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The relationship between eosinophils and the development of Ag-induced pulmonary pathologies, including airway hyper-responsiveness, was investigated using mice deficient for the secondary granule component, major basic protein-1 (mMBP-1). The loss of mMBP-1 had no effect on OVA-induced airway histopathologies or inflammatory cell recruitment. Lung function measurements of knockout mice demonstrated a generalized hyporeactivity to methacholine-induced airflow changes (relative to wild type); however, this baseline phenotype was observable only with methacholine; no relative airflow changes were observed in response to another nonspecific stimulus (serotonin). Moreover, OVA sensitization/aerosol challenge of wild-type and mMBP-1/−/− mice resulted in identical dose-response changes to either methacholine or serotonin. Thus, the airway hyper-responsiveness in murine models of asthma occurs in the absence of mMBP-1. The Journal of Immunology, 2000, 165: 5509–5517.

A growing body of literature now exists suggesting that the release of eosinophil secondary (sometimes referred to as specific) granule proteins (ESGPs) is critical to eosinophil-mediated activities and may be responsible for many of the observed pathologies associated with allergic respiratory disease. Potential deleterious effects of ESGPs include not only activities leading to airway damage and lung dysfunction (e.g., AHR) (10), but also direct effects on several other cell types. On a molar basis, the most abundant human ESGPs are eosinophil major basic proteins (MBPs). MBPs are small cationic proteins (~12 kDa; pl 10–12) that colocalize with the characteristic electron-dense crystalline core of eosinophil secondary granules (11). In the mammalian orders examined to date, including Primata (humans) and Rodentia (mice), MBPs are encoded by two genes that share considerable sequence identity (12–14). The relative function(s) of each MBP in a given species is not known. For example, the reported differences in biological activities between the human MBPs (hMBP-1 and -2) are nominal, and assessments of the proteins stored in the granule show that nearly all the MBP in eosinophils is hMBP-1 (~10:1) (13). Moreover, many clinical studies have shown that the deposition of hMBP-1 is a diagnostic marker of the inflammation occurring in the lungs of asthmatics, and accompanies airway epithelial damage, lung remodeling, and perturbations of lung function (15).

The correlative association between AHR and MBP-1 (and therefore eosinophils) has been documented in several mammalian species, including humans (15–18). Several studies have defined a potential causative mechanism resulting in AHR by linking eosinophil effector function, mediated by MBP-1 release, and muscarinic receptor dysfunction (17–20). However, the link between eosinophils and AHR using mouse models of pulmonary inflammation is more tenuous and controversial. For example, many studies disassociate AHR from the recruitment (and presumably activation) of eosinophils to the lung. These studies can be divided into three groups: 1) studies that demonstrate that airway eosinophilia can occur without AHR (21); 2) mouse models that show that AHR occurs in the absence of a significant airways eosinophilia (22–26); and 3) studies that disassociate the kinetics of eosinophil recruitment to the lung and the development of AHR (27). Moreover, several studies in the mouse link AHR directly with eosinophilia.
with T cell activities independently of eosinophil effector function (25, 27–32). Therefore, while a preponderance of evidence suggests that eosinophil effector functions are responsible for Ag-induced AHR, data from in vivo studies using mouse models have, in some cases, been in conflict with this hypothesis.

To define the specific role(s) of mMBP-1 in the development of Ag-induced AHR, we created a gene knockout mouse line deficient for this protein. These mice display baseline perturbations of airway reactivity (relative to wild-type littersmates) in response to methacholine, but show no homeostatic phenotype when airway reactivity is assessed by serotonin provocation. Furthermore, regardless of the nonspecific stimulus used to assay reactivity, the loss of mMBP-1 did not obviate Ag-induced AHR in an OVA sensitization/challenge model. These data suggest that although mMBP-1 expression, even at the low levels observed in naive mice, is sufficient to alter uniquely muscarinic receptor function(s), the AHR occurring in murine models of asthma is not causatively linked to mMBP-1 release from eosinophils.

