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Mouse mast cell protease -6 and MHC are involved in the development of experimental asthma[1]

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Abstract

Allergic asthma is a complex disease with a strong genetic component where mast cells play a major role by the release of pro-inflammatory 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. While OVA-sensitized and challenged wild type mice displayed a striking airway hyperresponsiveness (AHR), mMCP-6$^{-/-}$ mice had less AHR that was comparable to 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 (BAL) 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 while mMCP-6 plays a key role in the development of AHR.

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Introduction

Multiple genes determine disease susceptibility for human asthma e.g. genes involved in antigen 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 amongst the cells infiltrating 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 antigen exposure because the pre-formed 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, e.g. via IgE-mediated crosslinking of the high affinity FcεRI receptors with antigen. Mast cell tryptase has been linked to allergic airway responses through increased levels in 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 pro-inflammatory mediator in various conditions (11-13), the present study is to our knowledge the first investigation in a mouse model of allergic airway inflammation using mice genetically devoid of mMCP-6. Here, a model of OVA-induced allergic lung inflammation was used in which development of AHR, airway inflammation and goblet cell metaplasia was mast cell-dependent based on comparisons between the $Kit^{W/W\text{-}v}$ and $Kit^{W\text{-}sh/W\text{-}sh}$ mice and their wild type 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. While 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 while the MHC genes contribute to the development of inflammatory Th2 responses.

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-2bc* mice. The mMCP-$6^{-/-}$/*H-2bc* 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-2d* knockout mice (referred 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 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 d 0 and d 14. Starting from d 28, they were challenged with 1% OVA-aerosol in PBS for 30 min on three 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 hours after the last the challenge.

Computer analyses

The mouse genome database (MGI: www.informatics.jax.org) and the GRCm38 map was 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 were made using the UCSC genome browser v282 (http://genome.ucsc.edu/).

Airway hyperresponsiveness

Mice were anaesthetized with 100 mg/kg of Ketamine, 20 mg/kg of Xylazine and 3 mg/kg of 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 of 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 (Sigma-Aldrich, 10 μl of 3.125, 6.25, 12.5, 18, 25 mg/ml dissolved in sterile PBS). 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 d 0, 8, 21, 28 or the heart (d 31) and the sera were analyzed for OVA-specific IgE and total IgE Abs by ELISA. After clotting, the sera were centrifuged at 11200 rcf for 5 min to remove the remaining clots and red blood cells. 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% NaN_3 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 $MgCl_2 \times 6H_2O$, 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 $OD_{405} \pm SEM$.

Lung cell preparation

On d 31 (24 h after the last challenge), the mice were euthanized by isoflurane and the lungs were perfused with 10 ml of 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 units of collagenase type IV (Gibco, Paisley, Scotland, UK) 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 rcf 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 hemacytometer.

Bronchoalveolar lavage

On d 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 × 1ml 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 hemacytometer.

Flow cytometry

Flow cytometry was performed with conjugated Abs on a 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-PECy5 (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/CD32 (2.4G2) from BD Biosciences; IL4-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, UK); 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 pre-incubation with anti-CD16/32. For intracellular staining of IL-4, re-stimulation of mononuclear cells was performed as described (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. Semi-quantification of the inflammation in Fig. 2*D* 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 semi-quantify the difference in goblet cell hyperplasia, stained goblet cells were enumerated 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-calibre, preterminal bronchi (diameter 200-220 μm) were examined, since 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 mm basal lamina. Because of large inter-experimental variation in the airway inflammation and in the goblet cell hyperplasia of wild type mice in response to OVA-sensitization and challenge, the means of the wild type response were normalized to 1.0 in each experimental and the other groups 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. 2*C*.

Statistical analysis

All data except the data shown in Fig. 2 were analyzed using unpaired two-tailed Student's 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 ± 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 to the BALB/c strain. They were carrying the 129/Sv MHC (*H-2bc*) and not the BALB/c MHC (*H-2d*), due to close proximity of the mMCP-6 and MHC loci on chromosome 17 (Fig. 1*A*). 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 Material 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 a H-2Kd specific Ab recognizing the first locus in the H-2 complex and a 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 I-Ab negative but positive for H2-Kd, I-Ad and H2-Dd (Fig. 1*B*).

