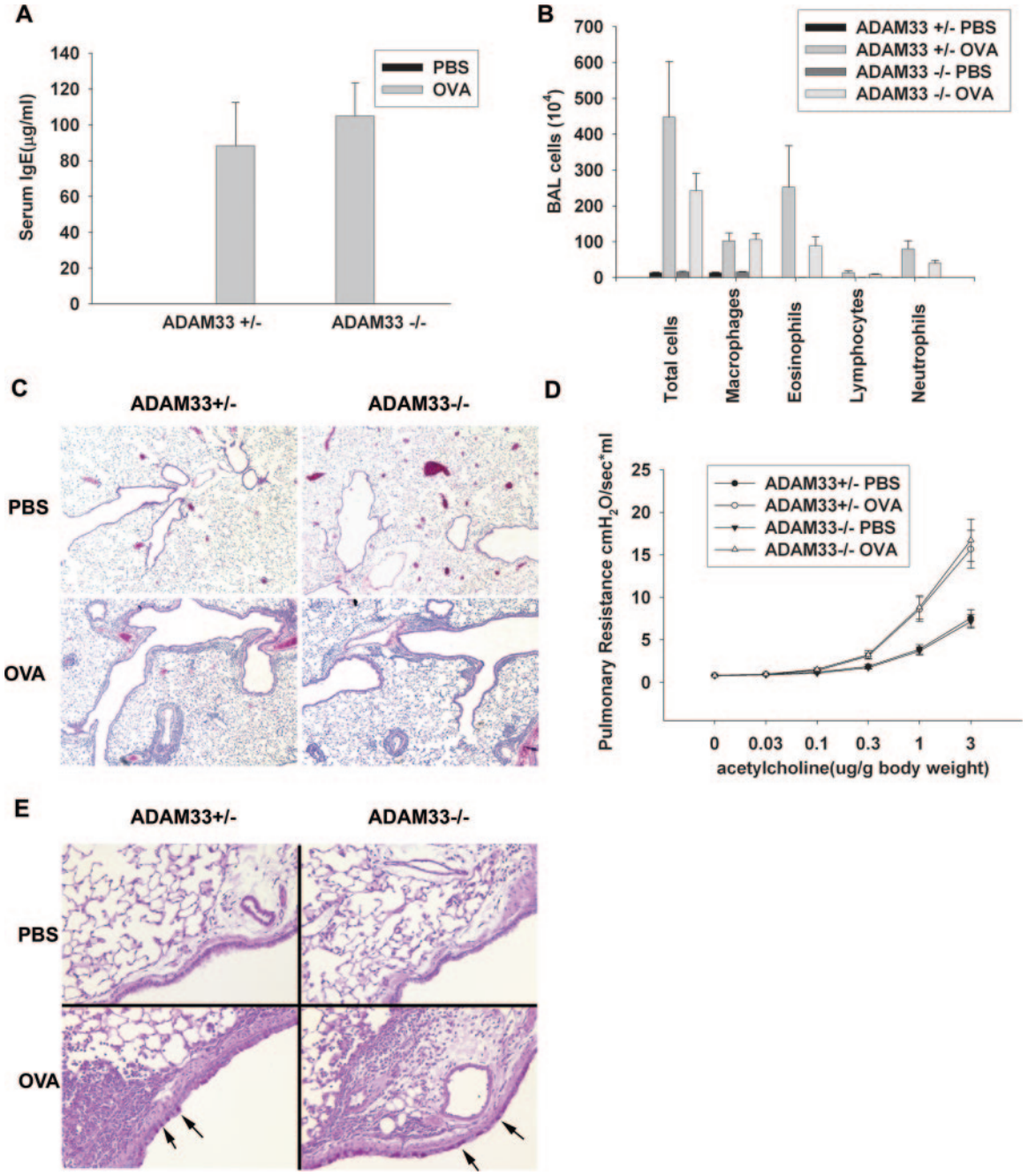

FIG. 3. Phenotypic features of asthma in mice are not significantly affected by loss of ADAM33. (A) ADAM33-heterozygous mice and ADAM33 knockout mice were sensitized and challenged with PBS or OVA. Serum was analyzed for IgE content by enzyme-linked immunosorbent assay. Values represent means ± standard errors of the means for 10 mice per group ($P < 0.05$ compared with PBS sensitization and challenge). (B) Total cell counts and counts for macrophages, eosinophils, neutrophils, and lymphocytes in bronchoalveolar lavage fluid are shown. (C) H&E staining for representative lung sections. (D) Invasive measurement of airway reactivity to intravenous acetylcholine. Results are means ± standard errors of the means ($n = 4$ to 5). (E) Periodic acid-Schiff staining for mucus in representative lung sections. Magenta-staining epithelial cells are positive for mucus (indicated by arrow).

the ability of these mice to mount an allergic humoral response, we measured serum OVA-specific IgE levels (Fig. 3A). OVA administration induced increases in IgE production in ADAM33 knockout mice similar to those seen for OVA-treated littermate control mice.

Loss of ADAM33 does not affect allergen-induced inflammation. Bronchoalveolar lavage was performed to assess the effect of allergen sensitization and challenge on inflammatory cell recruitment. OVA-sensitized and -challenged mice developed dramatic increases in eosinophils, neutrophils, and macrophages. The average eosinophil and neutrophil cell numbers in ADAM33$^{-/-}$ mice were slightly lower than those in ADAM33$^{+/-}$ mice; however, there were no statistically significant differences between them (Fig. 3B). H&E staining of

lungs from OVA-sensitized and -challenged mice confirmed the presence of a robust inflammatory response in ADAM33$^{-/-}$ mice (Fig. 3C). There were large numbers of infiltrating inflammatory cells (predominantly eosinophils) around the conducting airways and pulmonary veins in OVA-sensitized mice, but these responses did not differ between ADAM33-null mice and controls.

Loss of ADAM33 does not affect baseline or allergen-induced airway hyperreactivity. Airway reactivity was measured in sedated and paralyzed mechanically ventilated mice after allergen or sham challenge. There were no differences in airway reactivity between wild-type and ADAM33$^{-/-}$ mice, either at baseline or after OVA sensitization and challenge (Fig. 3D).

