Genetic Variation Determines Mast Cell Functions in Experimental Asthma


*J Immunol* published online 13 May 2011

http://www.jimmunol.org/content/early/2011/05/13/jimmunol.1100676
Genetic Variation Determines Mast Cell Functions in Experimental Asthma

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Mast cell-deficient mice are a key for investigating the function of mast cells in health and disease. Allergic airway disease induced as a Th2-type immune response in mice is employed as a model to unravel the mechanisms underlying inception and progression of human allergic asthma. Previous work done in mast cell-deficient mouse strains that otherwise typically mount Th1-dominated immune responses revealed contradictory results as to whether mast cells contribute to the development of airway hyperresponsiveness and airway inflammation. However, a major contribution of mast cells was shown using adjuvant-free protocols to achieve sensitization. The identification of a traceable genetic polymorphism closely linked to the KitW-sh allele allowed us to generate congenic mast cell-deficient mice on a Th2-prone BALB/c background, termed C.B6-KitW-sh. In accordance with the expectations, C.B6-KitW-sh mice do not develop IgE- and mast cell-dependent passive cutaneous anaphylaxis. Yet, unexpectedly, C.B6-KitW-sh mice develop full-blown airway inflammation, airway hyperresponsiveness, and mucus production despite the absence of mast cells. Thus, our findings demonstrate a major influence of genetic background on the contribution of mast cells in an important disease model and introduce a novel strain of mast cell-deficient mice. The Journal of Immunology, 2011, 186: 000–000.
opment of allergic airway disease, a model for human allergic asthma. This is in sharp contrast to previous findings demonstrating that mast cells are required to elicit airway inflammation and airway hyperresponsiveness (AHR) in WBB6F1-KitW\textsuperscript{sh}/KitW\textsuperscript{v} and C57BL/6-KitW\textsuperscript{sh}/KitW\textsuperscript{sh} mice. Thus, in complex disease models, the function of mast cells can be dictated by the genetic environment.

**Materials and Methods**

**Mice**

Genetically mast cell-deficient KitW\textsuperscript{sh}/KitW\textsuperscript{v} mice on a C57BL/6 background were kindly provided by Marcus Maurer (Department of Dermatology, Charité, Berlin, Germany) and bred in our own animal facility. Congenic mast cell-deficient C.B6-Kit\textsuperscript{W-sh} mice were generated by backcrossing heterozygous Kit\textsuperscript{w-sh} mice at least nine times on BALB/c. Mast cell-deficient C.B6-Kit\textsuperscript{W-sh} mice and wild-type littermates were obtained by intercrossing heterozygous mice from the 9th and 10th backcrosses. Mice were used at the age of 10–12 wk, and the animal procedures were conducted in accordance with the institutional guidelines.

**PCR and DNA sequencing**

To detect the polymorphism linked to KitW\textsuperscript{sh}, specimens of biopsies were genotyped by PCR using Phusion DNA Polymerase (Finnzymes, Espoo, Finland) and the following oligonucleotides: Kit\textit{for}, 5’-ACG TGA ATT CTC GCC TTA CAG ACC AAG ATC C-3’ and Kit\textit{rev} 5’-ACG TGA ATT CAT TAA TGC AGT TCT CAT GAG -3’. The polymorphism that is linked to the KitW\textsuperscript{sh} mutation was identified by DNA sequencing of the PCR products.

**Passive cutaneous anaphylaxis**

Induction of PCA was conducted as previously described (13). In brief, C.B6-Kit\textsuperscript{W-sh} mice and congenic wild-type littermates were sensitized by intradermal injection of 1 μg murine monoclonal anti-DNP IgE (clone SPE-7; Sigma-Aldrich) in 50 μl PBS into the right ear. The left ears received injections of 50 μl PBS as control. Mice were challenged 24 h later by i.v. injection of 50 μg DNP-HSA in 100 μl PBS, including 0.5% Evans blue, into the tail vein (all reagents from Sigma-Aldrich). Mice were killed 30 min after i.v. injection, ears were removed and weighted, and after extraction with formamidine (Sigma-Aldrich), dye content was determined by photometric analysis.