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

Airway hyperresponsiveness 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. 2*A*). OVA-challenge of sensitized wild type mice resulted in an increase in airway resistance (Fig. 2*B*). However, the airway resistance was significantly lower in mMCP-6$^{-/-}$ than in wild type mice (Fig. 2*B*). 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 mucus production (data not shown). However, a moderate inflammation was seen in sensitized wild type and mMCP-6$^{-/-}$ mice after challenge (Fig. 2*C*, left and middle). The lung inflammation was blindly scored and semi-quantified by estimating the percentage of bronchovascular bundles associated with cell infiltrations. However, no differences were found between wild type and mMCP-6$^{-/-}$ mice (Fig. 2*C* left and middle, Fig. 2*D*). In contrast, sensitized mMCP-6$^{-/-}$/*H-2bc* mice only showed mild signs of inflammation after challenge (Fig. 2*C* right, Fig. 2*D*). Further, sensitized and challenged wild type and mMCP-6$^{-/-}$ mice had a higher degree of activated mucus-producing goblet cells than mMCP-6$^{-/-}$/*H-2bc* mice (Fig. 2*E*).

To quantify the level of lung inflammation, eosinophils (CD45^{+}, CD11c$^{-/lo}$, Siglec-F^{+} cells) in BAL were analyzed using flow cytometry (Fig. 3*A*) (18). Sensitized and challenged wild type mice had a striking eosinophilia (Fig. 3*B*, *C*). The mMCP-6$^{-/-}$ mice had a small but significant decrease in BAL eosinophils (Fig. 3*B*) compared to the wild type mice whereas the mMCP-6$^{-/-}$/*H-2bc* mice had dramatically fewer eosinophils (Fig. 3*C*). To summarize, 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$^{-/-}$/H-2bc mice whereas mMCP-6$^{-/-}$ mice have intact responses after sensitization and challenge

As 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 due to 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 to wild type mice after sensitization and challenge (Fig. 4*A*). In sharp contrast, sensitized and challenged mMCP-6$^{-/-}$/*H-2bc* had a significant ~70% reduction in mononuclear cells and CD3$^+$CD4$^+$ cell per lung as compared to wild type mice treated in parallel (Fig. 4*B*). The percentage of IL-4$^+$ Th cells was around 2-3% in sensitized and challenged wild type and mMCP-6$^{-/-}$ lungs (Fig. 4*A*, *B*). In contrast, the mMCP-6$^{-/-}$/*H-2bc* mice treated in parallel had only around 1% IL-4$^+$ Th cells (Fig. 4*B*). The receptor for IL-33, ST2, is expressed by Th2 cells (19). While sensitized and challenged mMCP-6$^{-/-}$ mice had similar levels of ST2$^+$ Th cells (Fig. 4*A*), mMCP-6$^{-/-}$/*H-2bc* mice had less ST2$^+$ Th cells than the wild type controls (Fig. 4*B*).

Since 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 d 21 whereas the mMCP-6$^{-/-}$ mice displayed a similar development of OVA-specific IgE as the wild type mice (Fig. 4*C*). Furthermore, the mMCP-6$^{-/-}$ and wild type mice had similar levels of total IgE at most time points (Fig. 4*D* left). However, for unknown reasons the levels on d 8 were attenuated in the mMCP-6$^{-/-}$ mice in one out 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. 4*D* right). These results indicate that while 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 are largely derived from experiments in which mice devoid of mast cells have a significantly milder “asthma” phenotype than their wild type 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$^{W\text{-}sh/W\text{-}sh}$ and Kit$^{W/W\text{-}v}$) 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 lacking 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 dispensable also 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, BAL eosinophils were analyzed by flow cytometry. These analyses demonstrated that mMCP-6$^{-/-}$ mice had a small decrement in BAL eosinophils compared to wild type mice.

