Video Article

# Analyzing the Functions of Mast Cells *In Vivo* Using '*Mast Cell Knock-in*' Mice

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## Abstract

Mast cells (MCs) are hematopoietic cells which reside in various tissues, and are especially abundant at sites exposed to the external environment, such as skin, airways and gastrointestinal tract. Best known for their detrimental role in IgE-dependent allergic reactions, MCs have also emerged as important players in host defense against venom and invading bacteria and parasites. MC phenotype and function can be influenced by microenvironmental factors that may differ according to anatomic location and/or based on the type or stage of development of immune responses. For this reason, we and others have favored *in vivo* approaches over *in vitro* methods to gain insight into MC functions. Here, we describe methods for the generation of mouse bone marrow-derived cultured MCs (BMCMCs), their adoptive transfer into genetically MC-deficient mice, and the analysis of the numbers and distribution of adoptively transferred MCs at different anatomical sites. This method, named the '*mast cell knock-in'* approach, has been extensively used over the past 30 years to assess the functions of MCs and MC-derived products *in vivo*. We discuss the advantages and limitations of this method, in light of alternative approaches that have been developed in recent years.

## Video Link

The video component of this article can be found at https://www.jove.com/video/52753/

## Introduction

Mast cells (MCs) are hematopoietic cells that arise from pluripotent bone marrow progenitors[1-3]. Following bone marrow egression, MCs progenitors migrate into various tissues where they develop into mature MCs under the influence of local growth factors[1-3]. Tissue-resident MCs are strategically located at host-environment interfaces, such as the skin, the airways and the gastrointestinal tract, where they behave as a first line of defense against external insults[3-6]. MCs are often sub-classified based on their "baseline" phenotypic characteristics and their anatomic locations. In mice, two types of MCs have been described: "connective tissue-type" MCs (CTMCs) and mucosal MCs (MMCs)[1-3,7,8]. CTMCs are often located around venules and near nerve fibers, and reside in serosal cavities, while MMCs occupy intraepithelial locations in the gut and respiratory mucosa[1-3].

Numerous methodologies have been applied to study biological functions of MCs[9-13]. Many groups have focused on *in vitro* approaches using either cell lines (such as the human MC lines HMC1[14] or LAD2[15,16]), *in vitro* derived MCs (such as human peripheral blood-derived MCs[17], or mouse bone marrow-derived cultured MCs [BMCMCs][18], fetal skin-derived cultured MCs [FSCMCs][19] and peritoneal cell-derived MCs [PCMCs][20]) or *ex vivo* isolated MCs from different anatomical sites. All these models are widely used to study molecular details of MC biology, such as signaling pathways involved in MC activation. However, an important aspect of MCs biology is that their phenotypic and functional characteristics (*e.g.*, cytoplasmic granule protease content or response to different stimuli) can be modulated by anatomical location and microenvironment[2,7]. Since the exact mixture of such factors that are encountered *in vivo* may be difficult to reproduce *in vitro*, we favor using *in vivo* approaches to gain insights into MCs functions[9].

Several mouse strains with genetic MC deficiency exist, such as the widely used WBB6F$_1$-*Kit* $^{W/W-v}$ or C57BL/6-*Kit* $^{W-sh/W-sh}$ mice. These mice lack expression and/or activity of KIT (CD117), the receptor for the main MC growth factor stem cell factor (SCF)[21,22]. As a result, these mice have a profound MC deficiency but also have additional phenotypic abnormalities related to their c-*kit* mutations (in the WBB6F$_1$-*Kit* $^{W/W-v}$ mice) or to the effects of the large chromosomal inversion that results in reduced c-*kit* expression (in the C57BL/6-*Kit* $^{W-sh/W-sh}$ mice)[9,10,12,23]. More recently, several strains of mice with c-*kit*-independent constitutive MC deficiency have been reported[24-26]. All these mice and some additional new types of inducible MC-deficient mice have been recently reviewed in detail[9,10,13].

