

The naïve airway hyperresponsiveness of the A/J mouse is *Kit*-mediated

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There is a wide variation among humans and mice in airway hyperresponsiveness (AHR) in the absence of allergen sensitization, i.e., naïve AHR. Because mast cell (MC) activation is thought to mediate AHR in atopic asthmatic subjects, we asked whether MCs mediate naïve AHR in A/J mice. We generated an A/J congenic strain lacking c-Kit by introgression of the *Wv* mutation, which resulted in the elimination of MCs and the abrogation of naïve AHR. Imatinib, which disrupts *Kit* signaling, also abrogated AHR in A/J mice. Remarkably, introduction of the *Vga9 Mitf* mutation into the A/J background resulted in the ablation of MCs but did not ameliorate AHR. These results indicate that c-Kit is required for development of AHR in an MC-independent fashion.

Asthma is an inflammatory syndrome of the lower airways and one of the most common chronic diseases in developed countries. Genetic predisposition, environmental exposure, and lifestyle are all thought to contribute to the development and severity of asthma (1). Physiologically, asthma is characterized by episodic, variable intrathoracic airflow obstruction that is reversible with bronchodilator medications and by airway hyperresponsiveness (AHR), defined as increased sensitivity to bronchoconstriction induced by contractile agonists such as histamine and methacholine (MCh) (2–4). AHR is at least partly genetically determined and may be a predisposing factor for the development of asthma (5). In laboratory mice, AHR can be experimentally induced by systemic immunization to an antigen (often chicken egg ovalbumin) followed by inhalation challenge, which elicits robust bronchovascular inflammation and T-helper type 2 (Th2) cytokine production (6, 7). In such allergen-driven models, the increase in AHR reflects the influences of IL-13 and target genes expressed by bronchial epithelial cells (8). However, in the absence of antigen sensitization, the C57BL/6J (B6) and BALB/c strains of mice that are typically used in these studies are only weakly reactive to MCh. Furthermore, these mouse strains show almost no reactivity to the endogenous bronchoconstrictors that contract human airways more potently than MCh (e.g., histamine, leukotrienes, and prostaglandins) and that are involved in the pathophysiology of asthma exacerbations (9). Thus, the Th2-driven pathways that mediate AHR in antigen-driven mouse models are not clearly relevant to the pathophysiology of AHR in humans, which is of particular note given the failure of any anti-Th2 cytokine therapy (e.g., blockade of IL-4, IL-5, IL-13, or IgE) to alter AHR in clinical studies (10–12).

Unlike other hematopoietic cell types implicated in asthma pathophysiology, mast cells (MCs) are constitutive tissue residents. They develop in situ from CD13⁺CD34⁺KIT⁺ hematopoietic bone marrow progenitors, which migrate to peripheral tissues, including the skin, mucosa, and airway, where they terminally differentiate into mature MCs with prominent secretory granules and high-level expression of the high-affinity Fc receptor for IgE, FcεRI (13). Analysis of mouse mutants has demonstrated that the constitutive residence of MCs in tissues requires expression of c-Kit and the local availability of stem cell factor (SCF), a membrane-bound growth factor expressed by normal fibroblasts and endothelial cells. Mice carrying mutations

of the dominant white-spotting locus (*W*) encoding the *Kit* gene, the Steel locus (*S*) encoding SCF, and the microphthalmia-associated transcription factor locus (*Mitf*) encoding MITF, a downstream effector of *Kit*, are profoundly deficient in MCs (14–16). These mutant murine models highlight the critical role of SCF, c-Kit, and their downstream signaling and transcriptional targets in the development of MCs.

MCs store abundant amounts of inflammatory mediators in their granules, including neutral serine proteases. When activated, MCs release these proteases, along with histamine, and generate newly formed lipid mediators (leukotrienes, prostaglandins, etc.) and cytokines, all with potential actions at airway smooth muscle. In asthmatic individuals who are sensitized to allergen (atopic asthmatics), MC activation through FcεRI can be experimentally induced by allergen inhalation challenge, which results in bronchoconstriction and a biphasic decrement in airflow, with an early (15–30 min) and late (6–12 h) asthmatic response, the latter of which is associated with substantial potentiation of AHR to MCh (2). The fact that both early and late asthmatic responses are sensitive to pharmacologic agents that block FcεRI-dependent MC activation implies a pathway by which MCs can potentiate AHR (17). Of note, however, is that AHR can exist as an isolated phenotype in nonasthmatic individuals, can precede the clinical onset of asthma, and is also present in nonatopic asthmatics (18). The question of whether MCs play a role in asthma independent of IgE and sensitization to allergen remains largely unexplored.

A/J mice differ from most laboratory strains in that they display a high degree of reactivity to MCh, an airway smooth muscle contractile agonist, in the absence of sensitization and airway challenge (naïve AHR) (19). A number of studies, including our own, have identified regions of the genome associated with the expression of naïve AHR (6, 20–22). However, little is known about the biology underlying naïve AHR and, specifically, whether MCs could be playing a role. The analysis we report here demonstrates that MCs are not required to mediate AHR. A/J mice have increased tissue MC numbers and serum mouse MC protease 1 (mMCP-1) in the absence of allergen, but this finding does not correlate with AHR. Genetic or pharmacologic depletion of MCs through c-Kit perturbations abrogate AHR, whereas *Mitf* mutation-induced MC depletion does not. Together, these data indicate that c-Kit plays a role in mediating naïve AHR in an MC-independent manner.

