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Mouse Mast Cell Protease-6 and MHC Are Involved in the Development of Experimental Asthma

Yue Cui,^{*,1} Joakim S. Dahlin,^{*,1} Ricardo Feinstein,[†] Lora G. Bankova,^{‡,§}
Wei Xing,^{‡,§,2} Kichul Shin,^{‡,§,3} Michael F. Gurish,^{‡,§} and Jenny Hallgren^{*}

Allergic asthma is a complex disease with a strong genetic component where mast cells play a major role by the release of proinflammatory mediators. In the mouse, mast cell protease-6 (mMCP-6) closely resembles the human version of mast cell tryptase, β -tryptase. The gene that encodes mMCP-6, *Tpsb2*, resides close by the *H-2* complex (MHC gene) on chromosome 17. Thus, when the original mMCP-6 knockout mice were backcrossed to the BALB/c strain, these mice were carrying the 129/Sv haplotype of MHC (mMCP-6^{-/-}/H-2bc). Further backcrossing yielded mMCP-6^{-/-} mice with the BALB/c MHC locus. BALB/c mice were compared with mMCP-6^{-/-} and mMCP-6^{-/-}/H-2bc mice in a mouse model of experimental asthma. Although OVA-sensitized and challenged wild type mice displayed a striking airway hyperresponsiveness (AHR), mMCP-6^{-/-} mice had less AHR that was comparable with that of mMCP-6^{-/-}/H-2bc mice, suggesting that mMCP-6 is required for a full-blown AHR. The mMCP-6^{-/-}/H-2bc mice had strikingly reduced lung inflammation, IgE responses, and Th2 cell responses upon sensitization and challenge, whereas the mMCP-6^{-/-} mice responded similarly to the wild type mice but with a minor decrease in bronchoalveolar lavage eosinophils. These findings suggest that inflammatory Th2 responses are highly dependent on the MHC-haplotype and that they can develop essentially independently of mMCP-6, whereas mMCP-6 plays a key role in the development of AHR. *The Journal of Immunology*, 2014, 193: 000–000.

Multiple genes determine disease susceptibility for human asthma, for example, genes involved in Ag presentation (*HLA-DR*, *HLA-DQ*, *HLA-DP*), genes that regulate Th2 differentiation and effector function (e.g., *GATA-3*, *IL4*, *IL13*), and IgE-mediated activation of mast cells and basophils (*FCER1B*) (1). Mast cells are among the cells that infiltrate the asthmatic lung and contribute to the acute and the chronic phases of the allergic reaction (2). Mast cells are specialized to react quickly to Ag exposure because the preformed mediators are stored in their active form inside their granules. The stored granule-associated mediators, including histamine, proteoglycans, and several kinds of proteases such as tryptase, are released to the extracellular milieu upon mast cell activation, for example, via IgE-mediated cross-linking of the high-affinity Fc ϵ RI receptors

with Ag. Mast cell tryptase has been linked to allergic airway responses through increased levels in bronchoalveolar lavage (BAL) fluid from asthmatics (3), through its ability to degrade airway neuropeptides (4), and through its ability to work as a mitogen for airway epithelial and smooth muscle cells (5, 6). Moreover, tryptase inhibitors successfully block the inflammatory responses in sheep (7) and mouse (8) models of allergic airway inflammation.

Human β -tryptase shows a close sequence and structural resemblance to mouse mast cell protease-6 (mMCP-6) (9). In the lung and trachea, mMCP-6 is expressed by both connective tissue and mucosal type of mast cells (10). Although studies have implicated mMCP-6 as a proinflammatory mediator in various conditions (11–13), this study is, to our knowledge, the first investigation in a mouse model of allergic airway inflammation using mice genetically devoid of mMCP-6. In this study, a model of OVA-induced allergic lung inflammation was used in which development of airway hyperresponsiveness (AHR), airway inflammation, and goblet cell metaplasia was mast cell-dependent based on comparisons between the Kit^{W/W-v} and Kit^{W-sh/W-sh} mice and their wild type (WT) controls (14). The mMCP-6^{-/-} mice, created using 129/Sv embryonic stem cells (15) and backcrossed for 10 generations to the BALB/c background, still carried the MHC region of 129/Sv (mMCP-6^{-/-}/H-2bc) and not that of BALB/c mice. mMCP-6^{-/-} mice carrying the same MHC locus as the BALB/c mice were obtained after further backcrossing with selection for both the mMCP-6 null allele and the *H-2d* allele. Although the OVA-sensitized and challenged mMCP-6^{-/-}/H-2bc mice were largely protected from the development of all the features of allergic airway inflammation, the mMCP-6^{-/-} mice had no apparent reduction in lung inflammation, or in Th2 cells or IgE-responses. Despite this, the mMCP-6^{-/-} mice had significantly attenuated methacholine-induced AHR and slightly reduced BAL eosinophilia. Thus, mMCP-6 is needed for the development of AHR, whereas the MHC genes contribute to the development of inflammatory Th2 responses.

^{*}Department of Medical Biochemistry and Microbiology, Uppsala University, SE-751 23 Uppsala, Sweden; [†]Department of Pathology and Wildlife Diseases, The National Veterinary Institute, 75189 Uppsala, Sweden; [‡]Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Boston, MA 02115; and [§]Department of Medicine, Harvard Medical School, Boston, MA 02115

¹Y.C. and J.S.D. contributed equally in this study.

²Current address: Department of Pathology, University of Massachusetts Memorial Health Care, Worcester, MA.