Despite the minor effect of loss of mMCP-6 on pulmonary inflammation, AHR to increasing doses of methacholine was reduced by approximately 40% in the OVA-sensitized and challenged mMCP-6$^{-/-}$ mice. Protease-activated receptor -2 (PAR-2) is expressed in smooth muscle, epithelial and endothelial cells in the lung and has been shown to mediate AHR and inflammation in a mouse model experimental asthma (22). Human β-tryptase, which share 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 a N-terminal glycosylation site that makes human PAR-2 a poor substrate for human beta tryptase (27). PAR-2 is a more likely substrate for mMCP-6 since 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 wild type 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 mega bp, 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 to wild type (*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 due to the difference in MHC, since the mMCP-6$^{-/-}$ mice had inflammatory Th2-responses that were comparable to the wild type 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 to the wild type 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 since the proteins encoded by these genes are critical for antigen 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. Out 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 none or minor roles in our study. Apart from mMCP-6, mMCP-7 is a second mouse tryptase 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* 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 antibody responses (31, 32) as well as lung Th cell responses (31) and eosinophilic inflammation (32) in comparison to 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 while 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 upon 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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Abbreviations used in this article

AHR	Airway hyperresponsiveness
BAL	Bronchoalveolar lavage
OVA	Ovalbumin

PAR-2	Protease-activated receptor -2
PAS	Periodic Acid Schiff
SNP	Single nucleotide polymorphism
Th	T helper

References

1. Vercelli D. Discovering susceptibility genes for asthma and allergy. Nat. Rev. Immunol. 2008; 8:169–182. [PubMed: 18301422]
2. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature. 2008; 454:445–454. [PubMed: 18650915]
3. Schwartz LB. Tryptase: a clinical indicator of mast cell-dependent events. Allergy Proc. 1994; 15:119–123. [PubMed: 7926709]
4. Tam EK, Caughey GH. Degradation of airway neuropeptides by human lung tryptase. Am. J. Respir. Cell Mol. Biol. 1990; 3:27–32. [PubMed: 1694672]
5. Brown JK, Tyler CL, Jones CA, Ruoss SJ, Hartmann T, Caughey GH. Tryptase, the dominant secretory granular protein in human mast cells, is a potent mitogen for cultured dog tracheal smooth muscle cells. Am. J. Respir. Cell Mol. Biol. 1995; 13:227–236. [PubMed: 7626290]
6. Cairns JA, Walls AF. Mast cell tryptase is a mitogen for epithelial cells. Stimulation of IL-8 production and intercellular adhesion molecule-1 expression. J. Immunol. 1996; 156:275–283. [PubMed: 8598474]
7. Clark JM, Abraham WM, Fishman CE, Forteza R, Ahmed A, Cortes A, Warne RL, Moore WR, Tanaka RD. Tryptase inhibitors block allergen-induced airway and inflammatory responses in allergic sheep. Am. J. Respir. Crit. Care Med. 1995; 152:2076–2083. [PubMed: 8520778]
8. Oh SW, Pae CI, Lee DK, Jones F, Chiang GK, Kim HO, Moon SH, Cao B, Ogbu C, Jeong KW, Kozu G, Nakanishi H, Kahn M, Chi EY, Henderson WR Jr. Tryptase inhibition blocks airway inflammation in a mouse asthma model. J. Immunol. 2002; 168:1992–2000. [PubMed: 11823536]
9. Hallgren J, Pejler G. Biology of mast cell tryptase. An inflammatory mediator. FEBS J. 2006; 273:1871–1895. [PubMed: 16640553]
10. Xing W, Austen KF, Gurish MF, Jones TG. Protease phenotype of constitutive connective tissue and of induced mucosal mast cells in mice is regulated by the tissue. Proc. Natl. Acad. Sci. U. S. A. 2011; 108:14210–14215. [PubMed: 21825171]
11. McNeil HP, Shin K, Campbell IK, Wicks IP, Adachi R, Lee DM, Stevens RL. The mouse mast cell-restricted tetramer-forming tryptases mouse mast cell protease 6 and mouse mast cell protease 7 are critical mediators in inflammatory arthritis. Arthritis Rheum. 2008; 58:2338–2346. [PubMed: 18668540]
12. Shin K, Nigrovic PA, Crish J, Boilard E, McNeil HP, Larabee KS, Adachi R, Gurish MF, Gobezie R, Stevens RL, Lee DM. Mast cells contribute to autoimmune inflammatory arthritis via their tryptase/heparin complexes. J. Immunol. 2009; 182:647–656. [PubMed: 19109198]
13. Hamilton MJ, Sinnamon MJ, Lyng GD, Glickman JN, Wang X, Xing W, Krilis SA, Blumberg RS, Adachi R, Lee DM, Stevens RL. Essential role for mast cell tryptase in acute experimental colitis. Proc. Natl. Acad. Sci. U. S. A. 2011; 108:290–295. [PubMed: 21173247]
14. Reuter S, Heinz A, Sieren M, Wiewrodt R, Gelfand EW, Stassen M, Buhl R, Taube C. Mast cell-derived tumour necrosis factor is essential for allergic airway disease. Eur. Respir. J. 2008; 31:773–782. [PubMed: 18094004]
15. Shin K, Watts GF, Oettgen HC, Friend DS, Pemberton AD, Gurish MF, Lee DM. Mouse mast cell tryptase mMCP-6 is a critical link between adaptive and innate immunity in the chronic phase of Trichinella spiralis infection. J. Immunol. 2008; 180:4885–4891. [PubMed: 18354212]