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Results

Kit-Mediated Depletion of MCs in Hyperresponsive Mice Ameliorates AHR. The W^v allele of *Kit* (officially termed Kit^{W^v} but referred to as W^v hereafter for simplicity) carries a missense mutation that results in the synthesis of a functionally impaired c-Kit receptor tyrosine kinase (23). We generated congenic mice carrying the W^v mutation on the hyperresponsive A/J background by serial backcrosses for more than 10 generations, selecting for W^v by genotype analysis (Fig. S1A). The establishment of a congenic line was confirmed by genome-wide SNP genotype analysis (Fig. S1B).

We attempted to assess AHR in MC-deficient A/J mice by intercrossing $W^v/+$ carriers, but only 10% of the expected number of homozygous A/J $^{W^v/W^v}$ mice were obtained, demonstrating that the mutation is poorly tolerated on this genetic background. However, the fact that the AHR phenotype is dominantly inherited, and A/J $\times$ C57BL/6J F1 (F1) mice are just as hyperresponsive as the parental A/J mice (Fig. 1), permitted us to test the effect of MC depletion on AHR. Intercrosses between A/J $^{W^v/+}$ and B6 $^{W^v/+}$ mice generated homozygous mutant progeny in Mendelian ratios, and our analysis was done comparing F1 $^{+/+}$ and F1 $^{W^v/W^v}$ mice. Compared with A/J and B6 parental strains, F1 $^{W^v/W^v}$ mice had an MCh-induced peak airway resistance similar to that of normoresponsive B6 mice (Fig. 1). That is, the W^v mutation completely abrogated the AHR conferred by the A/J genetic background. Histological analysis confirmed that F1 $^{W^v/W^v}$ mice were devoid of MCs in all tissues examined, including trachea and spleen (Fig. 2A and B).

Hyperresponsive Mice Have Increased Tissue MCs That Do Not Correlate with AHR. In addition to MC deficiency, the W^v mutation causes macrocytic anemia, lack of hair pigmentation, and sterility. To determine whether the reduction in AHR observed in F1 $^{W^v/W^v}$ mice was caused specifically by the lack of MCs, we determined whether adoptive transfer of bone marrow-derived MCs (BMMCs) would restore the F1 AHR phenotype. However, the effects of the BMMC transfer on AHR proved to be inconclusive because it turned out that all mouse cohorts had a reduced MCh-induced airway resistance compared with that

normally found in A/J mice (Fig. S2A). This result prompted a phenotypic evaluation of A/J and B6 control mice equivalent in age to mice that had undergone adoptive transfer, which revealed that A/J mice at 28–32 wk old have a significantly reduced AHR phenotype compared with 8- to 10-wk-old mice (Fig. S2B), which is consistent with previous studies (24). This reduction in A/J AHR phenotype with age decreased the sensitivity of the analysis and precluded the ability to identify significant differences between groups receiving BMMCs. Additionally complicating the analysis was our finding that few MCs engrafted the airways of the F1 $^{W^v/W^v}$ mice receiving F1 or B6 BMMCs. MCs were found to be abundant in the spleen and bone marrow, suggesting that the adoptive transfer was successful, but the MCs did not migrate appropriately to target tissues (Fig. S2C and D).

In naïve mice, MCs are sparse in tracheal cross-sections and are rarely observed in airways smaller than the mainstem bronchi. To increase the sensitivity of our MC counts, we developed an immunofluorescence assay by using a whole tracheal mount. Using this method, we determined that the A/J and F1 control mice had markedly more MCs in the trachea than did the B6 mice (Fig. 2A). Likewise, splenic MCs were significantly increased in A/J compared with both F1 and B6 mice (Fig. 2B). The observed increased airway MCs are in a prime location to directly affect AHR in our model, and splenic MCs are reported to mediate physiologic functions at distal organs in certain disease models (25). Therefore, we sought to determine whether MC number in either of these tissues could be correlated with increased AHR. To this end, we generated a cohort of (A/J $\times$ B6) $\times$ B6 N2 (N2) mice segregating the AHR trait by backcrossing A/J $\times$ B6 F1 progeny to B6 mice. We then phenotyped N2 animals and determined tracheal and spleen MC numbers. The MCh-induced AHR phenotype was not strongly correlated to tracheal or splenic MC number in the N2 population ($R^2 = 0.0765$ and 0.1233 , respectively) (Fig. 2C and Fig. S3). This finding suggests that AHR is not a simple function of MC numbers in the tissues of A/J mice, which is consistent with our observation that F1 mice have increased airway reactivity (Fig. 1) but do not have elevated splenic MCs (Fig. 2B).

Imatinib Treatment Reduces the Naïve AHR Phenotype in A/J Mice. As an alternative means to assess the role of MCs in the mediation of the naïve AHR phenotype, we tested whether direct pharmacological inhibition of c-Kit would alter airway reactivity in A/J mice (26, 27). Imatinib acts to prevent SCF-induced c-Kit autophosphorylation and thus the subsequent downstream signaling necessary for MC development. The c-Kit receptor can also mediate an endogenous MC activation pathway that is independent of IgE and antigen (28). A/J and B6 mice were treated with 50 mg/kg imatinib i.p. for 21 d with a protocol described by Dietz et al. (26), and MCh-induced AHR was measured. Treated A/J mice had a significantly reduced AHR phenotype compared with untreated mice (Fig. 3A). Treatment of B6 mice with imatinib had no effect on MCh-induced AHR.