Here, we describe methods for the generation of mouse bone marrow-derived cultured MCs (BMCMCs), their adoptive transfer into MC-deficient mice, and the analysis of the numbers and distribution of adoptively transferred MCs at different anatomical sites. This so-called *'mast cell knock-in'* method can be used to assess the functions of MCs and MC-derived products *in vivo*. We discuss the advantages and limitations of this method, in light of alternative approaches that have been developed in recent years.

## Protocol

All animal care and experimentation were conducted in compliance with the guidelines of the National Institutes of Health and with the specific approval of the Institutional Animal Care and Use Committee of Stanford University.

# 1. Generation and Characterization of Bone Marrow-derived Cultured Mast Cells (BMCMCs).

Note: Donor BMCMCs should be generated from bone marrow cells of the same genetic background as the recipient MC-deficient mice. Male-derived donor BMCMCs are not suitable for engraftment of female mice. Female-derived donor BMCMCs will successfully engraft into both male and female recipients.

1. Bone Marrow Extraction.
    1. Pour sterile phosphate-buffered saline (PBS) into 6 well culture plate (1 well per mouse) and place on ice.
    2. Soak the dissection instruments in 70% ethanol for sterilization.
    3. Euthanize donor mice by carbon dioxide ($CO_2$) inhalation followed by cervical dislocation, then spray the mice with 70% ethanol at the sites of manipulation.
    4. Use sterile dissection instruments to extract the femur, tibia and fibula bones without cutting any bone epiphyses.
    5. While securing the bones with a forceps, scrape off all tissue from bones (and discard fibula) using sterile scissors (or scalpel blades). Place bones in PBS on ice until all bones are collected.
    6. Carry out all remaining steps in sterile tissue culture hood. Using sterile instruments, secure bones with forceps and cut off both epiphyses to expose the medullary cavity.
    7. Using 3 ml syringes and 30 G needles, and cold flushing medium (Dulbecco Modified Eagle Medium [DMEM] supplemented with 10% fetal calf serum [FCS, heat-inactivated], 2 mM L-glutamine, 1% antibiotic-antimycotic solution, 50 µM B-mercaptoethanol), flush the red bone marrow into a Petri dish.
    8. Use the same syringe to dissociate flushed bone marrow cell clusters by repeated gentle aspiration and ejection.
    9. Pool femoral and tibial bone marrow from each mouse into one 15 ml centrifuge tube. Fill the tube with cold flushing medium to wash bone marrow cells and centrifuge at 400 x g for 5 min at 4 °C.

2. BMCMCs Culture.
    1. Remove supernatant and recover pelleted bone marrow cells in 10 ml of culture medium (DMEM supplemented with 10% fetal calf serum [FCS, heat-inactivated], 2 mM L-glutamine, 1% antibiotic-antimycotic solution, 50 µM B-mercaptoethanol and 20% WEHI-3 cell-conditioned medium [as a source of IL-3; alternatively use recombinant mouse IL-3 at 10 ng/ml]).
    2. Place cells in tissue culture flask of appropriate size (*e.g.*, T25 for 1 mouse, T75 for 2 mice, *etc.*).
    3. 1-2 days after plating cells, transfer medium and suspension cells (leaving debris and adherent cells sticking to bottom of flask) to a new flask and add fresh culture medium (10 ml/mouse).
    4. During the following weeks, feed cells every 3-4 days (add 10 ml of culture medium per mouse). Maintain cell density between 2.5 x $10^5$-1 x $10^6$ cells/ml. Transfer non-adherent cells to a new flask once a week until no adherent cells are present in the culture flask. Test maturity of cells (see below) before use for *in vitro* assays or engraftment into MC-deficient mice.
    NOTE: Complete differentiation of BMCMCs will take 4-6 weeks.