16. Getahun A, Dahlstrom J, Wernersson S, Heyman B. IgG2a-mediated enhancement of antibody and T cell responses and its relation to inhibitory and activating Fc gamma receptors. J. Immunol. 2004; 172:5269–5276. [PubMed: 15100265]
17. Dahlin JS, Feinstein R, Cui Y, Heyman B, Hallgren J. CD11c+ cells are required for antigen-induced increase of mast cells in the lung. J. Immunol. 2012; 189:3869–3877. [PubMed: 22972929]
18. Stevens WW, Kim TS, Pujanauski LM, Hao X, Braciale TJ. Detection and quantitation of eosinophils in the murine respiratory tract by flow cytometry. J. Immunol. Methods. 2007; 327:63–74. [PubMed: 17716680]
19. Meisel C, Bonhagen K, Lohning M, Coyle AJ, Gutierrez-Ramos JC, Radbruch A, Kamradt T. Regulation and function of T1/ST2 expression on CD4+ T cells: induction of type 2 cytokine production by T1/ST2 cross-linking. J. Immunol. 2001; 166:3143–3150. [PubMed: 11207266]
20. Nakae S, Ho LH, Yu M, Monteforte R, Iikura M, Suto H, Galli SJ. Mast cell-derived TNF contributes to airway hyperreactivity, inflammation, and TH2 cytokine production in an asthma model in mice. J. Allergy Clin. Immunol. 2007; 120:48–55. [PubMed: 17482668]
21. Waern I, Jonasson S, Hjoberg J, Bucht A, Abrink M, Pejler G, Wernersson S. Mouse mast cell protease 4 is the major chymase in murine airways and has a protective role in allergic airway inflammation. J. Immunol. 2009; 183:6369–6376. [PubMed: 19841188]
22. Schmidlin F, Amadesi S, Dabbagh K, Lewis DE, Knott P, Bunnett NW, Gater PR, Geppetti P, Bertrand C, Stevens ME. Protease-activated receptor 2 mediates eosinophil infiltration and hyperreactivity in allergic inflammation of the airway. J. Immunol. 2002; 169:5315–5321. [PubMed: 12391252]
23. Hallgren J, Lindahl S, Pejler G. Structural requirements and mechanism for heparin-dependent activation and tetramerization of human betaI- and betaII-tryptase. J. Mol. Biol. 2005; 345:129–139. [PubMed: 15567416]
24. Hallgren J, Karlson U, Poorafshar M, Hellman L, Pejler G. Mechanism for activation of mouse mast cell tryptase: dependence on heparin and acidic pH for formation of active tetramers of mouse mast cell protease 6. Biochemistry. 2000; 39:13068–13077. [PubMed: 11041873]
25. Molino M, Barnathan ES, Numerof R, Clark J, Dreyer M, Cumashi A, Hoxie JA, Schechter N, Woolkalis M, Brass LF. Interactions of mast cell tryptase with thrombin receptors and PAR-2. J. Biol. Chem. 1997; 272:4043–4049. [PubMed: 9020112]
26. Mirza H, Schmidt VA, Derian CK, Jesty J, Bahou WF. Mitogenic responses mediated through the proteinase-activated receptor-2 are induced by expressed forms of mast cell alpha-or beta-tryptases. Blood. 1997; 90:3914–3922. [PubMed: 9354658]
27. Compton SJ, Sandhu S, Wijesuriya SJ, Hollenberg MD. Glycosylation of human proteinase-activated receptor-2 (hPAR2): role in cell surface expression and signalling. Biochem. J. 2002; 368:495–505. [PubMed: 12171601]
28. Bohm SK, Kong W, Bromme D, Smeekens SP, Anderson DC, Connolly A, Kahn M, Nelken NA, Coughlin SR, Payan DG, Bunnett NW. Molecular cloning, expression and potential functions of the human proteinase-activated receptor-2. Biochem. J. 1996; 314(Pt 3):1009–1016. [PubMed: 8615752]
29. Nawijn MC, Piavaux BJ, Jeurink PV, Gras R, Reinders MA, Stearns T, Foote S, Hylkema MN, Groot PC, Korstanje R, Oosterhout AJ. Identification of the Mhc region as an asthma susceptibility locus in recombinant congenic mice. Am. J. Respir. Cell Mol. Biol. 2011; 45:295–303. [PubMed: 20971879]
30. Fischer Lindahl K. On naming H2 haplotypes: functional significance of MHC class Ib alleles. Immunogenetics. 1997; 46:53–62. [PubMed: 9148789]
31. Zosky GR, Larcombe AN, White OJ, Burchell JT, von Garnier C, Holt PG, Turner DJ, Wikstrom ME, Sly PD, Stumbles PA. Airway hyperresponsiveness is associated with activated CD4+ T cells in the airways. Am. J. Physiol. Lung Cell Mol. Physiol. 2009; 297:L373–379. [PubMed: 19482896]
32. Brewer JP, Kisselgof AB, Martin TR. Genetic variability in pulmonary physiological, cellular, and antibody responses to antigen in mice. Am. J. Respir. Crit. Care Med. 1999; 160:1150–1156. [PubMed: 10508801]