**Histology**

To assess mast cell numbers in murine skin, ears were removed, fixed, and embedded in paraffin. Sections were used for fluorescence staining of tissue mast cells with avidin-Alexa 488 (Molecular Probes, Invitrogen, Karlsruhe, Germany) as detailed previously (14). Avidin is a heparin-binding protein that specifically binds to mast cell granules. Mast cell numbers were expressed as cells per millimeter basement membrane (BM) of the airways. To assess mast cell numbers in lung tissue, lungs were fixed by immersion in formalin and embedded in paraffin. Sections were used for fluorescence staining of tissue mast cells with avidin-Alexa 488 (Molecular Probes, Invitrogen, Karlsruhe, Germany) and bred in our own animal facility. Congenic mast cell-deficient C.B6-Kit\textsuperscript{W-sh} mice were generated by backcrossing heterozygous Kit\textsuperscript{w-sh} mice at least nine times on BALB/c. Mast cell-deficient C.B6-Kit\textsuperscript{W-sh} mice and wild-type littermates were obtained by intercrossing heterozygous mice from the 9th and 10th backcrosses. Mice were used at the age of 10–12 wk, and the animal procedures were conducted in accordance with the institutional guidelines.

**Induction of allergic airway inflammation**

Mice were sensitized by i.p. injection of 10 μg OVA in 0.1 ml PBS on days 0, 2, 4, 6, 8, 10, and 12. On days 40, 43, and 46, mice were challenged intranasally with 20 μl PBS containing 200 μg OVA (Sigma-Aldrich) or 20 μl PBS alone (16). Twenty-four hours after the last challenge, airway resistance in response to methacholine (MCh), lavage, and fixation of the lungs were assessed. Additionally, serum was obtained for determination of OVA-specific Ig titers.

**Measurement of airway reactivity and bronchoalveolar lavage**

Measurements of airway resistance were performed on anesthetized, intubated, and mechanically ventilated mice (FlexiVent; Scireq, Montreal, QC, Canada) in response to increasing doses of inhaled MCH (3.125, 6.25, 12.5, 25, and 50 mg/ml for BALB/c; 6.25, 12.5, 25, 50, and 100 mg/ml for C57BL/6). Measurements of airway resistance were performed every 15 s after each nebulization step until a plateau phase was reached. After assessment of airway reactivity, lungs were lavaged with PBS (1 ml), and differential and total cell counts were made as previously described (17). For differential cell counts, cytocentrifuged preparations were fixed and stained with the Microscopy Hemacolor set (Merck, Darmstadt, Germany).

**Statistical analysis**

In the vertical scatter plots, medians are represented by black bars in figures. Statistical differences were determined using Student’s t test. Differences in responsiveness to MCh were assessed by repeated-measures ANOVA. Comparisons for all pairs were performed by the Tukey–Kramer honest significant difference test. The numbers of eosinophils, histology score, and PAS-positive cells were initially analyzed by nonparametric ANOVA (Kruskal–Wallis Test) for overall differences. A p value <0.05 was considered to be significant. Values for all measurements are expressed as the means ± SD.

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**FIGURE 1.** Identification of a traceable DNA polymorphism linked to Kit\textsuperscript{W-sh}. A, PCR with a single primer pair yields products of distinct sizes upstream of c-kit in C57BL/6, BALB/c, and C57BL/6-Kit\textsuperscript{W-sh} mice. B, Schematic representation (not to scale) of the KitW\textsuperscript{sh} mutation on chromosome 5. Arrows indicate 5’ and 3’ breakpoints leading to the inversion of ~3 Mbp upstream of c-kit and interruption of corin, as published recently (18). The length polymorphism of PCR products maps to a region very close to the 3’ breakpoint and is based on the integration of short repetitive sequences in KitW\textsuperscript{sh}. An additional base exchange is indicated by an asterisk.
Results

Identification of a traceable polymorphism linked to the Kit\textsuperscript{W-sh} mutation and generation of congenic mast cell-deficient C.B6-Kit\textsuperscript{W-sh} mice

In an attempt to establish a PCR procedure discriminating Kit\textsuperscript{W-sh} and wild-type Kit\textsuperscript{+} alleles, we used a primer walking strategy to identify the 3' breakpoint of the Kit\textsuperscript{W-sh} inversion in C57BL/6-Kit\textsuperscript{W-sh} mice. This breakpoint was previously reported to be \( \sim 72 \) kbp upstream of the c-kit transcriptional start side. We discovered a length polymorphism that maps very closely (<1 kbp) to the 3' breakpoint of Kit\textsuperscript{W-sh} recently located by Nigrovic et al. (18). The length polymorphism observed in PCR reactions amplifying a 0.8–1.1 kbp fragment of genomic DNA is based on additional motifs GGA and GAA found adjacent to the 3' breakpoint of Kit\textsuperscript{W-sh} (Fig. 1).