Materials and Methods

Targeting of the mMBP-1 locus and the generation/propagation of knockout mice

A mMBP-1 gene targeting vector was constructed using genomic DNA fragments flanking a 1.55-kb region that includes exons 2, 3, and 4 of this gene (12). The targeting vector replaces these exons with a neomycin resistance cassette (PGK-neo) and also includes a herpes simplex virus thymidine kinase gene (HSV-TK) as a negative selection marker (Fig. 1A). G1-129 embryonic stem (ES) cells (4 × 10⁵) (33) were electroporated with 25 μg of linearized DNA and subjected to positive selection for PGK-neo with G418 (400 μg/ml) and negative selection for the presence of the HSV-TK marker with ganciclovir (2 μM). Clones containing heterozygous disruptions of the gene were identified by Southern blot analysis with a probe (Probe 1) flanking the 5′ homologous region (i.e., not contained within the targeting construct). The presence of a single integration event was verified by hybridization with a neo-derived DNA fragment (Probe 2). Gene-targeted cells were injected into C57BL/6 blastocysts to create chimeric animals that were subsequently bred to 129/SvJ mice, allowing the transmission of this allele on a 129 background (129/Ola/Hsd × 129/ SvJ). All mice were maintained in microisolator cages housed in a specific-pathogen-free animal facility. The sentinel cages within this animal colony were negative for viral Abs and the presence of known mouse pathogens. Protocols and studies involving animals were conducted in accordance with National Institutes of Health and Mayo Institutional guidelines.

Mice carrying the mMBP-1-targeted allele were genotyped using PCR and DNA recovered from tail biopsies. Animals were identified as wild type (mMBP-1+/+), heterozygous (mMBP-1+/-), or homozygous (mMBP-1−/−) using a three-primer strategy. The location and orientation of the primers is shown in Fig. 1A: primer 1, 5′-ATGGCAGTGTGACGACGGT AAAGG-3′; primer 2, 5′-CAGATGAAAGACGAGACCTC-3′; and primer 3, 5′-GAACACGGCTGTTGGCTG-3′. Primers 1 and 2 anneal uniquely to the wild-type locus and produce an 874-bp PCR amplicon. Primers P1 and P3 anneal to the targeted locus and produce a 683-bp PCR amplicon. Southern blots were prepared with GeneScreen Plus membranes (NEN, Boston, MA) and hybridized with the 32P-labeled DNA probes identified in Fig. 1.

Determination of mMBP-1 gene expression

RT-PCR to identify potential mMBP-1 transcripts from specific tissues were performed using template cDNA derived from the reverse transcription of 1 μg of total RNA (bone marrow, liver, lung, and spleen). PCR was performed using a primer set from exon 2 (P4, 5′-TCTTCTAGCGG GCTTCTTACTGAGG-3′) and exon 6 (P5, 5′-GACACAGTGAGT GACGCCAGTG-3′) to generate a 631-bp amplicon. The reaction program consisted of an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C (30 s), 60°C (1 min), 72°C (2 min), and a final 72°C (5-min) extension period. Parallel PCR using primers derived from the β-actin gene showed that all samples of RNA were reverse transcribed.

Electron microscopy of peripheral eosinophils

Eosinophils for microscopy were generated from peritoneal cavity exudates of mice sensitized/challenged with Mesocystoides corti protein extract (12). Exudate cells (20–40% eosinophils) were collected by lavage and concentrated by low speed centrifugation (8 min/1000 rpm, 4°C). The leukocytes in this pellet were resuspended directly in Trump’s fixative (4% formaldehyde, 0.5% glutaraldehyde, and 84 mM NaH₂PO₄, pH 7.2) and stored at room temperature before application to electron microscoty grids.