33. Sawaguchi M, Tanaka S, Nakatani Y, Harada Y, Mukai K, Matsunaga Y, Ishiwata K, Oboki K, Kambayashi T, Watanabe N, Karasuyama H, Nakae S, Inoue H, Kubo M. Role of mast cells and basophils in IgE responses and in allergic airway hyperresponsiveness. J. Immunol. 2012; 188:1809–1818. [PubMed: 22250079]

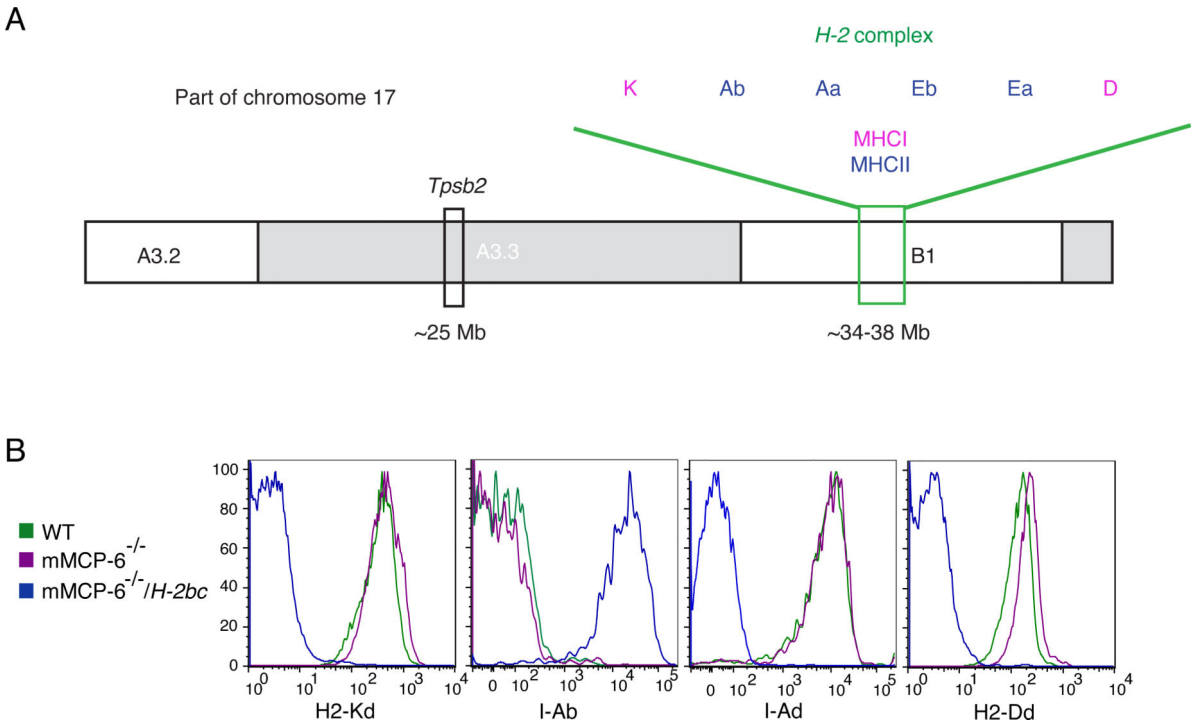

Figure 1.

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 MHCI and 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.