Interestingly, the size of this PCR product also differs between BALB/c and C57BL/6 strains, which allowed us not only to follow the fate of the Kit\textsuperscript{W-sh} allele in backcrosses from C57BL/6 to BALB/c but also to distinguish it from the autochthonous wild-type allele in BALB/c. Unfortunately, due to its highly repetitive character, we were not able to get reliable data from the corresponding sequence in BALB/c (data not shown). However, based on this genotyping strategy, we were able to generate the congenic strain designated C.B6-Kit\textsuperscript{W-sh}. Histological examinations revealed that these mice are indeed devoid of mast cells and do not show passive cutaneous anaphylaxis (Fig. 2).

Influence of the Kit\textsuperscript{W-sh} mutation and the genetic background on hematopoietic cell populations

Despite the absence of mast cells, the presence of CD117-expressing cells in bone marrow and spleen is not impaired by the Kit\textsuperscript{W-sh} mutation, yet the marrow of C57BL/6 mice shows a marked increase in CD117-positive cells in general (Fig. 3A). This strain-specific increase is also evident in the spleens of C57BL/6 mice, although less impressive. In accordance with previously published findings (18), the percentage of CD117-positive cells is increased in the spleens of C57BL/6 mice bearing the Kit\textsuperscript{W-sh} mutation (Fig. 3A). A slight but statistically significant expansion of CD117-expressing cells is also found in C.B6-Kit\textsuperscript{W-sh} spleens. However, this increase in CD117-positive

FIGURE 2. C.B6-Kit\textsuperscript{W-sh} mice are devoid of mast cells and do not develop passive cutaneous anaphylaxis. A, Paraffin-embedded ears and lungs, previously fixed with formaldehyde, were used for fluorescence staining of mast cells with avidin-Alexa 488. For the detection of peritoneal mast cells, peritoneal lavage was performed and stained for FcεRI and CD117 double-positive cells. B, Mice were sensitized by intradermal injection of DNP-specific IgE in the right ear and challenged 24 h later by i.v. injection of 0.5% Evans blue in combination with DNP-HSA. Animals were killed 30 min after the injection of dye and Ag, ears were removed and weighted, and after extraction, dye content was determined by photometric analysis. Shown are five to eight mice per group. n.d., not detectable.
cells does not apply to the bone marrow. Furthermore, the percentages of FcεRI and CD49b-positive basophils in the marrow of all strains are comparable (Fig. 3B). Interestingly, this does not hold true for the blood, in which the KitW-sh mutation leads to a significant increase in basophil counts in BALB/c but to a decrease in C57BL/6.

Further analyses of KitW-sh mutant mice on BALB/c and C57BL/6 background, compared with their respective congenic littersmates, revealed no differences in the composition of CD4 and CD8 T cell compartments in thymus, spleen, lymph nodes, and blood. In addition, CD11c-expressing dendritic cells were also present in comparable proportions (data not shown). With regard to CD19+ B cells and in agreement with available data (mouse phenome database at http://phenome.jax.org), we found slightly elevated numbers in bone marrow, blood, lymph nodes, and spleens from C57BL/6 compared with BALB/c mice. Yet, the KitW-sh mutation moderately decreases the numbers of CD19-positive cells in both strains (Fig. 3C).

Analyses of cells coexpressing Gr-1 and CD11b revealed that this population is generally expanded in BALB/c mice as compared with C57BL/6 (Fig. 3D). On the C57BL/6 background, the KitW-sh mutation causes a higher percentage of Gr-1/CD11b double-positive cells in spleen but not in blood and marrow. This situation is comparable in C.B6-KitW-sh mice, yet the expansion of Gr-1/CD11b double-positive cells in spleen is even more pronounced (Fig. 3D).