³Current address: Division of Rheumatology, Seoul Metropolitan Government–Seoul National University Boramae Medical Center, Seoul, Korea.

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Address correspondence and reprint requests to Dr. Jenny Hallgren, Department of Medical Biochemistry and Microbiology, Uppsala University, Box 582, SE-751 23 Uppsala, Sweden. E-mail address: jenny.hallgren@imbim.uu.se

Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; mMCP-6, mouse mast cell protease-6; PAR-2, protease-activated receptor -2; PAS, periodic acid–Schiff; SNP, single nucleotide polymorphism; WT, wild type.

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Materials and Methods

Mice

mMCP-6^{-/-} mice (15) were backcrossed to the BALB/c background by screening for the mMCP-6 negative allele for 10 generations to generate mMCP-6^{-/-}/H-2*b*c mice. The mMCP-6^{-/-}/H-2*b*c mice were then backcrossed to the BALB/c strain for two more generations, and the heterozygous pups that were I-Ad⁺ (reacted with an Ab recognizing the I-A^d MHC class II alloantigen) were identified by flow cytometry. This analysis was done on B220⁺ blood cells. I-Ad⁺ mMCP-6^{+/-} mice were intercrossed and their I-Ad⁺ pups genotyped by PCR for homozygous loss of mMCP-6 to obtain mMCP-6^{-/-}/H-2*d* knockout mice (referred to as mMCP-6^{-/-} in the *Results* and *Discussion*). BALB/c mice were originally from Bomimice (Ry, Denmark). All mice were bred in-house, and the experiments were conducted under approval of the Animal Ethics Committee, Uppsala, Sweden.

OVA-sensitization and challenge

The mice were injected with 20 µg OVA (Grade V; Sigma-Aldrich, St. Louis, MO) in 100 µl PBS i.p. on days 0 and 14. Starting from day 28, they were challenged with 1% OVA-aerosol in PBS for 30 min on 3 consecutive days. The mice were killed by an overdose of isoflurane or anesthetized for measurements of airway resistance and then killed by cervical dislocation 24–48 h after the last challenge.

Computer analyses

The mouse genome database (Mouse Genome Informatics: <http://www.informatics.jax.org>) and the GRCh38 map were used to obtain single nucleotide polymorphism (SNP) information of the 129/Sv and the BALB/cJ mice genomes between the end of the *Tpsb2* gene and the start of the *H-2k2* gene. The chromosomal localization of the *Tpsb2* and *H-2k2* genes was made using the UCSC genome browser v282 (<http://genome.ucsc.edu/>).

Airway hyperresponsiveness

Mice were anesthetized with 100 mg/kg ketamine, 20 mg/kg xylazine, and 3 mg/kg acepromazine by i.p. injection. A cannula was inserted into the exposed trachea when the reflexes were lost. AHR was measured as the transpulmonary resistance using a whole-body plethysmograph (BUXCO, Wilmington, NC). Mice were ventilated and connected to a preamplifier and computer controller. A second i.p. injection of 0.3 mg ketamine was administered after the end of the surgery. The maximum stroke volume was 0.25 ml, and the respiratory frequency was set to 160 strokes/min. Airway responsiveness was determined in response to increasing doses of aerosolized methacholine (10 µl of 3.125, 6.25, 12.5, 18, 25 mg/ml dissolved in sterile PBS; Sigma-Aldrich). The area under curve resistance for each methacholine concentration was measured and expressed as the mean percentage change over PBS challenge baseline.

ELISA

Blood was collected from the tail artery on days 0, 8, 21, and 28, or the heart (day 31) and the sera were analyzed for OVA-specific IgE and total IgE Abs by ELISA. After clotting, the sera were centrifuged at 11,200 relative centrifugal force for 5 min to remove the remaining clots and RBCs. The sera were collected and kept in -20°C. For all ELISAs, 96-well plates (Immunolon 2HB; Thermo, Milford, MA) were incubated with 2 µg/ml anti-mouse IgE (BD Biosciences, San Diego, CA) overnight at 4°C. Plates were washed with 0.05% Tween/PBS after incubation. The washing was typically performed three times between steps. Dry milk or BSA (Sigma-Aldrich) was used for blocking nonspecific binding sites on the plates. Sera were diluted in dilution buffer (PBS containing 0.05% Tween, 0.02% Na₃, and 0.25% dry milk or 5 mg/ml BSA) to appropriate concentrations and incubated in the blocked plates overnight at 4°C or 2 h at room temperature. Biotinylated OVA-2, 4,6-trinitrophenyl (16) in dilution buffer, followed by streptavidin-alkaline phosphatase in dilution buffer were used for detection of OVA-specific IgE. Alkaline phosphatase-conjugated anti-mouse IgE (Southern Biotech, Birmingham, AL) was added for detection of total IgE. Finally, substrate (*p*-nitro-phenylphosphate; Sigma Aldrich) diluted in diethanolamine buffer (1.0 M diethanolamine, 50 mM MgCl₂ × 6H₂O, pH 9.8) was added and the plate was incubated in the dark for up to 2.5 h. Absorbance was measured at 405 nm, and the Ab titer was expressed as OD₄₀₅ ± SEM.