OVA-induced allergic airway inflammation

Assessments of lung function and histopathology were performed on OVA-sensitized/saline-treated vs OVA-challenged 8- to 16-wk-old mice. All mice were immunized by two i.p. injections (100 μl) of OVA (20 μg; crude grade IV, Sigma) complexed with 2.25 mg of Injekt Alum (Al(OH)₃/Mg(OH)₂; Pierce, Rockford, IL) on days 0 and 14. Sensitized mice were then challenged with an OVA aerosol (1% OVA prepared in saline) for 20 min on days 24, 25, and 26 using an ultrasonic nebulizer (De Vilbiss, Somerset, PA); control animals received a saline-only aerosol. The mice were assessed for pulmonary cellular infiltrates, histopathologies, and lung function on day 28.

Assessments of allergic airway inflammation: pulmonary cellular infiltrates and histological changes in the lung

The methods and analyses assessing cellular infiltrates associated with bronchoalveolar lavage (BAL) and collagen digests of whole lungs were conducted as previously (33, 34). Histopathologic changes in the lung were determined from mucus content of the airway epithelium, were assessed from tissues excised and fixed in 10% formalin (lungs were inflated with a fixed volume (0.5 ml) of saline). The lung samples were washed free of formalin with 1× PBS and subsequently dehydrated through an ethanol series before equilibration in xylene and embedding in paraffin. Sections (4 μm) were mounted on either slides and stained with either periodic acid-Schiff or counterstained with hematoxylin/methylgreen) or alcian blue, pH 2.5 (counterstained with nuclear fast red). Parasagittal sections were analyzed by brightfield microscopy. The mucus content in the airway epithelium of mice from different groups was based on the evaluation of ~40 airways (both proximal and distal)/mouse (n = 5 animals/group). Relative comparisons of mucus content were made between cohorts of animals using an integrated program (Image-Pro Plus; identifying the data as a mucus index: [average alcin blue staining intensity of the airway epithelium] × (area of airway epithelium staining with alcin blue)]/[total area of the conducting airway epithelium] × total number of airways assessed.

Immunohistochemical detection of eosinophils in paraffin-embedded tissue

Sections of lung tissue (4 μm) were assessed for the infiltration of eosinophils using mAbs specific for individual secondary granule proteins. mAbs were raised against native mMBP-1 and the collective group of murine eosinophil-associated RNases (mEARs) using protein purified from peripheral blood eosinophils derived from an IL-5 transgenic line of mice (35). Individual Abs were selected on the basis of reactivity to native protein in an ELISA format as well as their utility as a detection reagent using formalin-fixed paraffin sections. The specificity of the mMBP-1 (rat mAb 14.7.4) and mEARs (rat mAb 32.1.3) protein A-Sepharose-purified Abs was determined by competition assays with purified native protein. Immunohistochemical staining was performed with dianibenzidine-peroxidase dation reagents according to the manufacturer’s instructions (Vector, Burlingame, CA). Briefly, lung sections were deparaffinized and washed in PBS (1× PBS), and endogenous peroxidase activity was quenched in a methanol buffer (80% methanol/0.6% hydrogen peroxide) in preparation for Ab staining. Methanol-peroxide-treated sections were washed with 1× PBS, digested (10 min, 25°C) with pepsin (Zymed, San Francisco, CA), and blocked by incubation at room temperature in 1.5% normal goat serum for 30 min. The blocked sections were treated with the mAbs at final concentrations of 0.4 μg/ml (diluting the Abs in 1.5% normal goat serum/PBS for 1 h at room temperature). The slides were subsequently washed free of primary Ab with several changes of 1× PBS, and a secondary reagent (polyclonal rabbit anti-rat IgG Abs conjugated to HRP (Dako, Carpinteria, CA)) was bound at 13 μg/ml (room temperature, h) before histochemical development using dianibenzidine-peroxidase dase. The sections were counterstained with methyl green in preparation for photomicroscopy.