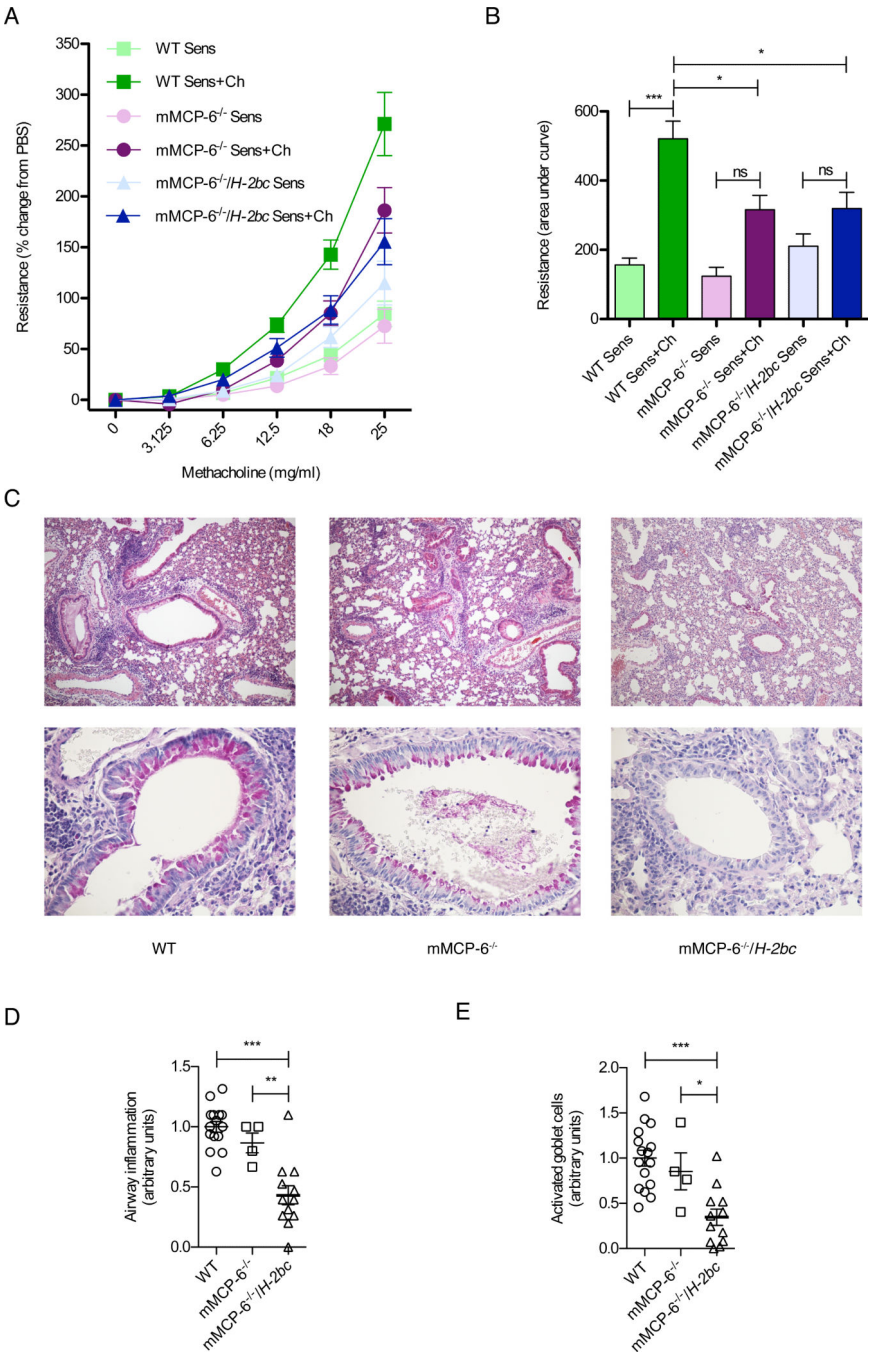

Figure 2.

AHR is reduced in sensitized and challenged mMCP-6$^{-/-}$ and mMCP-6$^{-/-}$/*H-2bc* mice. (A, B) Airway resistance towards increasing concentrations of aerosolized methacholine was measured in OVA-sensitized (Sens) mice or OVA-sensitized and challenged (Sens + Ch) mice. The graphs represent pooled data from 5 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. *, and *** indicate p values of <0.05, 0.001, respectively and 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, original magnification ×10) or PAS stain (down, original magnification ×40). The number of bronchovascular bundles associated with inflammatory cells (D) and the number of activated goblet cells/mm (E) were semi-quantified in three experiments and the mean response