Taken together, these results suggest: 1) higher numbers of neutrophils in the blood of BALB/c mice as compared with C57BL/6; 2) in both strains, the numbers of neutrophils in marrow and blood are hardly affected by the KitW-sh mutation; and 3) massive expansion of splenocytes coexpressing Gr-1 and CD11b caused by the KitW-sh mutation on both genetic backgrounds.

C.B6-KitW-sh mice develop full-blown airway inflammation and AHR despite the absence of mast cells

We deployed an established mast cell-dependent murine model for allergic airway disease based on i.p. immunization with Ag in the absence of adjuvant (16, 19). Repeated challenge of the lungs with the Ag then ultimately leads to cellular infiltration, enhanced mucus production, and AHR to bronchoconstrictors, renowned hallmarks of allergic asthma.

In the following experiments, we compared BALB/c mice with their congenic mast cell-deficient C.B6-KitW-sh littersmates. Control animals were immunized i.p. but received PBS instead of the Ag for challenge. In parallel, all experiments were performed using the C57BL/6-KitW-sh strain and wild-type littersmates. This served as an internal control, and indeed, sensitized and challenged C57BL/6-KitW-sh showed less airway reactivity and inflammation compared with sensitized and challenged C57BL/6. This is in line with already published work (15–17, 19).

As depicted in Fig. 4A, immunization and Ag challenge elicits a strong influx of inflammatory cells into the lungs as demonstrated by increased cell counts in bronchoalveolar lavage (BAL). Infiltrating cells mainly consist of eosinophils and neutrophils. Importantly, there is no difference between mast cell-deficient C.B6-KitW-sh and mast cell-sufficient BALB/c mice, neither qualitatively nor quantitatively. H&E staining of fixed lung slices to score tissue inflammation also reveals inflammatory infiltrates surrounding airways and blood vessels, indiscriminate of whether mast cells are present in mice with BALB/c background (Fig. 4B). In contrast, and as previously reported (16, 17), cellular infiltration of BAL and lung tissue is strongly reduced in mast cell-deficient C57BL/6-KitW-sh mice as compared with their wild-type littersmates (Fig. 4A, 4B).

Analyses of Ag-specific production of Igs and Th2 cytokine

FIGURE 3. Hematopoietic cells in KitW-sh mutant mice and congenic wild-type littersmates on BALB/c and C57BL/6 backgrounds. A, Percentages of cells expressing CD117 in bone marrow and spleen are shown. n = 8–13 mice per group. B, Percentages of basophils defined as FcεRI and CD49b double-positive cells in bone marrow and blood are depicted. n = 5–13 animals per group. C, CD19-positive B cells; n = 5–21 mice per group. D, Cells coexpressing Gr-1 and CD11b; n = 10–23 animals per group.
expression of ex vivo-restimulated CD4+ T cells revealed no differences between BALB/c and C.B6-KitW-sh mice (data not shown). This is in agreement with previous reports showing that mast cells are not required for allergen-specific B cell and T cell responses in both WBB6F1-KitW/KitW- and C57BL/6-KitW-sh mice (17, 19).

PAS staining for mucus-producing goblet cells lining the airways also shows comparable metaplasia following sensitization and Ag challenge in BALB/c and C.B6-KitW-sh mice (Fig. 4C).

Yet, from the data shown in Fig. 4C, it can also be concluded that mucus production in this model strongly depends on the presence of mast cells in C57BL/6 mice (15).

Besides these inflammatory parameters, we also investigated the reactivity of sensitized mice to inhalation with MCh (Fig. 5). Increasing doses of MCh augment airway reactivity in both BALB/c and C.B6-KitW-sh mice (Fig. 5A). In comparison, C57BL/6 mice require higher doses of MCh, and the overall response is
lower (Fig. 5B). However, the absence of mast cells abrogates AHR in C57BL/6-Kit\textsuperscript{W-sh} mice in agreement with published work (16, 17).