Examination of tissue from imatinib-treated A/J mice showed significantly reduced MC numbers in the trachea but not in the spleen (Fig. 3B and C). Because c-Kit plays a role in MC activation and degranulation (29, 30), we also examined the level of the chymase mMCP-1 in the serum, a readout that reflects MC activation (31). Interestingly, A/J vehicle control mice had dramatically elevated serum mMCP-1 levels compared with B6 vehicle control mice (Fig. 3D). Imatinib treatment induced a significant reduction in serum mMCP-1 levels in A/J mice, whereas B6 mMCP-1 levels remained unchanged after treatment with imatinib (Fig. 3D). To examine whether MC activation could be correlated to MCh responsiveness, we determined serum mMCP-1 levels in N2 mice and found that these were not strongly correlated with the peak airway resistance to MCh (Fig. 2D).

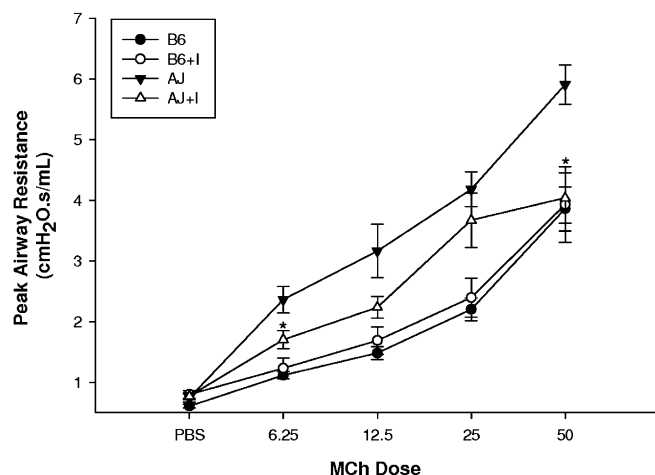


Fig. 1. Genetic depletion of tissue MCs in congenic A/J $\times$ B6 F1 $^{W^v/W^v}$ mice abrogates AHR. B6 $^{W^v/+}$ were crossed with A/J mice to create F1 $^{W^v/+}$ progeny. The F1 $^{W^v/+}$ mice were then backcrossed to A/J mice for 10 generations, creating A/J N10 $^{W^v/+}$ mice. These mice were then crossed to B6 $^{W^v/+}$ mice to create F1 wild-type and F1 $^{W^v/W^v}$ mice for study. A/J mice had an increased airway resistance compared with B6 mice, and F1 mice had a naïve AHR phenotype equivalent to their parental A/J strain. F1 $^{W^v/W^v}$ mice displayed an airway resistance similar to normoresponsive B6 mice. Values represent mean $\pm$ SE; $n =$ at least 10 in each group.

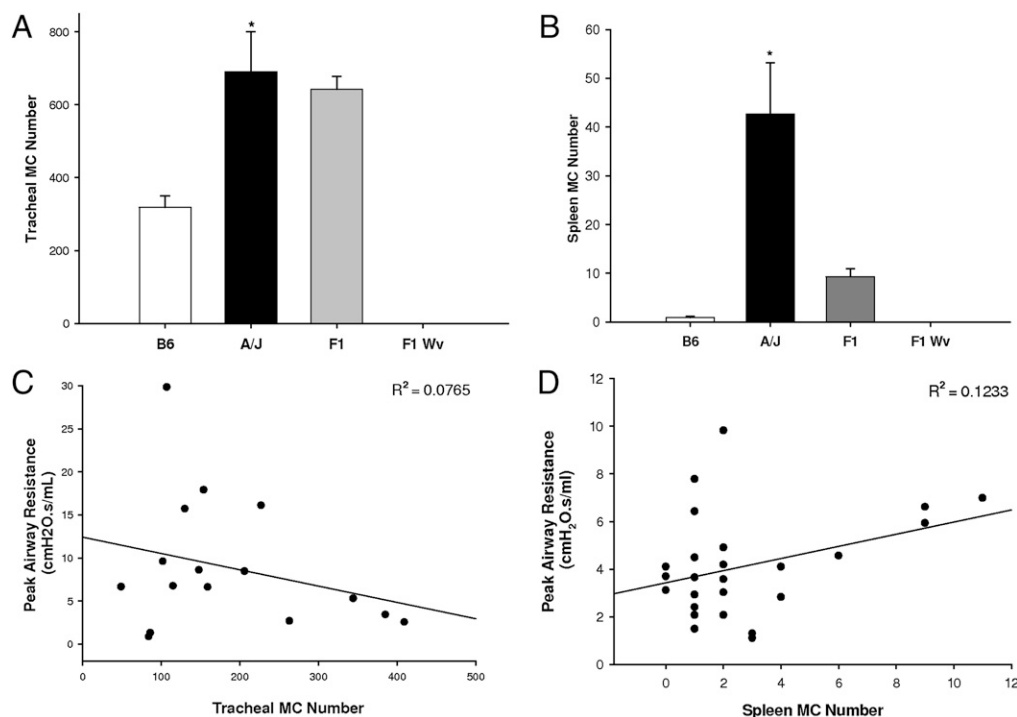


Fig. 2. Correlation of tissue MC numbers to AHR and serum mMCP-1. (A and B) $F1^{Wv/Wv}$ mice lack tissue MCs in all tissues evaluated, including trachea and spleen. Hyperresponsive A/J and F1 mice have a tracheal and splenic mastocytosis compared with normoresponsive B6 mice. However, F1 have significantly less splenic MCs than A/J mice. (C and D). Tracheal MCs and serum mMCP-1 values are not correlated with AHR in N2 mice. Values represent mean $\pm$ SE; $n > 10$ in each group for MC counts; $n > 16$ in each group for tissue MCs and mMCP-1 correlation studies.

