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

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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 (FCERIB) (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 FceRI 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 KitW/W−/− and KitW−/− 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.

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; mMCP-6, mouse mast cell protease-6; PAR-2, protease-activated receptor-2; P4AS, 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-2b mice. The mMCP-6−/−H-2b 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-Ad 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-2d knockout mice (referred to as mMCP-6−/− in the Results and Discussion). BALB/c mice were originally from Bommice (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 measurement of airway resistance and then killed by cervical dislocation 24–48 h after the last the challenge.

Computer analyses

The mouse genome database (Mouse Genome Informatics: http://www.informatics.jax.org) and the GRCm38 map were used to obtain single nucleotide polymorphism (SNP) information of the 129/Sv and the BALB/cI mice genomes between the end of the Tpoβ2 gene and the start of the H-2Kβ gene. The chromosomal localization of the Tpoβ2 and H-2Kβ 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 transmural 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 made using the UCSC genome browser v282 (http://genome.ucsc.edu/).

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 1,200 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% NaN3, 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-nitrophenylphosphate; 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 pMCP-6 AND MHC IN ALLERGIC AIRWAY RESPONSES

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
Results

Mapping of genetic differences between mMCP-6−/−/129/Sv and mMCP-6−/−/H-2bc 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 H-2Kd, I-Ab, and H-2-Dd, but positive for I-Ab, whereas the mMCP-6−/− mice (and the BALB/c mice) were negative for I-Ab, but positive for H-2-Kd, I-Ad, and H-2-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−/−/129/Sv 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 mucus 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−/−/129/Sv 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 mucus-producing goblet cells than mMCP-6−/−/H-2bc mice (Fig. 2E).

To quantify the level of lung inflammation, we analyzed eosinophils (CD45+, CD11c−/0, 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 mucus production.

Th2 responses and IgE levels are reduced in mMCP-6−/−/129/Sv 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.
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 2](http://www.jimmunol.org/)

**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.
cated 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<sup>W-sh/W-sh</sup> and Kit<sup>W/W-</sup>) 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<sup>–/–</sup> 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 reduced by ~40% in the OVA-sensitized and -challenged mMCP-6<sup>–/–</sup> 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 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<sup>–/–</sup> 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<sup>–/–</sup>/H-2(bc) mice had reduced OVA-specific and total IgE levels upon sensitization and challenge. In addition, mMCP-6<sup>–/–</sup>/H-2(bc) 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<sup>–/–</sup>/H-2(bc) mice was likely because of the difference in MHC, because the mMCP-6<sup>–/–</sup> 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-A<sup>b</sup>-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<sup>–/–</sup>/H-2(bc) 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<sup>–/–</sup> mice (15). Thus, differential mMCP-7 expression cannot explain the differences between the mMCP-6/H-2bc
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

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

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Disclosures
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References