Lung cell preparation

On day 31 (24 h after the last challenge), the mice were euthanized by isoflurane and the lungs were perfused with 10 ml PBS via the right ventricle. The lung samples were collected in RPMI 1640 complete medium (RPMI

1640 containing 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µg/ml gentamicin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10 mM HEPES, 50 µM 2-ME, 1 mM sodium pyruvate, and 10% heat-inactivated FCS; Sigma-Aldrich) and finely chopped into pieces, followed by digestion with 1800 U Collagenase type IV (Life Technologies, Paisley, U.K.) in 10 ml RPMI 1640 complete. Three consecutive collagenase digestions of lung tissue were performed at 37°C for 20 min for the two first digestions and 30 min for the final digestion. The released lung cells were spun on 44/67% Percoll (Sigma-Aldrich) gradients at 500 relative centrifugal force for 20 min. The mononuclear cells were harvested from the interface of the gradients and washed in RPMI 1640 complete medium. Viable cell counts were determined by trypan blue exclusion on a hemocytometer.

Bronchoalveolar lavage

On day 32 (48 h after the final challenge), mice were euthanized by an overdose of isoflurane, the chest carefully opened, and a tube connected to a 1-ml syringe was inserted into the trachea. The lungs were carefully filled and emptied with 3 × 1 ml PBS. The cells were pelleted by centrifugation and resuspended in 2% FCS in PBS. The total cell numbers were determined by trypan blue exclusion on a hemocytometer.

Flow cytometry

Flow cytometry was performed with conjugated Abs on an LSRII cytometer (BD Biosciences) or FACScan (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR). Abs used included (all anti-mouse): CD4-FITC (L3T4), CD3-PE-Cy5 (17A2), IA^d-PE (AMS-32.1), IA^b-Biotin (KH74), CD11c-allophycocyanin (HL3), CD45-FITC (30-F11), Siglec-F-PE (E50-2440), purified CD16/32 (2.4G2) from BD Biosciences; IL-4-PE (11B11), CD4-PE (GK1.5), B220-PE-Cy5 (RA3-6B2), H-2Kd-PE (SF1-1.1.1) from eBioscience (San Diego, CA); CD3-Alexa 488 (500-A2) from Caltag (Buckingham, U.K.); ST2-biotin (DJ8) from MD Bioproducts (Zürich, Switzerland); H-2Dd-PE (34-2-12) from Biolegend (San Diego, CA). Cells were stained in FACS buffer (2% FCS in PBS, pH 7.4) after preincubation with anti-CD16/32. For intracellular staining of IL-4, restimulation of mononuclear cells was performed as described previously (17).

Histological analysis

Mice were killed by an overdose of isoflurane, and their airways (trachea and lung lobes) were carefully removed. The tissues were fixed overnight in 4% paraformaldehyde and embedded in glycolmethacrylate or in paraffin. For glycol methacrylate-embedded tissues, 2.5 µm sections were made followed by staining with H&E or periodic acid-Schiff (PAS) for goblet cells. Analyses were made using a Leica DM LB2 microscope (Leica Microsystems, Wetzlar, Germany) and a Leica HCX PL Flutotar ×40/0.75 objective lens. For photography, a Nikon DXM1200 camera (Tokyo, Japan) was used together with the Nikon ACT-1 (version 2.70) acquisition software. Semiquantification of the inflammation in Fig. 2D in the lung parenchyma was made by counting the number of bronchovascular bundles associated with inflammatory cells out of a total of 4–15 randomly visualized bundles per section. To semiquantify the difference in goblet cell hyperplasia, we enumerated stained goblet cells in at least three independent bronchovascular bundles from the lung sections obtained for each animal in the different experimental groups. The length of basal lamina of corresponding bronchus was measured by ImageJ. Only the comparable large-caliber, preterminal bronchi (diameter 200–220 µm) were examined, because minimal changes occur in terminal bronchioles. The data were initially expressed as the average of goblet cell counts stained in each bronchus in each section per millimeter basal lamina. Because of large interexperimental variation in the airway inflammation and in the goblet cell hyperplasia of WT mice in response to OVA-sensitization and challenge, the means of the WT response were normalized to 1.0 in each experiment, and the other groups were adjusted with the same factor. For paraffin-embedded tissues, lung specimens were fixed in 10% neutral-buffered formalin overnight. Tissues were routinely processed, embedded in paraffin, sectioned at 4 µm, and stained with H&E or PAS stain. A Nikon E600 microscope and a Nikon DXM1200 were used for the initial histological examinations and photography in Fig. 2C.

Statistical analysis

All data except the data shown in Fig. 2 were analyzed using unpaired two-tailed Student *t* test. The data shown in Fig. 2 were analyzed using one-way ANOVA with a Tukey's test for multiple comparisons. All statistical analyses were made using GraphPad Prism version 5.0d for Mac OS X (GraphPad Software, San Diego, CA; <http://www.graphpad.com>). Data were considered significant if *p* < 0.05 and were denoted according to the

following scheme: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. All data are reported as the mean $\pm$ SEM.

Results

Mapping of genetic differences between mMCP-6^{-/-}/H-2bc and mMCP-6^{-/-} mice

During the progress of this study, the mMCP-6-deficient mice backcrossed to BALB/c were discovered to have another genetic difference in comparison with the BALB/c strain. They were carrying the 129/Sv MHC (*H-2bc*) and not the BALB/c MHC (*H-2d*), because of close proximity of the mMCP-6 and MHC loci on chromosome 17 (Fig. 1A). Hence the mMCP-6^{-/-}/H-2bc mice were further backcrossed to the BALB/c strain, and the pups were screened for the mMCP-6 null allele and I-Ad allele (see *Materials and Methods*). The resulting I-Ad⁺ mMCP-6^{-/-} mice and the mMCP-6^{-/-}/H-2bc mice were screened for other differences in the MHC locus using an H-2Kd-specific Ab recognizing the first locus in the H-2 complex and an H-2Dd-specific Ab recognizing the D region in the end of the H-2 complex. We found that the mMCP-6^{-/-}/H-2bc mice were negative for H2-Kd, I-Ad, and H2-Dd, but positive for I-Ab, whereas the mMCP-6^{-/-} mice (and the BALB/c mice) were negative for I-Ab, but positive for H2-Kd, I-Ad, and H2-Dd (Fig. 1B).