Mitf-Mediated Depletion of MCs Does Not Ablate AHR. We have shown that genetic and pharmacological disruption of MC physiology significantly reduced AHR in A/J mice. However, these interventions were based solely on perturbing *Kir* and c-Kit at the genetic and protein levels, respectively. Therefore, we sought to disrupt MCs in hyperresponsive mice through alternative genetic means to better understand the role MCs play in naïve AHR. The murine *mi* locus encodes MITF, a basic helix-loop-helix leucine zipper transcription factor important for pigmentation, neural development, bone formation, fertility, and hematopoiesis (32). Mice that are homozygous carriers of the *Vga9* allele of *Mitf* do not express MITF protein and are white and microphthalmic. These mice have been shown to lack MCs in all tissue except the skin, where their numbers are substantially reduced (32, 33).

We introduced the *Vga9* mutation onto a hyperresponsive background by crossing $B6^{Vga9/Vga9}$ and A/J mice to generate $F1^{+/+Vga9}$ progeny. $F1^{+/+Vga9}$ mice were then intercrossed to generate both $F2^{+/+}$ and $F2^{Vga9/Vga9}$ mice for analyses. F2 mice have variable AHR phenotypes similar to what is seen in an N2 backcross population of these strains (21). Introduction of the *Vga9* mutation did not alter the distribution of F2 AHR phenotypes (Fig. 4 A and B). Thus, depletion of MCs through MITF genetic perturbation did not eliminate the F2 high-MCh responders. Because it was possible that the F2 mixed genetic background could contribute to the lack of reduction in AHR, we generated an A/J congenic line containing the *Vga9* mutation. We introduced the *Vga9* mutation onto the A/J genetic background through serial backcross until more than 90% of A/J homozygosity was achieved at the N4 generation, as assessed by SNP genotype analysis. Select $N4^{Vga9/+}$ mice were then crossed to $B6^{Vga9/+}$ mice to generate $F1^{Vga9/Vga9}$ and $F1^{+/+}$ mice for testing (Fig. S4). The F1 generation was used to ensure that murine genetic backgrounds were consistent between *Wv* and *Mitf*

studies. As expected, $F1^{Vga9/Vga9}$ mice did not contain MCs in any of the tissue examined compared with $F1^{+/+}$ controls, as evidenced by mMCP-6 and chloroacetate esterase staining (Fig. S4). Unlike the results we obtained with a comparable analysis of A/J $\times$ B6 $F1^{Wv/Wv}$ mice, MCh-induced peak airway resistance in $F1^{Vga9/Vga9}$ mice was not significantly reduced (Fig. 4C).

Discussion

In contrast to human airways, mouse airways generally have few MCs in airways smaller than the trachea and large bronchi, and the strains used most commonly for mechanistic studies of AHR show little reactivity to MCh in the naïve state. Moreover, the AHR that is induced by Th2-driven inflammatory pathways in classical ovalbumin-driven mouse asthma models either has no requirement for MCs (34) or has an MC requirement only when adjuvants are eliminated during the sensitization step.

It has been clearly shown that the A/J strain of mice demonstrates AHR in the absence of allergen sensitization (21). We and others have shown that this is a heritable trait and have mapped quantitative trait loci that correlate with AHR (6, 20–22). Our previously published data indicate that multiple loci are associated with inheritance of increased airway reactivity. In the present study, we performed complementary analyses to test the hypothesis that naïve AHR in the A/J mouse is mediated by MCs. We introduced the *Wv* mutation into the A/J background by serial backcross for more than 10 generations, which, in the absence of marker-assisted breeding approaches, is the conventional requirement for generation of a congenic strain. We were able to circumvent the poor viability found for *Wv* homozygous mice on the A/J background by comparing $F1^{+/+}$ mice (which are hyperresponsive because this phenotype is inherited as a dominant trait) to $F1^{Wv/Wv}$ homozygous mutant mice. The anticipated loss of tissue MCs that occurred with the introduction of the homozygous *Wv* mutation was accompanied by a striking

MC turnover, tissue-specific differences in c-Kit-independent factors capable of maintaining MC survival, and incomplete efficacy of the pharmacologic approach to c-Kit inhibition. Nonetheless, the marked reduction of serum mMCP-1 levels in the imatinib-treated A/J mice indicates that imatinib was sufficient to substantially attenuate the constitutive, *Kit*-dependent pathway for MC activation in the A/J strain. Moreover, the fact that the extent of mMCP-1 reduction failed to correlate with the degree of MCh reactivity supports the conclusion that mMCP-1 (or associated MC-derived mediators) are not effectors of naïve AHR.

Although our observation that genetic and pharmacological perturbation of c-Kit resulted in a significant decrement in naïve AHR was consistent with a role for MCs in mediating AHR, it did not exclude a non-MC *Kit*-dependent mechanism. Introducing the *Mitf* mutation onto the F1 background provided another means to assess whether MCs are required for mice to demonstrate naïve AHR. This evaluation was initially done by comparing a cohort of F2 mice that were also segregating the *Mitf* mutation (Fig. 4). As expected, the F2 mice that were wild type at the *Mitf* locus showed a distribution of airway responsiveness similar to that seen in other F2 and N2 populations. Surprisingly, the distribution in the F2 mice homozygous for *Mitf* was not different; that is, genetic ablation of MCs did not preclude the expression of AHR. Because this analysis was not identical to our original study of F1 mice carrying the *W^v* mutation on a congenic background, we replicated that analysis by introducing the *Mitf* mutation onto the A/J genetic background by using a marker-assisted breeding strategy, which allowed the comparison of the effect of the *W^v* mutation to the *Mitf* mutation in the context of the same F1 genetic background. Remarkably, naïve AHR was not reduced in F1 mice that lacked MCs as a consequence of genetic disruption of *Mitf* (Fig. 4). Thus, c-Kit function, but not functional MCs, is required for naïve AHR in A/J mice.