Loss of ADAM33 does not affect allergen-induced mucus metaplasia. To determine if ADAM33 is important for the mucus metaplasia induced by allergen challenge, we analyzed periodic acid-Schiff-stained sections of lungs for mucus content. PBS-challenged mice had little if any mucus visible in the airways. OVA-challenged mice produced substantial amounts of mucus. There was no apparent difference between ADAM33 knockout and control mice (Fig. 3E).

DISCUSSION

We generated mice deficient in ADAM33 to evaluate the potential roles of this protein in development, fertility, and regulation of airway reactivity and allergic inflammation. Based on established roles for several ADAM family members in the development of a wide variety of tissues and of at least three family members in fertility, it was reasonable to hypothesize that ADAM33 might also contribute to these processes. However, we could not identify any nonredundant role for ADAM33 in either development or fertility.

Asthma is a complex disorder in which major genetic and environmental factors interact to both initiate the disease and modify its progression. The genetics of asthma are now being characterized using gene discovery and functional genetic methodologies. In 2002, Van Eerdewegh et al. reported fine mapping of the ADAM33 gene as an asthma and airway hyperreactivity gene on chromosome 20p13 by using a genome-wide scan from 460 Caucasian families (39). Several studies have confirmed the association of ADAM33 with asthma (18, 25, 40); however, Lind et al. could not find any association in Puerto Rican or Mexican populations (27). There was no association seen in Icelandic and United Kingdom populations in another study from Blakey et al. (5). Raby et al. did not demonstrate the association of ADAM33 single nucleotide polymorphisms with asthma or airway responsiveness, and only weak associations were observed with IgE levels and total eosinophilia (34). Furthermore, to date, no sequence variants for ADAM33 have been described as resulting in meaningful alterations in ADAM33 expression or function. The role of ADAM33 in asthma pathogenesis thus remains controversial. It remains formally possible that the reported associations with the ADAM33 gene could be due to linkage disequilibrium with disease-causing sequence variants in other nearby genes.