To find out whether there was strain variability between the 129/Sv and the BALB/c parental strains, we performed a computer database analysis of SNPs in the region between *Tpsb2* and the *H-2* complex. Only 8 SNPs of 499 found in this region of the genome were different between 129/Sv and BALB/c strains. Because only minor differences were found, the exact position of the recombination event was not identified.

AHR is reduced in sensitized and challenged mMCP-6^{-/-} and mMCP-6^{-/-}/H-2bc mice

As a measure of lung function, the airway resistance in response to increasing doses of aerosolized methacholine was measured (Fig. 2A). OVA-challenge of sensitized WT mice resulted in an increase in airway resistance (Fig. 2B). However, the airway resistance was significantly lower in mMCP-6^{-/-} than in WT mice (Fig. 2B). The airway resistance in sensitized and OVA-challenged

mMCP-6^{-/-}/H-2bc mice was at a similar level as the corresponding mMCP-6^{-/-} mice. Altogether, this suggests that mMCP-6 plays an important role in the development of AHR.

mMCP-6^{-/-}/H-2bc mice have attenuated inflammatory responses, whereas mMCP-6^{-/-} mice have intact responses after sensitization and challenge

Histological examination of the lungs from mice that were only sensitized showed no signs of inflammation or increased mucous production (data not shown). However, a moderate inflammation was seen in sensitized WT and mMCP-6^{-/-} mice after challenge (Fig. 2C, left and middle panels). The lung inflammation was blindly scored and semiquantified by estimating the percentage of bronchovascular bundles associated with cell infiltrations. However, no differences were found between WT and mMCP-6^{-/-} mice (Fig. 2C, left and middle panels, 2D). In contrast, sensitized mMCP-6^{-/-}/H-2bc mice showed only mild signs of inflammation after challenge (Fig. 2C, right panels, Fig. 2D). Further, sensitized and challenged WT and mMCP-6^{-/-} mice had a higher degree of activated mucous-producing goblet cells than mMCP-6^{-/-}/H-2bc mice (Fig. 2E).

To quantify the level of lung inflammation, we analyzed eosinophils (CD45⁺, CD11c^{-lo}, Siglec-F⁺ cells) in BAL using flow cytometry (Fig. 3A) (18). Sensitized and challenged WT mice had a striking eosinophilia (Fig. 3B, 3C). The mMCP-6^{-/-} mice had a small but significant decrease in BAL eosinophils (Fig. 3B) compared with the WT mice, whereas the mMCP-6^{-/-}/H-2bc mice had dramatically fewer eosinophils (Fig. 3C). In summary, the loss of mMCP-6 has a minor effect on OVA-induced airway inflammation. In contrast, the MHC-haplotype plays a major role for the development of eosinophilic inflammation and mucous production.

Th2 responses and IgE levels are reduced in mMCP-6^{-/-}/H-2bc mice, whereas mMCP-6^{-/-} mice have intact responses after sensitization and challenge

Because the development of allergic lung inflammation and AHR is related to the production of Th2 cytokines, attempts were made to measure cytokine production in BAL fluid after sensitization