Our study suggests that a perturbation in c-Kit function leads to two distinct phenotypic features in A/J mice: naïve AHR and MC dysregulation. The latter is reflected by increased basal MC numbers (particularly in the spleen and, to a lesser extent, in the airway) and MC activation (reflected by elevated serum mMCP-1). Importantly, our data demonstrate that the histological presence of MCs is not causally related to AHR, suggesting that c-Kit acts on additional targets to induce naïve AHR in A/J mice. Pulmonary dendritic cells (the most potent antigen-presenting cells) can express c-Kit in certain antigen-induced models of pulmonary inflammation, and dendritic cells from the lungs of A/J mice show an enhanced capacity to stimulate the development of pathogenic T-helper responses relative to dendritic cells from C3H mice (35). However, given that our studies were carried out in mice that had not been subjected to a specific antigen challenge regimen, it is unlikely that dendritic cells play a role in the A/J phenotype. Furthermore, previous studies documented that the naïve AHR of A/J mice cannot be conveyed to hyporesponsive strains by transfer of bone marrow nor can it be abrogated by transfers of marrow from hyporesponsive strains (36). These investigators also generated A/J mice deficient for B and T cells by creating congenic lines carrying mutations in *Rag1* and *Il4*, respectively, and found that deleting these lineages had no effect on naïve AHR.

The nonhematopoietic cell types that require normal c-Kit function include neural crest derivatives such as afferent neurons (37). Also, potentially of interest are interstitial Cajal-like cells (recently called telocytes), which are found in many tissues, including embryonic lungs (38, 39). These are c-Kit-expressing cells that may be related to the interstitial Cajal cells of the gut,

which mediate rhythmic activity in smooth muscle cells (40). The activity of these cells can be inhibited by imatinib (41).

We suspect that naïve AHR reflects a perturbation in a to-be-identified nonhematopoietic cell type whose function depends on c-Kit. Our findings suggest that the evident relationship between MCs and AHR in humans with asthma may also reflect separate processes that both involve a perturbation in c-Kit function. Moreover, our study suggests that c-Kit inhibition may have utility in the treatment of AHR that is separate from its effects on MC function.

Methods

Animals. A/J, B6, and C57BL/6J-*Kit^{W^v}* mice were obtained from Jackson Laboratories. To introgress the *Kit* mutation onto a hyperresponsive background, B6 *Kit^{W^v}* (abbreviated as B6^{W^v}) were crossed with A/J mice to create (A × B)F1 *Kit^{W^v}* (abbreviated as F1^{W^v}) progeny. The F1^{W^v} mice were then backcrossed to A/J mice for 10 generations, creating congenic A/J^{W^v} mice. Representative A/J^{W^v} mice were genotyped with a 1,440 SNP medium-density panel (Illumina) to confirm the presence of the *Kit* mutation and A/J genetic homozygosity (Fig. S1B). These mice were then crossed to B6^{W^v} mice to create F1^{+/+} and F1^{W^vW^v} mice for analyses. To introgress the *Mitf* mutation onto a hyperresponsive background, B6 mice carrying the *Mitf^{mi-Vga9}* allele were crossed with A/J mice to generate F1-*Mitf^{mi-Vga9}* (F1^{Vga9/+}) mice. The F1^{Vga9/+} mice were then intercrossed to generate F2^{+/+} and F2^{Vga9/Vga9} mice for AHR analyses. A SNP genotype-assisted breeding strategy was used to create congenic A/J^{Vga9/Vga9} mice. F1^{Vga9/+} mice were backcrossed to A/J mice for four generations, selecting for those carrying A/J alleles, to create N4 A/J *Mitf^{mi-Vga9}* (N4^{Vga9/+}) mice. N4^{Vga9/+} mice at this stage were genotyped with the 1,440 SNP panel and shown to be homozygous A/J at >90% of loci. N4^{Vga9/+} mice were crossed with B6^{Vga9/+} mice to generate homozygous (A/J × B6) F1^{mi-Vga9/Vga9} (F1^{Vga9/Vga9}) and wild-type F1^{+/+} mice for analysis. N2 animals were generated by backcrossing (A × B) F1 mice to B6 mice. All animals were housed in microisolation cages under viral antibody-free conditions.

Phenotype Analysis. Animals were phenotyped as adapted from Ackerman et al. (20). Briefly, pulmonary resistance after each inhaled MCh dose (5, 10, 20, or 50 mg/mL in sterile PBS) was determined by using the SCIREQ flexiVent system. See *SI Methods* for details.

Imatinib Treatment. A/J and B6 mice (8–10 wk old) were treated with imatinib (methanesulfonate salt; LC Laboratories) as adapted from Dietz et al. and Nakagawa et al. (26, 27). Briefly, mice received 50 mg/kg imatinib diluted in sterile water by i.p. injection for 21 d consecutively. See *SI Methods* for details.

Histology. The lungs, spleen, sternum, stomach, and small intestine were harvested after phenotypic analysis, rinsed in sterile PBS, and fixed in 4% paraformaldehyde in PBS for 3–6 h. Tissues were stained with chloroacetate esterase to demarcate MCs and counterstained with hematoxylin, and MC numbers were counted by using brightfield microscopy under 20× magnification. Tracheas were harvested after phenotypic analysis, fixed in 4% paraformaldehyde in PBS for 24 h, cut ventrally, and placed in 96-well plates. The tracheas were then permeabilized with 0.2% Triton X-100. After blocking with serum, tracheas were incubated with rabbit anti-mMCP-6. After incubation with a mixture of secondary goat anti-rabbit IgG and α-smooth muscle-FITC, tracheas were spread out on a glass slide with the adventitia upward, covered with Vectashield mounting media containing DAPI nuclear stain, and coverslipped. Demarcated MCs were then counted through the entire length of the trachea to generate a total tracheal MC number per animal. See *SI Methods* for details.