Our results indicate that ADAM33 is not required for normal function of airway smooth muscle in mice and does not regulate baseline airway reactivity. We were also unable to find any evidence supporting a role for ADAM33 in allergic sensitization or in the increased airway reactivity, mucus metaplasia, or airway inflammation induced by allergen challenge. Multiple methods have been used to measure airway responsiveness in mice. The method we used, based on measurements of airflow and pressure during forced oscillation maneuvers, is primarily a reflection of the caliber of the conducting airways. Unrestrained plethysmography, a noninvasive technique that requires little operator effort, has also been widely used. However, the interpretation of the changes in the pattern of breathing measured by this approach remains controversial (2, 13, 14, 31, 33). It has also been reported that administration of bronchoconstrictor drugs by inhalation may yield results different from those obtained by administration intravenously, as we did here (12). More importantly, murine models of asthma do not perfectly reproduce human asthma. Murine models do not perfectly mimic the role that mast cells appear to play in the human disease, nor do they perfectly mimic the airway wall remodeling that accompanies human asthma. Because mice have only a single submucosal gland in their airways, the glandular hyperplasia that is often a feature of human asthma cannot be effectively studied in the mouse. It therefore remains possible that alterations in expression or function of ADAM33 could play a role in human asthma.

Inbred mice show strain-specific variation with respect to various traits of asthma, such as airway inflammation and bronchial hyperresponsiveness (9). We did not find differences in any asthma-associated traits in 129/SVJae mice but cannot exclude the possibility that there would be effects of ADAM33 in other genetic backgrounds. Ackerman et al. found that the difference between two mouse strains (A/J and C57BL/6J) in airway hyperresponsiveness was not associated with any single locus but was significantly associated with an interaction of loci on chromosomes 2 and 6 (1). The mouse ADAM33 gene is located on chromosome 2. However, there was no difference between nearly 200 A/J and C57BL/6J single nucleotide polymorphisms in the ADAM33 genomic sequence (http://mousesnp.roche.com/). Together with our finding, these results suggest that the ADAM33 gene may not contribute to phenotypic features of asthma in mice.

Other ADAM proteins play important roles in protein ectodomain shedding. Many cell surface molecules, such as cytokines, cytokine receptors, cell adhesion molecules, and growth factors, are processed to convert them into physiologically active soluble derivatives. Proteases responsible for releasing cell surface molecules are classified as sheddases or secretases. ADAM33 belongs to a subfamily that includes ADAM19, ADAM12, and ADAM13, as determined by sequence homology, (16, 41). ADAM19 may participate in the shedding of β1-neuregulin (37). ADAM12 was reported to process HB-EGF and insulin-like growth factor binding proteins 3 and 5 in vivo (3, 28). ADAM33 was also found to be able to cleave α2-macroglobulin (15). However, it is still not clear what the physiological substrates of ADAM33 are and if ectodomain shedding by ADAM33 contributes in any meaningful way to human biology. Similarly, like most other ADAM proteins, ADAM33 contains an integrin-binding disintegrin domain that can serve as a ligand for integrin α9β1 (10). Integrin α9β1 binds to several other ligands, and mice lacking the α9 subunit (19) die within the first 12 days of life. It is thus clear that neither integrin depends principally on interaction

with ADAM33 for its major in vivo functions. Nonetheless, ADAM33 knockout mice and the cell lines derived from these mice should be useful tools to test and verify potential roles for this protein in other disease models and to identify and validate its proteolytic substrates and integrin interactions.

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