3. Assessment of BMCMC maturity.
    1. *Using flow cytometry.*
        1. Wash cells (5 x $10^4$-5 x $10^5$ cells/condition) with ice-cold FACS buffer (PBS, 0.5% FCS) in 5 ml polystyrene round bottom tube. Centrifuge at 400 x g for 5 min at 4 °C. Aspirate supernatant.
        2. Dilute anti-mouse CD16/32 (clone 93 or 2.4G2) monoclonal antibodies 1:200 (2.5 µg/ml) in FACS buffer. Add 10 µl to each pellet and resuspend by brief vortexing. Incubate 5 min on ice to block Fc binding.
        NOTE: Protect samples from direct light for all remaining steps.
        3. Dilute phycoerythrin (PE) conjugated anti-mouse Fc εRI α (clone MAR-1) 1:200 (1 µg/ml) and fluorescein isothiocyanate (FITC) conjugated anti-mouse KIT (CD117; clone 2B8) 1:200 (2.5 µg/ml) ("staining solution"), or the respective labeled isotype control antibodies in FACS buffer ("isotype control solution").
        4. Split half of the blocked cells from step 1.3.1.2) into an additional polystyrene round bottom tube and add 20 µl of "staining solution" in one tube and 20 µl of "isotype controls solution" in the other tube. Incubate 30 min on ice.
        5. Add 3 ml ice-cold FACS buffer and centrifuge at 400 x g for 5 min at 4 °C. Discard supernatant and resuspend the pellet in 300 µl FACS buffer containing 1 µg/ml propidium iodide (PI, for identification of dead cells). Proceed to flow cytometry analysis (see **Figure 2A**).

    2. Using toluidine blue staining.
        1. Wash 1 x $10^5$ cells once with PBS by centrifugation at 400 x g for 5 min at room temperature, then aspirate and resuspend cells in 200 µl of PBS.
        2. Transfer the cell suspension into a prepared cytofunnel attached to a microscope slide and proceed with cyto-centrifugation using a cytocentrifuge (40 x g for 5 min).
        3. Air dry slides for 10 min and draw a wax circle around cells using a PAP pen. Add 0.1% Toluidine blue solution to cover the cells. Incubate for 1 min. Wash slides in running tap water for 1 min, then air-dry slides for 10 min.
        4. Coverslip cells using mounting medium. Proceed to light microscope analysis (see **Figure 2B**)

## 2. Engraftment of Mast Cell-deficient Mice with BMCMCs.

1. Ear engraftment by intradermal (i.d.) injection.
   NOTE: For adoptive transfer of BMCMCs into the ear pinnae of MC-deficient mice, we recommend performing two injections of 1 x $10^6$ cells each (for a total of 2 x $10^6$ cells) per ear pinna. Intradermal engraftment of BMCMCs into the ear pinnae of MC-deficient mice has been used by many investigators in several models, including models of passive cutaneous anaphylaxis (PCA)[24,27], host defense against venoms[28], and chronic hypersensitivity (CHS) reactions[29,30].
   1. Count and resuspend cells at 4 x $10^7$ BMCMCs/ml (1 x $10^6$ BMCMCs/ 25 µl) in cold DMEM. Transfer BMCMCs solution into a 1 ml syringe equipped with a 30 G needle. Keep on ice until injection.
   2. Anesthetize 4-6 weeks old MC-deficient mice using isoflurane (2.5% v/v). Check depth of anesthesia by toe pinch, adjust isoflurane if indicated and continue to monitor breathing and toe pinch response throughout the procedure. Apply ophthalmic ointment with a Q-tip to prevent dryness of eyes.
   3. With the index finger, create vertical pressure on the dorsal face of the ear pinna to expose and stretch the ventral face.
   4. Perform two 25 µl i.d. injections of BMCMC solution into two different sites of the ventral face of the ear pinna, the first injection in the middle of the ear and the second injection toward the tip of the ear pinna. Wait 4-6 weeks after i.d. engraftment before performing *in vivo* experiments.
      Note: In our experience, by that time the number of MCs/$mm^2$ of dermis in the central part of the ear pinna generally is similar to that in the corresponding location in wild type mice, whereas the numbers of MCs/$mm^2$ in the periphery of the ear pinnae are typically substantially lower than those in the corresponding wild type mice[27,28]. This should be kept in mind when designing experiments with such MC-engrafted mice (*e.g.*, agents with possible effects on MC function should be injected into the central part of the ear pinnae, and the analysis of the effects of such treatments should also focus on that area).