Serum mMCP-1. Serum mMCP-1 levels were determined by using a commercially available ELISA kit (Moredun Scientific) according to the manufacturer's instructions.

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Supporting Information

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SI Methods

Phenotype Analysis. Animals were phenotyped as adapted from Ackerman et al. (1). Pulmonary resistance after each inhaled methacholine (MCh) dose (5, 10, 20, 50 mg/mL in sterile phosphate-buffered saline) was determined using the flexiVent system (SCIREQ, Montreal, QC, Canada). Mice were anesthetized with sodium pentobarbital [80 mg/kg intraperitoneal (i.p.)] diluted in sterile saline. Once mice were completely anesthetized as evidenced by no response after toe pinch, a tracheostomy was performed. An 18-gauge tubing adaptor (BD Biosciences, Franklin Lakes, NJ) was inserted into the trachea and secured with suture. Animals did not receive a paralytic agent but were studied in deep anesthesia so that no spontaneous breathing efforts were evident.

Mice were placed on the ventilator and standard ventilation was delivered at 10 mL/kg tidal volume. A lung recruitment maneuver was then performed by occluding the expiratory loop circuit for three breath cycles. After a 5-min equilibration period, sterile phosphate-buffered saline and subsequent MCh doses (6.25, 12.5, 25, and 50 mg/mL) were delivered by a nebulizer integrated into the inspiratory ventilator circuit. Pulmonary resistance was measured immediately after MCh delivery and measurements were made every 4 s for 2 min. Peak airway resistance per MCh dose (highest pulmonary resistance achieved during each MCh dose–baseline pulmonary resistance) was used for data analysis.

Mast Cell (MC) Culture and Adoptive Transfer. Bone marrow was flushed from the femurs of male C57BL/6J F1 (F1) and C57BL/6J (B6) mice with a 27-gauge needle attached to a 10-cc syringe containing sterile PBS. Bone marrow-derived MCs (BMMCs) were generated as previously detailed by Murakami et al. (2, 3). Briefly, bone marrow cells were cultured for 6–8 wk in T-25 culture flasks (Corning) containing 50% enriched medium (RPMI medium 1640 with 100 U/mL penicillin, 100 μ g/mL streptomycin, 10 μ g/mL gentamycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, and 10% FBS/50% WEHI-3 cell conditioned medium) and both IL-3 (10 ng/mL; Pepro-Tech) and stem cell factor (SCF; 100 ng/mL; PeproTech). After 6 wk, cells were stained with toluidine blue and safranin (as a surrogate for heparin content), and >95% of cells were BMMCs. For bone marrow adoptive transfers, BMMCs were suspended at 5×10^6 cells in 200 μ L of sterile PBS and injected into the tail vein of recipient mice. At 16–20 wk postinjection, phenotypic and histological analyses were carried out as described in *SI Methods*.

Imatinib Treatment 8–10-wk-old A/J and C57BL/J mice were treated with imatinib (methanesulphonate salt, LC Laboratories, Woburn, MA) as adapted from Deitz et al. and Nakagawa et al. (4, 5). Briefly, mice received 50 mg/kg imatinib diluted in sterile water by i.p. injection for 21 consecutive d. Control animals re-

ceived sterile water i.p. for 21 consecutive d. On day 22, animals were phenotyped as indicated above and peak airway resistance per MCh dose was recorded. After phenotypic analysis, mice were euthanized by cervical dislocation and blood, trachea, lungs, spleen, sternum, stomach, and small intestine were removed for future ELISA and histological analyses.

Histology The lungs, spleen, sternum, stomach, and small intestine, were harvested after phenotypic analysis, rinsed in sterile PBS, and fixed in 4% paraformaldehyde in PBS for 3–6 h. Tissues were embedded in JB-4 glycol methacrylate polymer with a commercially available kit according to manufacturer's instructions (Electron Microscopy Sciences, Hatfield, PA) and sectioned. Sections were stained with α -d-naphthol chloroacetate esterase (CAE) to demarcate MC and counterstained with Gill's II hematoxylin. Histological analyses were performed by a pathologist under blinded conditions. Tissue mast cell numbers were counted using brightfield microscopy under $20 \times$ magnifications. For each tissue, MC were counted in multiple fields (lung, 20 fields; trachea: mucosa, submucosa, smooth muscle, connective tissue, four fields per subcompartment; stomach: mucosa, submucosa, smooth muscle, five fields per subcompartment; small intestine: mucosa, submucosa, smooth muscle, five fields per subcompartment; spleen, five fields; sternum bone marrow, five fields) on multiple slides and averaged for each animal group.