in sensitized and challenged wild type mice were normalized to 1.0 in each individual experiment. One experiment was performed with all three genotypes. All mice are shown.

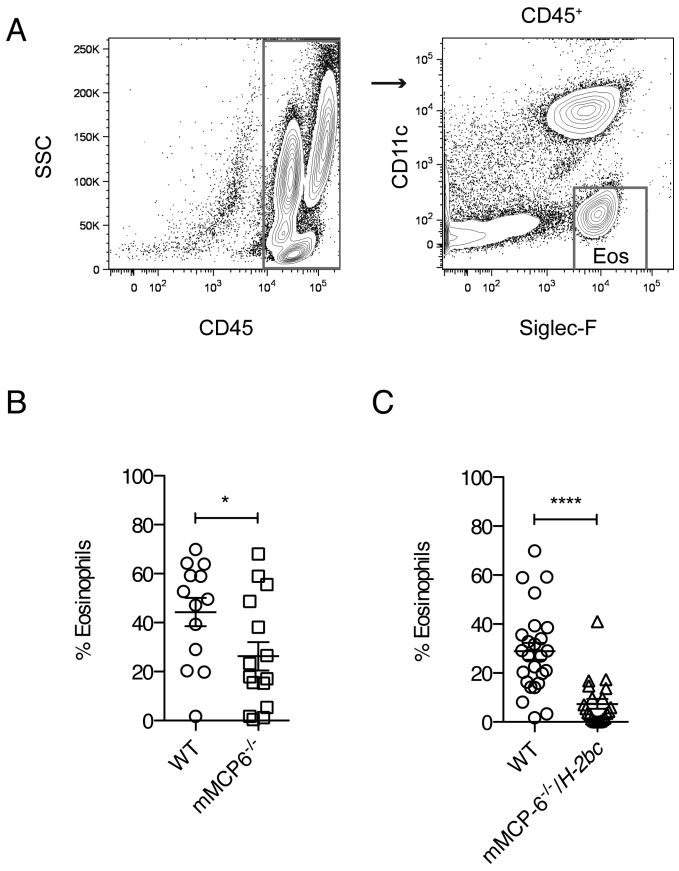

Figure 3.

BAL eosinophila is slightly reduced in sensitized and challenged mMCP-6$^{-/-}$ and dramatically reduced in mMCP-6$^{-/-}$/*H-2bc* mice. BAL eosinophils from sensitized and challenge mice were quantified using flow cytometry by the gating strategy shown in (A). The percentage of eosinophils in sensitized and challenged wild type (WT) and mMCP-6$^{-/-}$ mice (B) and WT and mMCP-6$^{-/-}$/*H-2bc* (C). The 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, 9 WT mice are represented in both (B) and (C). *, and **** indicate p values of <0.05 and <0.0001, respectively.