**Discussion**

Asthma is a complex syndrome with heterogeneous clinical characteristics; common symptoms are chronic inflammation with reversible airway obstruction and AHR. Besides allergen exposure, genetic factors, viral infection, air pollution, and tobacco smoke can contribute to the different phenotypes of the disease (20, 21). Thus, it appears unlikely that single animal models reflect all aspects of human disease, yet the dependence on CD4\textsuperscript{+} T lymphocytes required to deliver the Th2-type cytokine milieu is commonly accepted (22, 23). A critical variable contributing to disparate experimental outcomes in animal models surely is the lack of standardized protocols to elicit airway disease. In this context, the role of mast cells has been a matter of debate, as murine models employing OVA as Ag have shown contradictory results. Based on a number of studies, it can be concluded that the contribution of mast cells depends on the strength of sensitization to the Ag and subsequent provocation. In general, weaker immunizations without additional adjuvant i.p. or by inhalation followed by a minimal number of challenges depend on the presence of mast cells necessary for airway inflammation and AHR in both WBB\textsuperscript{6} F1-Kit\textsuperscript{W-sh}/Kit\textsuperscript{W-sh} and C57BL/6-Kit\textsuperscript{W-sh} mice (15–17, 19, 24–26). These results fit to observations in humans with active asthma in whom mast cells are predominantly localized within bronchial smooth muscle bundles and exhibit a degranulated (i.e., activated) phenotype (27, 28). Yet, mast cell numbers in the lungs are very low in naive mice as compared with humans, and infiltration of airway smooth muscle with mast cells is not found in these animals. However, pharmacological approaches targeting mast cells have not always been successful in the treatment of human asthma. Indeed, sodium cromoglycate used as a mast cell stabilizer has little effect in the treatment of human asthma. Indeed, sodium cromoglycate is possibly an inherited trait regulated separately from the inflammatory response (33, 34). BALB/c and C57BL/6 are widely used strains, showing polarized Th cell responses. BALB/c mice typically develop Th2-skewed immune responses, whereas C57BL/6 are prone to Th1-dominated immunity. In general, it has been reported that OVA challenge in sensitized mice induces higher levels of IgE and more pronounced AHR in BALB/c; conversely, C57BL/6 accumulate more eosinophils (35). In BALB/c, eosinophils typically accumulate around the airways, whereas in C57BL/6, they infiltrate the parenchyma (36, 37).

Mast cell-deficient BALB/c mice have not yet been reported but appeared to be advantageous to study the functions of these cells in an allergy-prone strain. Contrary to our expectations, mast cell deficiency has no effect on the development of airway disease in congenic BALB/c mice. This suggests that either mast cell functions differ between strains and/or other mechanisms compensate for the absence of mast cells. Indeed, it was shown that strain background (C57BL/6 versus 129/Sv) can influence mast cell activation in vitro and in vivo (38). Mast cells derived from BALB/c mice were reported to release higher amounts of mediators such as PGD\textsubscript{2} and IL-6 as compared with C57BL/6. Conversely, mast cells from the latter strain release higher amounts of preformed mediators in vitro (39). With respect to our own data reported in this study, C57BL/6 mice show slightly greater reactivity in IgE-mediated PCA, which cannot be due to higher mast cell numbers as these are comparable in the ear skin of C57BL/6 and BALB/c. Yet, increased release of preformed mediators upon mast cell degranulation in C57BL/6 mice might explain this apparent discrepancy. In the lungs, mast cell numbers are even slightly elevated in BALB/c, whereas these mice display a modest reduction in peritoneal mast cell numbers in comparison with C57BL/6. Obviously, strain-specific differences in the local tissue micro-milieu and/or in the targeted migration of mast cell precursors have an influence on mast cell numbers. It should also be noted that we do not see increased levels of mast cells after sensitization and challenge in either strain (data not shown). However, strain-specific contribution to airway disease was also reported for eosinophils, which are crucial for the development of AHR and inflammation in C57BL/6 but not in BALB/c (40–43). In addition, the airway epithelium was shown to be critical in OVA-induced airway inflammation in BALB/c mice but not in C57BL/6 (44). This also leads to the question of whether epithelial-derived cytokines such as IL-33 and IL-25, which are able to initiate the release of Th2-type cytokines by recently discovered natural Th cells, may compensate for the absence of mast cells. Compensatory functions are also conceivable for basophils as inducers of a Th2 response (45, 46). Mast cell-deficient mice on a BALB/c background might be a useful tool to investigate these partly
redundant and variable cellular and molecular mechanisms under-lying allergic airway disease.

In conclusion, animal models for asthma seem to mirror the complexity seen in the human disease, and results from such experiments should be interpreted carefully, as they may reflect borderline cases depending on both the model and the mouse strain used.

Disclosures
The authors have no financial conflicts of interest.

References