Tracheas were harvested after phenotypic analysis, rinsed in sterile PBS, fixed in 4% paraformaldehyde in PBS for 24 h, cut ventrally, and placed in 96-well plates. The tracheas were then permeabilized with 0.2 % Triton X-100 in PBS for 2 h on an orbital shaker. Nonspecific secondary and synthetic fluorophore binding sites were then blocked with a 1:1 mixture of goat (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and mouse (Vector Laboratories, Burlingame, CA) serum for 2 h. After blocking, tracheas were incubated with 200 μ L/well (1:500 dilution) of rabbit antimouse mast cell protease 6 (mMCP-6) overnight at 4 $^{\circ}$ C. Trachea were then washed three times in PBS and co-incubated in a mixture of secondary Alexa Fluor 568 goat anti-rabbit IgG (2 μ g/mL, Invitrogen, Carlsbad, CA) and α -smooth muscle-FITC (2.7 μ g/mL, Sigma-Aldrich, St. Louis, MO) antibodies for 2 h, covered, at 37 $^{\circ}$ C, on an orbital shaker. Tracheas were rinsed thoroughly in PBS, spread out on a glass slide with the adventitia upward, covered with Vectashield mounting media containing DAPI nuclear stain (Vector Laboratories, Burlingame, CA), and cover slipped. Tracheas were then viewed with the Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY) and images were acquired with the Hamamatsu C10800 digital camera and HC Image software 1.1.3.1 (Hamamatsu Corp., Bridgewater, NJ). Demarcated MCs were then counted through the entire length of the trachea to generate a total tracheal MC number per animal. Total MC counts were then averaged per animal group.

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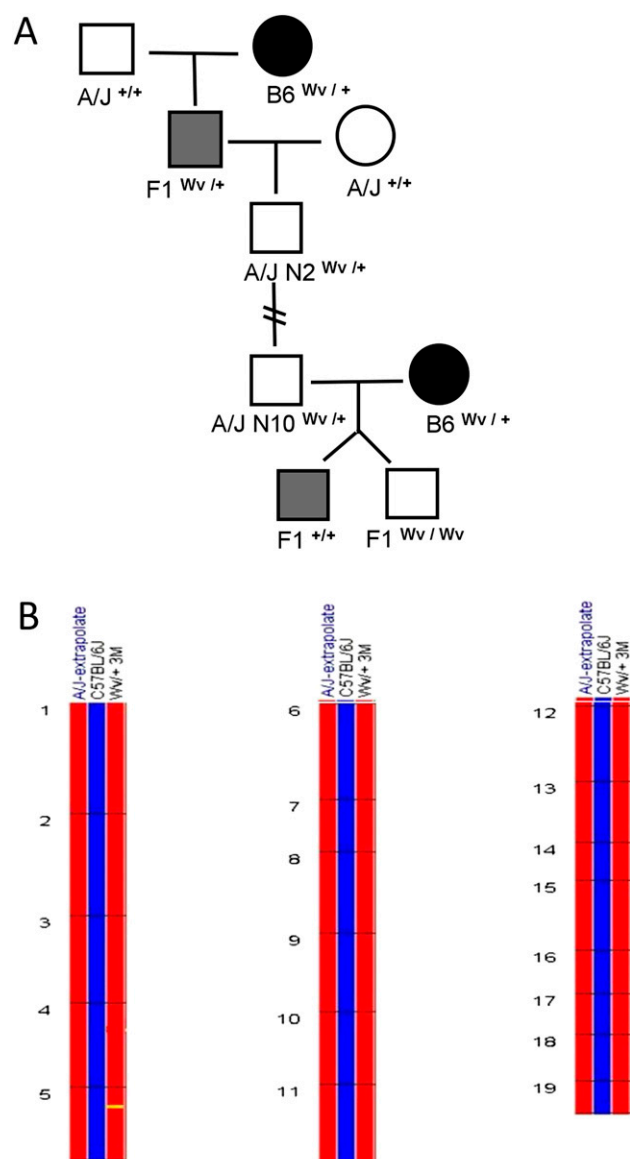


Fig. S1. Generation of $A/J^{Wv/+}$ congenic mice. (A) As described in *Methods and Results*, $B6^{Wv/+}$ mice were crossed with A/J mice to create $F1^{Wv/+}$ progeny. The $F1^{Wv/+}$ mice were then backcrossed to A/J mice for 10 generations, creating $A/J^{N10} Wv/+$ mice. (B) Representative A/J , $B6$, and $A/J^{Wv/+}$ parental mice were genotyped on a whole-genome panel to demonstrate A/J homozygosity. All informative markers except those at the *Kit* locus on chromosome 5 are A/J -derived. Red, A/J allele; blue, $B6$ allele; yellow, heterozygous allele.