Determination of airway reactivity in response to provocation with nonspecific stimuli

AHR was assessed by inducing airflow obstruction with either a methacholine or serotonin aerosol using a noninvasive protocol (34, 36). This method uses unrestrained conscious mice that are placed into the main chamber of a plethysmograph (Buxco Electronics, Troy, NY). Pressure differences between this chamber and a reference chamber were used...
to extrapolate minute volume, tidal volume, breathing frequency, and enhanced pause (PENH). PENH is a dimensionless parameter that is a function of total pulmonary airflow in mice (i.e., the sum of the airflows in the upper and lower respiratory tracts) during the respiratory cycle of the animal. This parameter closely correlates to lung resistance as measured by traditional invasive techniques using ventilated animals (36). Dose-response data were plotted as the percent baseline PENH vs the log_{10} of the methacholine or serotonin solution (milligrams per milliliter) used to generate the aerosol. There were no statistically significant differences (Tukey-Kramer highest significant difference test) in observed baseline PENH values (±SEM) between each cohort of animals used in these studies: wild-type (+/+), saline, 0.70 ± 0.07; wild-type (+/+), OVA, 0.63 ± 0.08; mMBP-1^{−/−}, saline, 0.51 ± 0.02; and mMBP-1^{−/−}, OVA, 0.52 ± 0.04.

**Statistical analyses**

Pairs of groups were compared using Student’s *t* tests. The *p* value for significance was set at 0.05, and values for all measurements are expressed as the mean ± SEM.

**Results**

**Generation of mMBP-1 gene knockout mice**

We previously reported the identification and cloning of mMBP-1, the murine orthologue of hMBP-1 (12). Genomic clones representing this locus were used to develop a targeting construct designed to replace exons 2, 3, and 4 of the mMBP-1 gene with a neomycin resistance cassette (Fig. 1) to replace exons 2, 3, and 4 of the mMBP-1 gene with a neomycin resistance cassette. These exons include the signal sequence associated with protein secretion, the propolypeptide precursor that is proteolytically cleaved, and the N-terminal 54 aa of the mature mMBP-1 protein stored in the secondary granule. The targeting construct was electroporated into GK129 ES cells, and clones heterozygous for the induced mMBP-1 mutation were identified by Southern blot as shown in Fig. 1B. Targeted ES cell clones were injected into C57BL/6J blastocysts, and highly chimeric male mice were bred with 129/SvJ females to generate germline mice. Homozygous mMBP-1 knockout mice on a 129 background were generated by interbreeding of heterozygous siblings (Fig. 1C). We have not observed any anomalies associated with the breeding of mMBP-1^{−/−} mice. Homozygous animals were produced from these sibling intercrosses at the expected Mendelian frequency, and matings of homozygous mice produced litters of a size and frequency equivalent to wild-type or heterozygous animals.

Gene activity was assessed using RT-PCR in the lung, liver, spleen, and bone marrow. These data show that homozygous mutant mice did not accumulate mMBP-1 transcripts in any of the tissues examined (Fig. 1D). Expression of the mMBP-1 gene was restricted to wild-type and mMBP-1^{+/−} mice, and as reported previously (35), steady state transcripts were limited to known sites of eosinophilopoiesis (i.e., bone marrow and spleen).

**Loss of mMBP-1 has ultrastructural effects on eosinophil secondary granules**

The number of secondary granules in mature eosinophils (derived from the peritoneal cavity of mice sensitized/challenged with a parasite (Mesocestoides corti) protein extract (12)) appeared to be unaffected by deletion of the mMBP-1 gene. The average number of granules per cross-sectional area, as determined by electron microscopy, was the same in wild-type (29.9 ± 3.6) and mMBP-1^{−/−} (32.1 ± 3.8) eosinophils. In addition, the size of the granules
in each group of mice (i.e., cross-sectional area) was indistinguishable. However, Wright-Giemsa-stained mMBP-1−/− eosinophils lacked much of the intense color associated with the secondary granules of these cells, suggesting that although the granules were present, they no longer had enough cationic character to bind eosin efficiently (data not shown). The identification of secondary granules in eosinophils is based primarily on electron microscopy showing the presence of an electron-dense crystalline core uniquely associated with this granule. In human eosinophils, electron immunogold staining using an anti-hMBP-1 Ab has shown that hMBP-1 localizes to the crystalline core (11). An examination of electron micrographs of eosinophils recovered from the peritoneal cavity of M. corti-sensitized/challenged mice revealed that mMBP-1-deficient animals lack the electron-dense core associated with secondary granules (Fig. 2). This observation shows that the core structure in mouse eosinophil granules is dependent on the presence of mMBP-1, but does not exclude the possibility that the core may also be dependent on other molecules contained within the secondary granule, including the other MBP gene family member (i.e., mMBP-2). It is also noteworthy that the secondary granules of heterozygous animals contain electron-dense cores that were identical with the granule cores from wild-type mice. Although we have no quantitative data with regard to mMBP-1 production in heterozygous mice, this observation suggests that core formation is not sensitive to the ~50% decrease in mMBP-1 synthesis that one might expect in mMBP-11/2 mice.