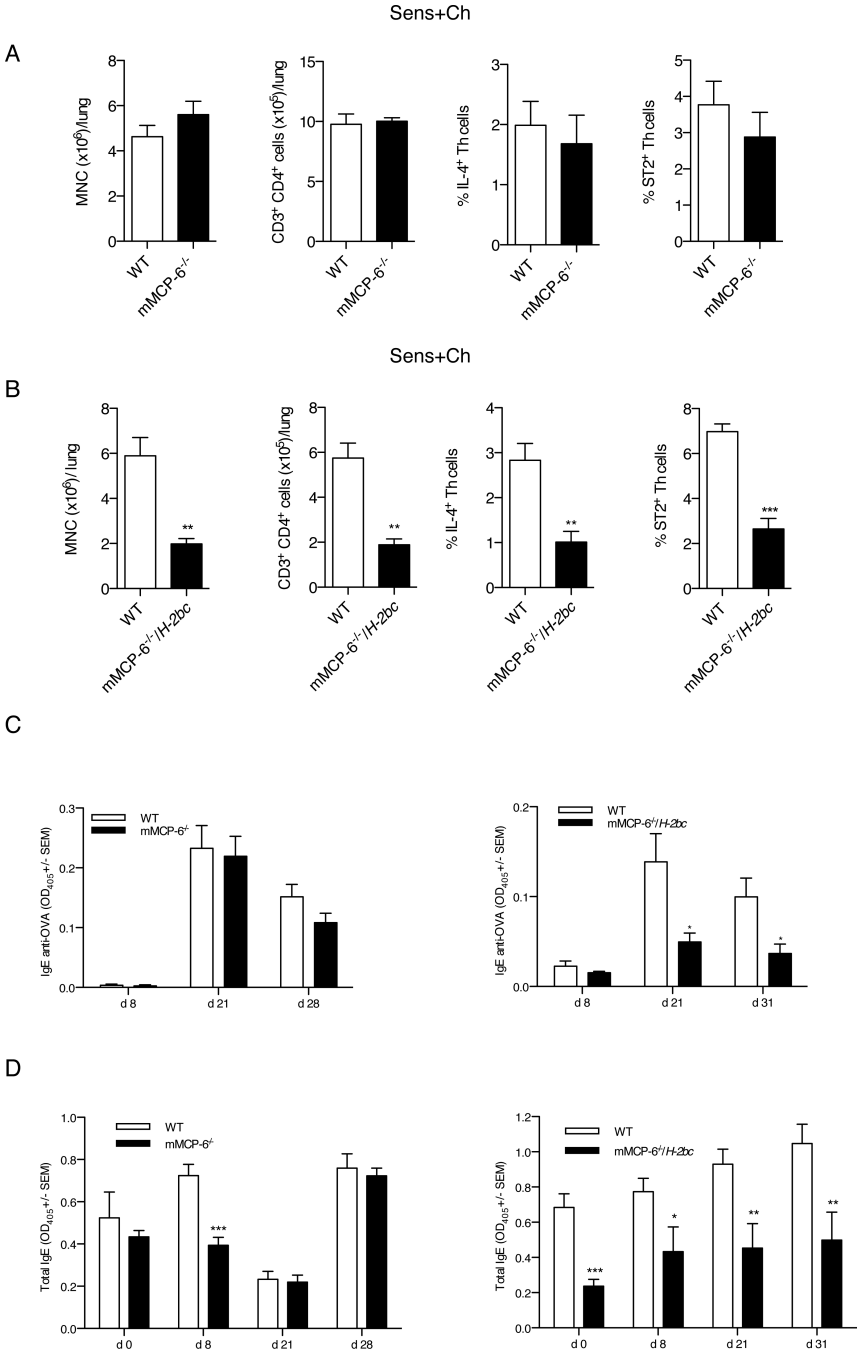

Figure 4.

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 wild type (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. The data are representative of two independent experiments with 4 mice in each group for (A) and three independent experiments with 3-4 mice in each group for (B). (C-D)Groups of mice (left, n=13-15, right,

n=6-7) were bled d 0 (prior to 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 was quantified by ELISA. The data shown in (C–D left) are representative of two independent experiments whereas the data shown in (C–D right) are representative of three independent experiments.