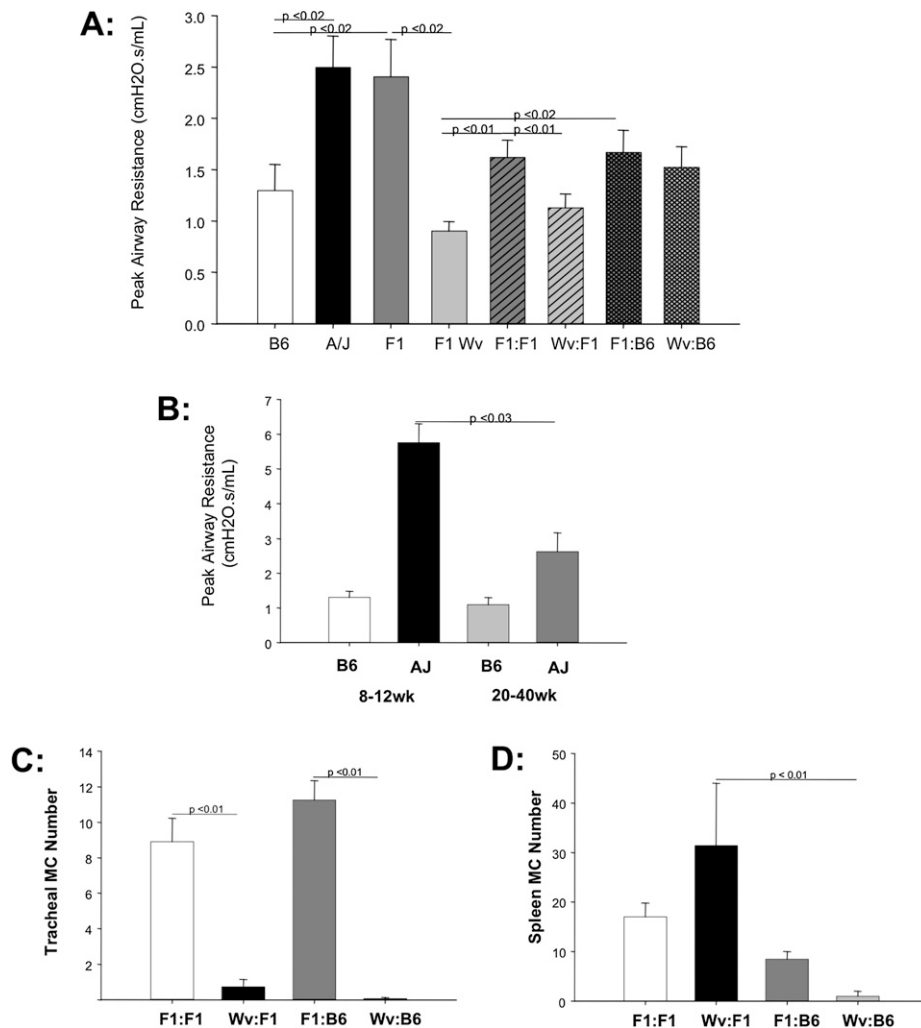


Fig. S2. Adoptive transfer of F1 or B6 BMMCs to $F1^{Wv/Wv}$ recipient mice. (A) BMMCs were transferred to mice via tail-vein injection. At 16–20 wk postinjection, methacholine (MCh)-induced peak airway resistance was determined. Adoptive transfer of either $F1^{+/+}$ or B6 BMMCs did not completely restore the MCh-induced airway resistance of $F1^{Wv/Wv}$ mice to the level of F1 control mice. In addition, all murine adoptive transfer groups had a reduced airway hyper-responsiveness (AHR) phenotype compared with A/J and F1 controls. F1:F1, F1 recipient mice received murine BMMCs from F1 donor mice. Wv:F1, F1 recipient mice received murine BMMCs from $F1^{Wv/Wv}$ donor mice. F1:B6, F1 recipient mice received murine BMMCs from B6 donor mice. Wv:B6, $F1^{Wv/Wv}$ recipient mice received murine BMMCs from B6 donor mice. Controls: B6, A/J, F1, and $F1^{Wv/Wv}$. (B) Peak airway resistance in B6 and A/J mice is age-dependent. B6 and, to a greater extent, A/J mice equivalent in age (20–40 wk) to mice that underwent adoptive transfer had a reduced AHR phenotype compared with younger (8–12 wk) mice. (C) Tissue MC quantification by chloroacetate esterase staining and brightfield microscopy revealed that BMMCs did not engraft to appreciable amounts in the tracheas of recipient $F1^{Wv/Wv}$ mice compared with F1 controls. (D) BMMCs were sequestered primarily in the spleens of $F1^{Wv/Wv}$ mice transferred with F1 BMMCs and were nearly absent from $F1^{Wv/Wv}$ receiving B6 BMMCs. Values represent mean $\pm$ SE; n = at least 8 in each group.

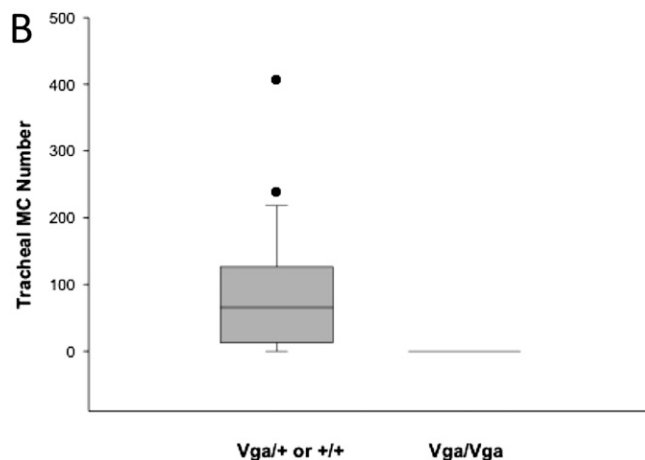
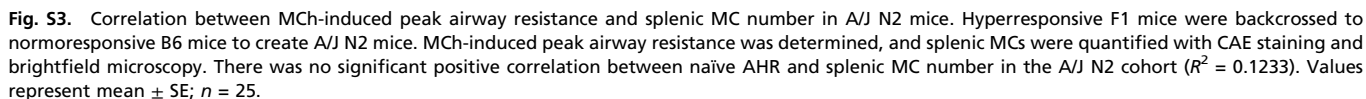


Fig. S4. Generation of $A/J^{Vga9/+}$ congenic mice. (A) $B6^{Vga9/Vga9}$ were crossed with A/J mice to create $F1^{Vga9/+}$ progeny. The $F1^{Vga9/+}$ mice were then backcrossed to A/J mice for four generations, creating $A/J\ N4^{Vga9/+}$ mice. These mice were then crossed to $B6^{Vga9/+}$ mice to create $F1$ wild-type and $F1^{Vga9/Vga9}$ mice for study. (B) Tracheal MC numbers in wild-type versus *Mitf* mutant mice. MCs were demarcated with anti-mouse MC protease 6 (mMCP-6) antibody and counted along the entire length of the trachea by florescent microscopy. $n > 8$ in each group.