OVA-induced recruitment of eosinophils to the lung is not dependent on mMBP-1 expression

The importance of mMBP-1 to eosinophil effector function was assessed using an established OVA model of allergic pulmonary inflammation, which included i.p. sensitization of the mice with OVA and an adjuvant, followed by several OVA aerosol challenges of the lung (see Materials and Methods). Fig. 3A compares the number and composition of leukocytes found in the BAL fluid of wild-type and mMBP-1−/− mice in response to an aerosol challenge with saline or OVA. Whereas aerosol challenge with saline alone had no effect on the homeostatic levels of cells in the lumen of either wild-type or mMBP-1−/− mice (i.e., eosinophils were not recruited to the lumen and comprise <1% of the cells in the air.

FIGURE 2. The secondary granules of mMBP-1−/− eosinophils are devoid of electron-dense core structures. Eosinophils from peritoneal cavity exudates of parasite Ag-sensitized/challenged mice (12) were fixed and subjected to electron microscopy. A, Electron photomicrographs showing clusters of secondary granules from wild type (+/+), mMBP-1−/−, and mMBP-1−− mice (original magnification, ×69,000). B, Single granules from mice of each genotype at high magnification (original magnification, ×512,500).

FIGURE 3. The OVA-induced eosinophilia of the airway lumen and interstitial regions are unaffected in mMBP-1−/− mice. A, The cellularity of BAL fluid from sensitized wild-type and mMBP-1−/− mice was assessed in response to either a saline or OVA aerosol challenge (n = 6–11 animals/group). The cellularity of each animal cohort is expressed as the product of the total number of cells recovered and the percentages of each cell type derived from differentials (Wright-stained cytospin preparations) of 300 cells. Data represent the mean ± SEM. B, Parenchymal leukocyte cellularity from sensitized wild-type and mMBP-1−/− mice (n = 6–11 animals/group) was assessed by collagenase digestion of perfused lungs. Data are expressed as the means (±SEM), counting a minimum of 300 cells/sample. MΦ, macrophage; Lym, lymphocyte; Eos, eosinophil; Neu, neutrophil; Mono, monocyte. * p < 0.05, wild-type/mMBP-1−/− saline control groups vs OVA-treated mice.
the pulmonary inflammation associated with OVA challenge in both wild-type and mMBP-1−/− mice was accompanied by the selective recruitment of eosinophils to the airway lumen. The recruitment of eosinophils to the airways of OVA-challenged mMBP-1−/− mice was indistinguishable from that in wild-type littermates. Eosinophil trafficking to the interstitial regions of the lung in mMBP-1−/− mice was also unaffected relative to that in wild-type littermates, as differential counts of leukocytes recovered from collagen-digested whole lungs showed equal numbers of eosinophils recruited to the lung in response to OVA challenge (Fig. 3B).