2. Peritoneal cavity engraftment by intraperitoneal (i.p.) injection.
   NOTE: Adoptive transfer of BMCMCs into the peritoneal cavity of MC-deficient mice will require one injection of 2 x $10^6$ cells per mouse. Such i.p. injections of BMCMCs into MC-deficient mice have been used by many groups to study the roles of peritoneal MCs in various models, including models of host defense against venoms[31] or bacterial sepsis[32,33].
   1. Count appropriate number of cells and resuspend at 1 x $10^7$ BMCMCs/ml (2 x $10^6$ BMCMCs/ 200 µl) in cold DMEM. Transfer BMCMCs solution into a 1 ml syringe equipped with a 25 G needle. Keep on ice until injection.
   2. Perform 1 injection of 200 µl BMCMCs solution into the peritoneal cavity of 4-6 week old MC-deficient mice. Wait 4-6 weeks after i.p. engraftment before performing *in vivo* experiments.

3. Engraftment by intravenous (i.v.) injection.
   NOTE:Adoptive transfer of BMCMCs by i.v. injection into MC-deficient mice will require one injection of 5 x $10^6$ cells per mouse. Intravenous (i.v.) injections of BMCMCs into MC-deficient mice have been used by many groups to study the roles of MCs in various disease models, including models of bladder infection[34], asthma[35], lung fibrosis[36], and antibody-mediated arthritis[37].
   1. Count appropriate number of cells and resuspend at 2.5 x $10^7$ BMCMCs/ml (5 x $10^6$ BMCMCs/200 µl) in cold DMEM. Transfer BMCMCs solution into a 1 ml syringe equipped with a 30 G needle. Keep on ice until injection.
   2. Anesthetize 4-6 weeks old MC-deficient mice using isoflurane (2.5% v/v). Check depth of anesthesia by toe pinch, adjust isoflurane if indicated and continue to monitor breathing and toe pinch response throughout the procedure. Apply ophthalmic ointment with a Q-tip to prevent dryness of eyes.
   3. Perform one injection of 200 µl of BMCMC solution into the tail vein (or, alternatively, the retro-orbital vein) of a MC-deficient mouse. Wait 12 weeks after i.v. engraftment before performing *in vivo* experiments.

## 3. Analysis of Engrafted Mast Cell-deficient Mice.

1. Ear engraftment analysis.
   1. At the end of the experiment, euthanize mice by $CO_2$ inhalation followed by cervical dislocation.
   2. Isolate the ear pinnae and fix in 10% (vol/vol) buffered formalin overnight at 4 °C. Embed fixed ear pinnae in paraffin, cut 4 µm sections of ear pinnae and mount on glass slides.
   3. Stain the slides with a 0.1% Toluidine blue solution for 1 min at room temperature. Wash slides in tap water for 1 min, then air-dry slides for 10 min at room temperature.
   4. Coverslip slides using mounting medium, then count the number of engrafted MCs per pinnae sections using a light microscope. Evaluate the engraftment efficiency by comparing the percentage and distribution of MCs in the ear skin of engrafted mice *versus* wild type mice.

2. Peritoneal cavity engraftment analysis.
   1. Evaluation of mast cells number in the peritoneal cavity.
      1. At the end of the experiment, euthanize mice by $CO_2$ inhalation followed by cervical dislocation. Remove carefully the ventral skin of the mice without breaking the peritoneal cavity.
      2. Inject 5 ml of cold or room temperature PBS in the peritoneal cavity using a 5 ml syringe equipped with a 25 G needle. Use cold PBS to reduce the risk of activating peritoneal cells (this is very important when evaluating peritoneal MC degranulation or levels of some MC-derived products in the peritoneal lavage fluid). Perform a massage of the abdomen for 20 sec to harvest peritoneal cells.
      3. Slowly aspirate the peritoneal lavage using a 5 ml syringe equipped with a 22 G needle. Record the volume of aspirated lavage (expect to recover up to 80% of injected volume).
      4. Transfer peritoneal lavage fluid into a 5 ml polystyrene round bottom tube and centrifuge at 400 x g for 5 min at 4 °C. Aspirate supernatant. Recover the pellet in 400 µl cold PBS.