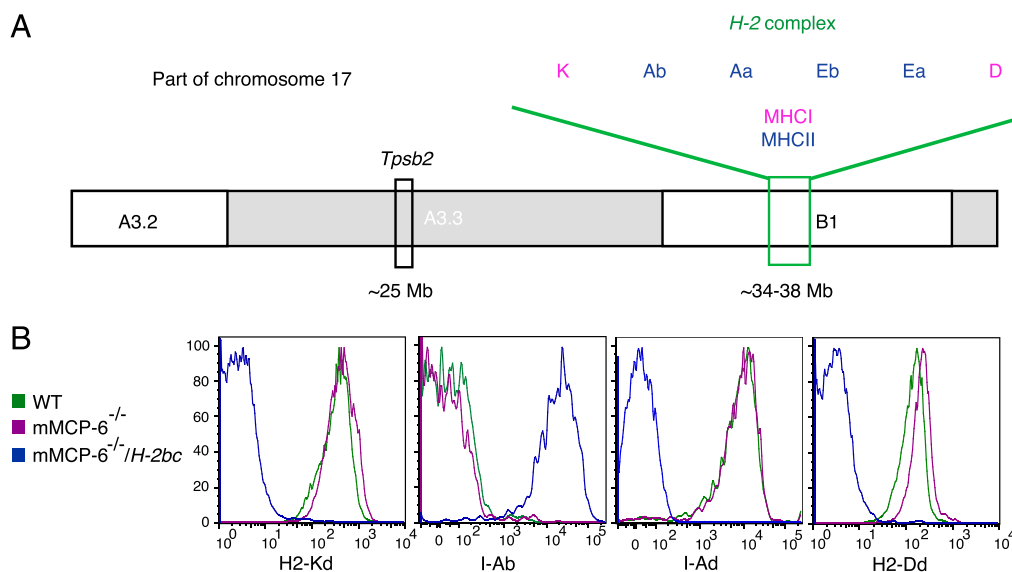
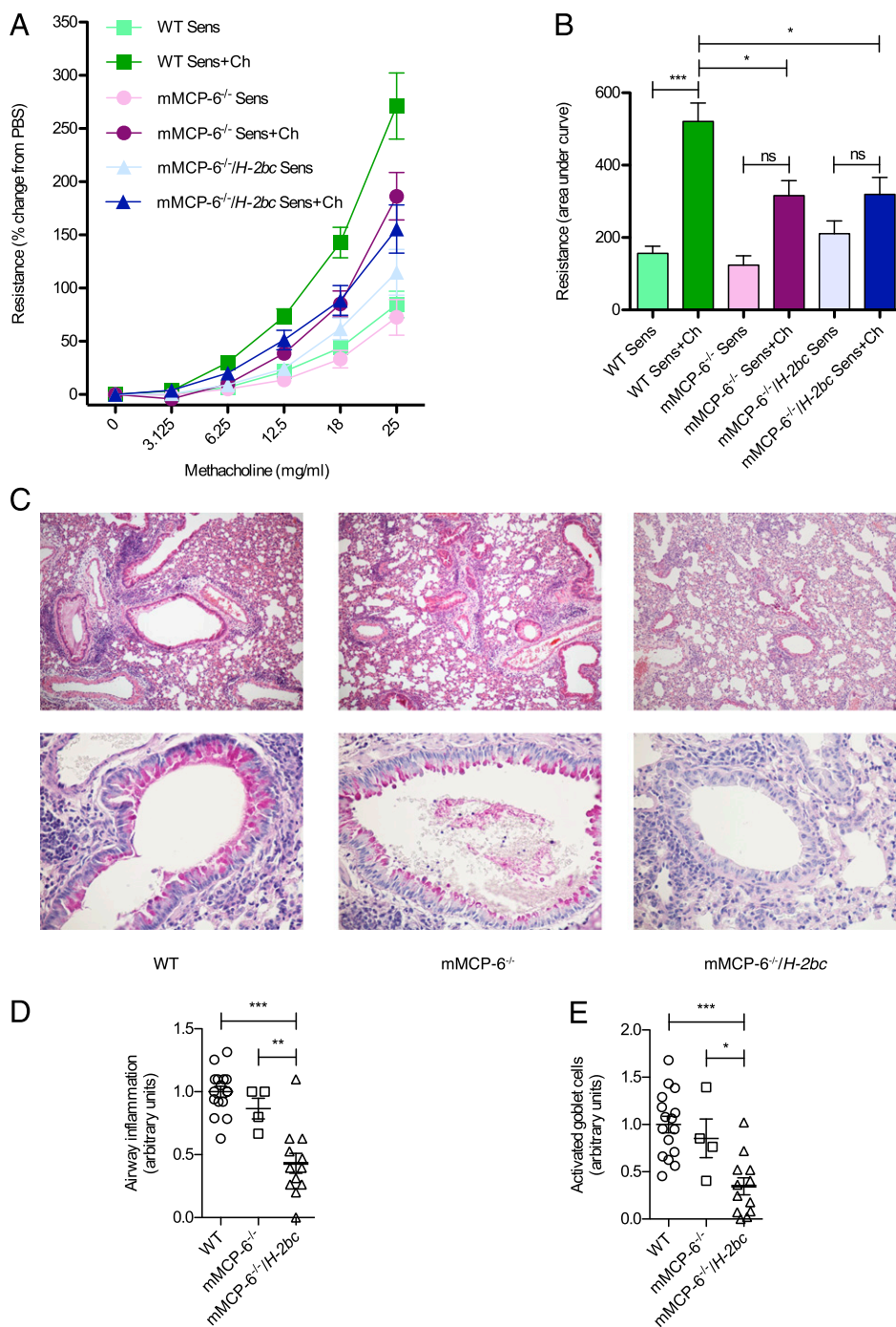


FIGURE 1. Mapping of the genetic differences in the *H-2* locus. **(A)** The gene coding for mMCP-6 (*Tpsb2*) is localized on chromosome 17 in A3.3 (~25 Mb). This is in close proximity to the *H-2* complex, located in B1 (~34–38 Mb), which codes for MHC class I (MHCI) and MHC class II (MHCII). **(B)** Mapping of the *H-2* complex using strain-specific Abs recognizing (from left) H2-Kd (MHCI type of BALB/c), I-Ab (MHCII type of 129/Sv), I-Ad (MHCII type of BALB/c), H2-Dd (MHCI type of BALB/c). This analysis was done on B220⁺ blood cells.

FIGURE 2. AHR is reduced in sensitized and challenged mMCP-6^{-/-} and mMCP-6^{-/-}/H-2bc mice. **(A)** and **(B)** Airway resistance toward increasing concentrations of aerosolized methacholine was measured in OVA-sensitized (Sens) mice or OVA-sensitized and challenged (Sens + Ch) mice. Graphs represent pooled data from five separate experiments. Each data point represents 7–20 mice. **(B)** The mean area under curve resistance was calculated and compared between each group of mice. **p* < 0.05 and ****p* < 0.001, respectively; ns indicates *p* > 0.05. **(C–E)** Lungs from OVA-sensitized and challenged mice were evaluated by histology. **(C)** Representative pictures of lung sections showing bronchoalveolar bundles stained with H&E (*top panels*, original magnification ×10) or PAS stain (*bottom panels*, original magnification ×40). The number of bronchovascular bundles associated with inflammatory cells **(D)** and the number of activated goblet cells/mm **(E)** were semiquantified in three experiments, and the mean response in sensitized and challenged WT mice was normalized to 1.0 in each individual experiment. One experiment was performed with all three genotypes. All mice are shown. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.