Immunohistochemistry using a rat mAb specific for mMBP-1 (rmAb 14.7.4) demonstrated that the eosinophils recruited to the lungs of knockout mice are, as expected, devoid of mMBP-1 (Fig. 4). These data also demonstrated that mMBP-1-containing eosinophils are a homeostatic resident population within the parenchyma of wild-type mice at baseline (i.e., OVA sensitized/saline challenged; Fig. 4A), that increases dramatically in response to an OVA aerosol provocation (Fig. 4B). In contrast, this mAb failed to detect the presence of mMBP-1-containing eosinophils in the lungs of knockout mice, either at baseline (Fig. 4C) or after an OVA aerosol provocation (i.e., OVA-sensitized/OVA-challenged; Fig. 4D). However, the spatial distribution of eosinophils within the pulmonary interstitium of mMBP-1−/− mice was nonetheless unaffected as a consequence of the absence of mMBP-1. Recruited eosinophils were identified in knockout mice by immunohistochemistry using a rat mAb specific for another eosinophil secondary granule protein group, the mEARs (rmAb 32.1.3). These data demonstrated that eosinophils were recruited to perivascular and peribronchial regions of the lungs of both OVA-treated wild-type and mMBP-1−/− mice with no difference in the relative distribution of these cells between either group of mice (Fig. 4, E and F).

Murine MBP-1-deficient mice show no difference relative to wild-type animals with respect to the histopathologies that develop in response to OVA challenge

OVA-treated wild-type and mMBP-1−/− mice each displayed the same airway changes, including bronchiolar-associated leukocyte aggregates, airway epithelial cell hypertrophy, and goblet cell hyperplasia (Fig. 5, A and C, vs Fig. 5, B and D, respectively). Furthermore, the periodic acid-Schiff-stained sections of Fig. 5 also show that airway epithelial mucus content of both wild-type and mMBP-1−/− mice increased dramatically in response to OVA sensitization/challenge. A quantitative assessment of airway epithelial mucus demonstrates that the extent of OVA-induced mucus production in wild-type and mMBP-1−/− mice is identical (Fig. 5E). In summary, mMBP-1−/− mice displayed no differences in OVA-induced pulmonary histopathology relative to wild-type animals, suggesting that many, if not all, histologic changes associated with allergic inflammation in mouse models are not contingent upon the release of mMBP-1 from eosinophils recruited to the lung.

The AHR that occurs in OVA sensitization/challenge models of asthma in mice is not dependent on mMBP-1

The relationship between the development of AHR and mMBP-1 expression was initially examined by assessing baseline methacholine responsiveness in naive animals. Surprisingly, the methacholine dose-response curves (means of single-animal measurements)
shown that the threshold dose for methacholine-induced airflow changes in wild-type mice was lower than the dose required to induce equivalent changes in knockout mice (Fig. 6A). Moreover, the absolute magnitude of these methacholine-induced changes was significantly higher in wild-type mice relative to mMBP-1−/− littermates. This baseline phenotype, however, was observable only with methacholine; no airflow changes were observed between wild-type and mMBP-1−/− mice in response to another nonspecific stimulus (serotonin provocation; Fig. 6B). The unique ability of methacholine to induce airflow perturbations in naive mMBP-1−/− mice suggests that mMBP-1 is a homeostatic ligand of one or more pulmonary muscarinic receptors.

Studies of airway reactivity in the knockout mice were extended further by examining the relative effects of Ag sensitization/challenge using an OVA model system. Fig. 7 compiles data comparing either methacholine (A) or serotonin (B) dose-dependent reactivity changes in OVA-sensitized/challenged wild-type and mMBP-1−/− mice (n = 8–10/group) relative to control groups. In response to methacholine provocation (Fig. 7A), three striking differences regarding the absolute values of the responses obtained from each genotype are apparent. 1) Saline-treated mMBP-1−/− mice were hyporeactive relative to saline-treated wild-type mice (effective dose 200% (ED200) = 36.2 ± 6.9 vs 12.5 ± 1.1 mg/ml, respectively; p < 0.03), confirming that in the absence of mMBP-1 the observed baseline reactivity in response to methacholine is attenuated relative to that in wild-type animals. 2) The threshold dose for methacholine-induced airflow changes in OVA-sensitized/challenged mMBP-1−/− mice was also two to three times the amount of methacholine necessary to produce the same changes in OVA-treated wild-type mice (ED200 = 11.0 ± 1.6 vs 4.3 ± 0.2 mg/ml, respectively; p < 0.03). 3) Airway reactivity in response to the methacholine dose inducing the maximal observed PENH difference between wild-type and mMBP-1−/− mice (i.e., 25 mg/ml) is markedly less (~50% decrease) in both saline- and OVA-treated mMBP-1−/− mice relative to the responses observed in wild-type mice (p < 0.02). The implication of these differences is that the relative shift in the methacholine dose-response curves is the same in both genotypes. OVA provocation (relative to a genotype-matched saline control group) of either wild-type (ED200 = 4.3 ± 0.2 vs 12.5 ± 1.1 mg/ml, respectively; p < 0.01) or mMBP-1−/− (ED200 = 11.0 ± 1.6 vs 36.2 ± 6.9 mg/ml, respectively; p < 0.01) mice resulted in an ~60% decrease in the threshold dose for methacholine-induced airflow changes. Thus, allergen-induced increases in methacholine-mediated airflow changes (i.e., AHR) occur in the absence of mMBP-1. This lack of an effect on allergen-induced airflow changes in mMBP-1 knockout mice was confirmed using serotonin as an alternative nonspecific stimulus (Fig. 7B). OVA-sensitized/challenged wild-type and mMBP-1−/− mice each displayed identical increases in serotonin-induced airflow changes relative to naive control groups of mice, which displayed no differences in their baseline responses.

**Discussion**

The disassociation of mMBP-1 expression by eosinophils and the development of Ag-induced pulmonary changes in a murine model of asthma would suggest that the release of this ESGP is not a
causative event associated with allergic inflammation in this model. The extent of this disassociation was broad, as the loss of mMBP-1 had no observable effect on Ag-induced eosinophil recruitment, airway histopathologies (including mucus overproduction and goblet cell metaplasia), and perturbations of lung function. The only observed effect of the mMBP-1 deficiency was a baseline attenuation of airway reactivity in response to methacholine provocation. However, this effect was unique to the methacholine provocation assay and did not occur with another nonspecific stimulus (i.e., exposure to serotonin). Moreover, this baseline effect in response to methacholine was independent of the number of eosinophils recruited to the lung and did not obviate the increase in Ag-mediated AHR. Because baseline eosinophil numbers are very low, and these cells are not activated, it is likely that only low levels of MBP would be present in wild-type mice, and thus only a fraction of the pulmonary M2 muscarinic receptors will be occupied (i.e., blocking the negative feedback loop regulating neureginal acetylcholine release). In this paradigm, the baseline equilibrium between MBP and pulmonary M2 receptors would shift dramatically during allergic inflammatory responses because of the increased availability of systemic sources of MBP and, more importantly, the availability of localized pulmonary sources of MBP (i.e., strategically located eosinophils within the lung). However, the data presented here are in conflict with this model, as Ag-induced increases in AHR occurred equally in the presence or the absence of mMBP-1. These observations evoke two mutually exclusive conclusions regarding ESGP effector function in this murine model: 1) functionally significant degranulation of eosinophils (as assessed by the release of mMBP-1) is not occurring in this OVA-mediated mouse model of asthma; or 2) degranulation by eosinophils is occurring, but the temporal and/or spatial release of ESGPs is functionally insignificant with respect to the development of AHR. Although qualitative data exist suggesting that mMBP is released as part of some OVA-mediated models (37),

FIGURE 6. The airway reactivity of naive mMBP-1−/− mice is attenuated relative to that in wild-type animals in response to methacholine, but not serotonin, provocation. The airway reactivity (percentage of the baseline PENH (y-axis)) of naive wild-type and mMBP-1−/− mice (n = 8 mice/group) was assessed by whole-body plethysmography as a function of increasing doses of nebulized methacholine/serotonin (x-axis). The airway reactivity in each panel was plotted (±SEM error bars) as the percent increase from the response observed after exposure to a saline aerosol. A, Methacholine dose-response assays show that the threshold dose for methacholine-induced airflow changes in wild-type mice was lower than the dose required to induce equivalent changes in mMBP-1−/− mice. Moreover, the absolute magnitude of these methacholine-induced changes was significantly higher in wild-type animals than in mMBP-1−/− littermates. B, Dose-dependent airflow changes were not observed between naive (i.e., baseline) wild-type and mMBP-1−/− mice in a serotonin-mediated provocation assay. *, p < 0.05, naive wild-type vs mMBP-1−/− mice.