and challenge. However, the levels were largely undetectable, perhaps because of the mild protocol used. Instead, IL-4⁺ CD3⁺ CD4⁺ cells (IL-4⁺ Th cells) in the lung were quantified using flow cytometry. mMCP-6^{-/-} mice displayed intact mononuclear cell yields, total lung CD3⁺CD4⁺ cell (Th cell) numbers, as well as similar levels of IL-4⁺ Th cells as compared with WT mice after sensitization and challenge (Fig. 4A). In sharp contrast, sensitized and challenged mMCP-6^{-/-}/H-2bc had a significant ~70% reduction in mononuclear cells and CD3⁺CD4⁺ cells per lung as compared with WT mice treated in parallel (Fig. 4B). The percentage of IL-4⁺ Th cells was around 2–3% in sensitized and challenged WT and mMCP-6^{-/-} lungs (Fig. 4A, 4B). In contrast, the mMCP-6^{-/-}/H-2bc mice treated in parallel had only around 1% IL-4⁺ Th cells (Fig. 4B). The receptor for IL-33, ST2, is expressed by Th2 cells (19). Although sensitized and challenged mMCP-6^{-/-} mice had

similar levels of ST2⁺ Th cells (Fig. 4A), mMCP-6^{-/-}/H-2bc mice had less ST2⁺ Th cells than the WT controls (Fig. 4B).

Because the mMCP-6^{-/-}/H-2bc mice appeared to have severely impaired Th2-induced airway responses, the IgE levels were measured in serum during the course of the protocol. The OVA-specific IgE response was attenuated in the mMCP-6^{-/-}/H-2bc mice from day 21, whereas the mMCP-6^{-/-} mice displayed a similar development of OVA-specific IgE as the WT mice (Fig. 4C). Furthermore, the mMCP-6^{-/-} and WT mice had similar levels of total IgE at most time points (Fig. 4D, left panel). However, for unknown reasons, the levels on day 8 were attenuated in the mMCP-6^{-/-} mice in one of two experiments. In contrast, the mMCP-6^{-/-}/H-2bc mice had less total IgE even before sensitization and continued to have lower levels during the course of the protocol (Fig. 4D, right panel). These results indi-

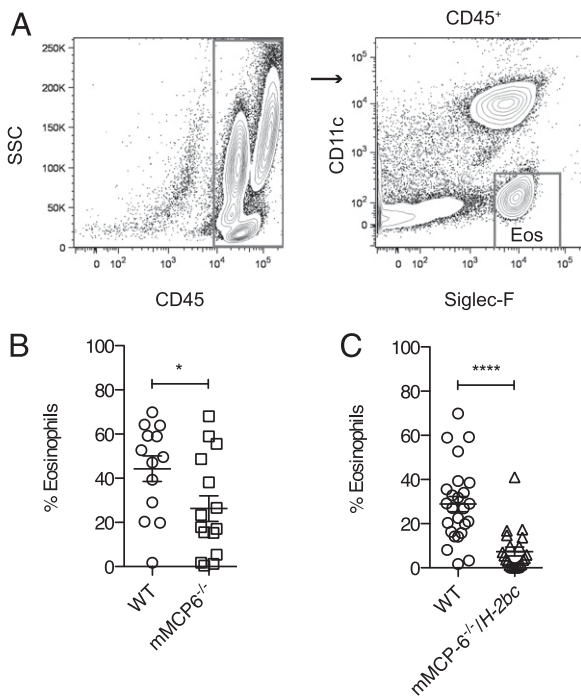


FIGURE 3. BAL eosinophilia is slightly reduced in sensitized and challenged mMCP-6^{-/-} and dramatically reduced in mMCP-6^{-/-}/H-2bc mice. BAL eosinophils from sensitized and challenged mice were quantified using flow cytometry by the gating strategy shown in (A). The percentage of eosinophils in sensitized and challenged WT and mMCP-6^{-/-} mice (B) and WT and mMCP-6^{-/-}/H-2bc (C). Data shown in (B) are pooled data from 3 separate experiments with in total 13 WT and 15 mMCP-6^{-/-} mice, whereas the data shown in (C) are from 5 separate experiments with 25 WT and 22 mMCP-6^{-/-}/H-2bc mice. All three genotypes were included in two of the experiments. Hence nine WT mice are represented in both (B) and (C). **p* < 0.05, *****p* < 0.0001, respectively.

cate that although mMCP-6 is dispensable for the Th2 cell and IgE responses, the MHC-haplotype is critical for the development of these parameters.

Discussion

Proof of the involvement of mast cells in models of experimental asthma is largely derived from experiments in which mice devoid of mast cells have a significantly milder “asthma” phenotype than their WT counterparts. Furthermore, the contribution of single mast cell mediators has been addressed previously (14, 20, 21). In our study, the role of mMCP-6 was studied in an OVA-induced pulmonary inflammation model using sensitization i.p. without adjuvant, followed by three daily OVA-aerosol challenges (14). In this model, mast cell-deficient mice (Kit^{W-sh/W-sh} and Kit^{W/W-v}) develop significantly less AHR, airway inflammation, and goblet cell metaplasia, whereas Ab responses are intact (14). As expected, IgE responses were also intact in the mice that lack mMCP-6. Strikingly, the overall inflammatory response as measured by goblet cell activation and cellular infiltrates, as well as the Th2 cell response in the lung, was intact, suggesting that mMCP-6 is also dispensable in this regard. Nevertheless, there was variation in the level of inflammation and goblet cell metaplasia between experiments. To compare the level of lung inflammation by a quantitative method, we analyzed BAL eosinophils by flow cytometry. These analyses demonstrated that mMCP-6^{-/-} mice had a small decrement in BAL eosinophils compared with WT mice.

Despite the minor effect of loss of mMCP-6 on pulmonary inflammation, AHR to increasing doses of methacholine was re-

duced by ~40% in the OVA-sensitized and -challenged mMCP-6^{-/-} mice. Protease-activated receptor-2 (PAR-2) is expressed in smooth muscle, epithelial cells, and endothelial cells in the lung, and has been shown to mediate AHR and inflammation in a mouse model of experimental asthma (22). Human β -tryptase, which shares sequence similarity (9) and biochemical properties (23, 24) with mMCP-6, has been implicated in the cleavage and activation of human PAR-2 (25, 26). Another study questioned this because of an N-terminal glycosylation site that makes human PAR-2 a poor substrate for human β tryptase (27). PAR-2 is a more likely substrate for mMCP-6 because mouse PAR-2 naturally lacks the N-terminal glycosylation site (28). Hence the level of PAR-2 protein was compared by Western blot analysis of homogenized lung tissue from sensitized and challenged mMCP-6^{-/-} and WT mice (data not shown). Two close bands of approximately the right size to be the intact and cleaved form of PAR-2 were detected. However, control experiments showed that one of the two bands was nonspecific and that only one form of PAR-2 could be detected. Thus, another experimental approach is necessary to evaluate the possible involvement of PAR-2 in the mMCP-6-mediated effects on AHR.

The MHC locus is associated with human asthma in most population studies (1). In the mouse, this locus resides on chromosome 17, close to the mMCP-6 gene locus (~25 Mbp, on chromosome 17). Another goal of our investigation was to study the contribution of the MHC locus on the development of features of allergic airway inflammation. In comparison with WT (*H-2d*) mice, the mMCP-6^{-/-}/H-2bc mice had reduced OVA-specific and total IgE levels upon sensitization and challenge. In addition, mMCP-6^{-/-}/H-2bc mice had a striking phenotype with largely suppressed eosinophilia, decreased airway inflammation, and goblet cell hyperplasia, and a diminished Th2 cell response. The suppressed response in mMCP-6^{-/-}/H-2bc mice was likely because of the difference in MHC, because the mMCP-6^{-/-} mice had inflammatory Th2 responses that were comparable with the WT controls. Our results are in agreement with a study of two strains that carry C57 type MHC genes on chromosome 17 including the *H-2b* haplotype of MHC on the BALB/c background using a similar model of experimental asthma (29). These strains had a suppressed inflammatory Th2 response in comparison with the WT controls, which suggested that the *H-2* genes were responsible for the diminished inflammatory Th2 response. The 129/Sv strain, which was used to create the mMCP-6 knockout, shares identical *I-Ab*-type loci in the classical *H-2* region, but there are major differences in the *H-2* T-region, and thus they are referred to as *bc* instead of *b* (30). Nevertheless, our results with the mMCP-6^{-/-}/H-2bc mice agree to a large extent with the study by Nawijn et al. (29) in that the type of MHC is very important for the magnitude of allergic airway inflammation. The importance of MHC genes is expected because the proteins encoded by these genes are critical for Ag presentation, which is a key step in the development and effector phase of adaptive immune responses.

There are also minor strain variations within the region between *Tpsb2* and the *H-2* complex according to our database analysis of SNPs from BALB/cJ and 129/Sv strains. Of 507 SNPs, 8 were different. These differences were found in introns, untranscribed regions, or were unannotated. Although we cannot rule out possible roles for these SNPs, we believe they play no or minor roles in our study. Apart from mMCP-6, mMCP-7 is a second mouse tryptase that is also related to human β -tryptase. The mMCP-7 gene (*Tpsab1*), which is lacking in C57BL/6 mice, resides just upstream of the *Tpsb2* in both 129/Sv and BALB/c strains, as well as in the mMCP-6^{-/-} mice (15). Thus, differential mMCP-7 expression cannot explain the differences between the mMCP-6/H-2bc