FIGURE 7. Murine MBP-1 does not mediate OVA-induced increases in airway hyper-responsiveness in response to nonspecific stimuli (i.e., AHR). Shown are methacholine (A) and serotonin (B) dose-response curves resulting from group mean data derived from four cohorts of animals (n = 8–10 mice/group), including naive and OVA-treated wild-type and mMBP-1−/− mice. Airway reactivity (percentage of the baseline PENH (y-axis)) in response to increasing doses of nebulized methacholine/serotonin (x-axis) was assessed by whole-body plethysmography. The ED200 is shown as an assessment of the threshold dose of methacholine required to induce a 2-fold increase in PENH from the observed baseline value. *, p < 0.05, naive mMBP-1−/− vs OVA-treated mMBP-1−/− mice.
this study does not demonstrate the extent of this phenomenon, nor does it causatively link the release of mMBP-1 with AHR. Thus, a review of the existing literature regarding the mouse models of inflammation would suggest that either there is OVA model variability associated with the relative contribution of eosinophil degranulation to the development of AHR or, unlike human eosinophils from asthma patients (38; see review in Ref. 39), functionally significant eosinophil degranulation does not occur in murine OVA models of respiratory inflammation (40, 41).

This apparent difference observed between MBP-1 effector function in patients with asthma and the murine models may reflect the chronic inflammatory character of this disease state in humans. Human MBP-1 deposition probably occurs over long periods of time (i.e., months/years) and cumulatively correlates with both AHR and histopathological airway epithelial changes, whereas extensive (and chronic) eosinophil degranulation does not occur in the 96-h challenge interval of most OVA protocols. However, our unpublished data assessing murine vs human eosinophil respiratory burst and release of EPO in vitro demonstrate that mouse eosinophil degranulation is greater than an order of magnitude less under conditions that elicit this response from human cells. These data imply that the incongruities between human patients and mouse models of asthma are probably not an issue of the OVA model, but reflect species differences in eosinophil effector function. We would propose that degranulation occurs more readily in human eosinophils and is required, because of unique effector functions that were/are evolutionarily advantageous to primates and not rodents. The resolution of this quandary will probably have significant implications regarding other roles of eosinophils in mouse models of human disease, as some, but not all, effector functions will have been conserved during mammalian evolution.

The data presented here do not preclude the participation of other ESGPs in the development of Ag-induced lung dysfunction. However, the demonstration that OVA-induced AHR in the mouse occurs independently of mMBP-1 suggests a parsimonious explanation of previous studies that showed a disassociation of eosinophil effector function and AHR; Ag-induced AHR in mouse models of asthma may not be causatively linked to the release of ESGPs. Studies in which AHR occurred in the absence of a significant airways eosinophilia (22–25) or that disassociated AHR from eosinophil recruitment to the lung (27) were apparently assessing an MBP-1-independent phenomena. This lack of an observed effect mediated by the release of the most abundant ESGP suggests that AHR is either dependent on other less abundant ESGPs, eosinophil effector functions other than degranulation, or is a pathophysiologic response mediated by the activity of another cell type(s). As noted in several studies, activated CD4+ T cells recruited to the lung in response to allergen challenge were likely candidates to mediate eosinophil-independent effects (25, 30, 31). Furthermore, genetic studies linking AHR and T cells (29, 32) or studies linking AHR and T cell-derived cytokines that have little or no direct effect on eosinophil proliferation and/or function (e.g., pulmonary expression of IL-13) (27, 28), further support a relationship between AHR and T cells. The implicit conclusion that arises is that a direct causative link between eosinophil degranulation (i.e., the release of ESGPs such as mMBP-1) and the development of AHR remains an elusive, and potentially unattainable, goal in the mouse.

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