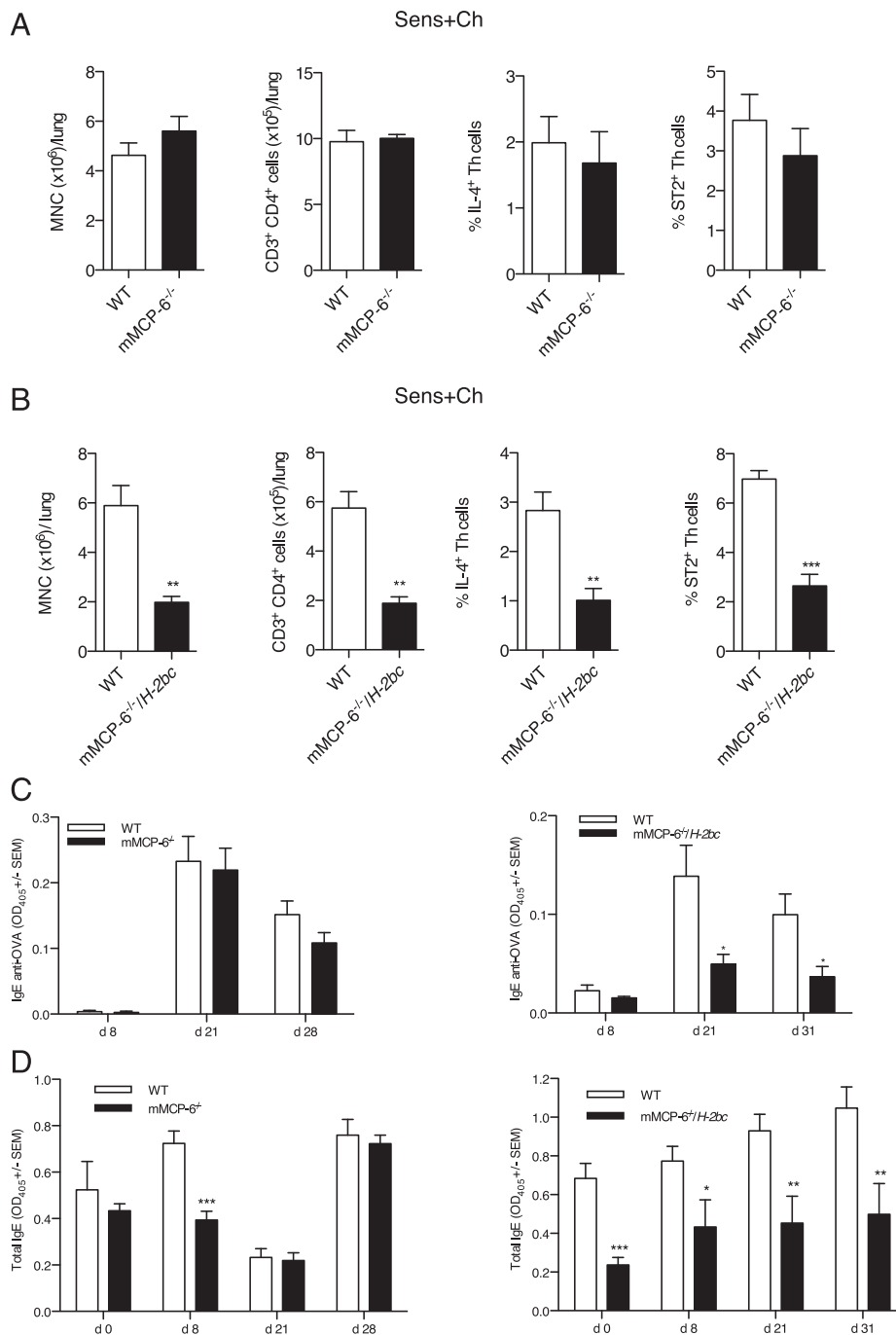


FIGURE 4. Th2 and IgE responses are impaired in sensitized and challenged mMCP-6^{-/-}/H-2bc mice. **(A)** The mean number of mononuclear cells (MNC), CD3⁺ CD4⁺ (Th) cells/lung, percent IL-4⁺ Th cells, and percent ST2⁺ Th cells were quantified in groups of sensitized and challenged WT and mMCP-6^{-/-} mice **(A)** or WT and mMCP-6^{-/-}/H-2bc **(B)** treated in parallel and analyzed 24 h after the final challenge. Data are representative of two independent experiments with four mice in each group for **(A)** and three independent experiments with three to four mice in each group for **(B)**. **(C and D)** Groups of mice (left, $n = 13-15$, right, $n = 6-7$) were bled days 0 (before sensitization), 8, 21, and 28, or 31 of the OVA protocol, and the levels of OVA-specific IgE **(C)** or total IgE **(D)** in serum were quantified by ELISA. Data shown in the *left panels* in **(C)** and **(D)** are representative of two independent experiments, whereas the data shown in the *right panels* of **(C)** and **(D)** are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

and the mMCP-6^{-/-} mice. Other studies using similar OVA models of experimental asthma have demonstrated that the 129/Sv (H-2bc) strain is hyporesponsive in terms of AHR, OVA-specific Ab responses (31, 32), as well as lung Th cell responses (31) and eosinophilic inflammation (32) in comparison with the BALB/c strain. The major factor for the reduced allergic lung inflammation is likely the difference in the H-2 region between these strains. Along with the inflammatory Th2 response, AHR is reduced in sensitized and challenged mice with the H-2b haplotype on the BALB/c background (29). However, in our study, AHR was reduced not only in mMCP-6/H-2bc mice but also in mMCP-6^{-/-} mice despite largely intact lung inflammation and intact Th2 cell and IgE responses. Thus, mMCP-6 increases the magnitude of AHR by a mechanism that does not affect the development of inflammatory Th2 responses to the same degree. Our data suggest

a clear mMCP-6-dependent and therefore mast cell-dependent effect on AHR. The importance of mast cells for development of AHR is supported by Sawaguchi et al. (33). In this study, mice were depleted of mast cells and basophils (Mas-TRECK mice) or basophils specifically (Bas-TRECK mice) during the challenge phase in an OVA model of allergic asthma. Similarly, they found that AHR, but not airway inflammation, was dependent on mast cells.

To summarize, our study demonstrates that the development of AHR is dependent on mMCP-6, whereas the development of lung inflammation, IgE, and Th2 responses are predominantly independent of mMCP-6 but highly dependent on the haplotype of MHC. Our findings also highlight the risk that neighboring genes to the gene that is knocked out or modified have a low probability of being changed to the right genotype on backcrossing when selecting only for the knocked out/modified gene, and that these

other genes can have a significant impact on the outcome of the experiments.

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Disclosures

The authors have no financial conflicts of interest.

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