5. Count total number of peritoneal cells using a hemocytometer chamber. Use half of the cell suspension for a flow cytometry analysis (as described in step 1.3.1), to evaluate the percentage and marker expression of engrafted MCs in the peritoneal cavity. Multiply the total number of peritoneal cells by the percentage of MCs to obtain absolute number of peritoneal MCs.
6. Perform a cytocentrifugation with the remaining cells as described in step 1.3.2.2. Air dry slides for 10 min at room temperature and draw a wax circle around cells using a PAP pen.
7. Cover cells with undiluted May-Grünwald Staining solution for 5 min, followed by washing in PBS for 5 min, both at room temperature. Cover cells with Giemsa stain diluted 1:20 with deionized water and incubate 20 min at room temperature. Wash slides 2 times in tap water for 1 min, then air-dry slides.
8. Coverslip cells using mounting medium, and calculate the percentage of engrafted MCs using a light microscope (by counting at least 400 total cells). Evaluate the engraftment efficiency by comparing the percentage of MCs in the peritoneal lavage of engrafted mice *versus* wild type mice.

2. Evaluation of mast cells in the mesenteric windows.
    1. Following the peritoneal lavage described in step 3.2.1, cut open the peritoneal membrane to expose the intestinal tract of the mice. Arrange 4 to 5 mesenteric windows per mouse onto a slide.
    2. Fix the slides for 1 hr in Carnoy solution (3:2:1 vol/vol/vol of ethanol, chloroform, and glacial acetic acid) at room temperature. Air-dry slides and remove the intestine (the fixed mesenteric windows will remain attached to the slides).
    3. Stain the preparations for 20 min at room temperature with Csaba (Alcian blue/Safranin O) staining solution.
        1. To make 500 ml of Csaba stain, prepare 500 ml of acetate buffer by mixing 100 ml of 1 M sodium acetate solution with 120 ml of 1 M HCl. Make up to 500 ml with deionized water and adjust pH to 1.42. Then, dissolve 90 mg Safranin [identifies 'mature MCs' in red], 1.8 g Alcian blue [identifies 'immature MCs' in blue] and 2.4 g ferric ammonium sulphate $NH_4Fe(SO_4)_2$ into 500 ml of acetate buffer.
    4. Coverslip slides using mounting medium, then count the number of engrafted MCs per mesenteric window using a light microscope. Evaluate the engraftment efficiency by comparing the percentage and distribution of MCs in the mesenteric windows of engrafted mice *versus* wild type mice.

3. I.v. engraftment analysis.
    1. At the end of the experiment, euthanize mice by $CO_2$ inhalation followed by cervical dislocation, and harvest tissue of interest (*e.g.* lung, skin or spleen) and fix overnight in 10% (vol/vol) buffered formalin at 4 °C.
    2. Embed fixed tissues in paraffin, cut 4 µm sections, and mount on glass slides. Stain the slides with a 0.1% Toluidine blue solution for 1 min at room temperature. Wash slides in tap water for 1 min, then air-dry slides for 10 min.
    3. Coverslip slides using mounting medium and evaluate number and distribution of engrafted MCs per tissue sections using a light microscope.

## Representative Results

An overview of the '*mast cell knock-in*' approach is shown in **Figure 1**, and includes the generation of BMCMCs, the number of cells that should be engrafted i.p., i.d. or i.v. into MC-deficient mice (the number can be varied if indicated based on the experimental design) and the interval between engraftment and experiment depending on the injection site (this interval also can vary, if indicated; *e.g.*, the content of stored mediators in MC cytoplasmic granules increases steadily with time[38]). **Figure 2** shows representative flow cytometry analyses and toluidine blue staining of BMCMCs after 1, 15 and 45 days of culture in DMEM medium containing in 20% WEHI-3 cell-conditioned medium as a source of IL-3. Note that BMCMCs cultured for 45 days are 95% pure, contain high numbers of cytoplasmic granules and can be used for engraftment experiments, while cells cultured for 15 days are not suitable for engraftment. **Figure 3** shows representative successful engraftment in the ear pinnae 4 weeks after i.d. engraftment (**Figure 3A**), in mesenteric windows (**Figure 3B**) and peritoneal cavity (**Figure 3C**) 6 weeks after i.p. engraftment, and in the lung (**Figure 3D**) 12 weeks after i.v. engraftment.

## Figure 1

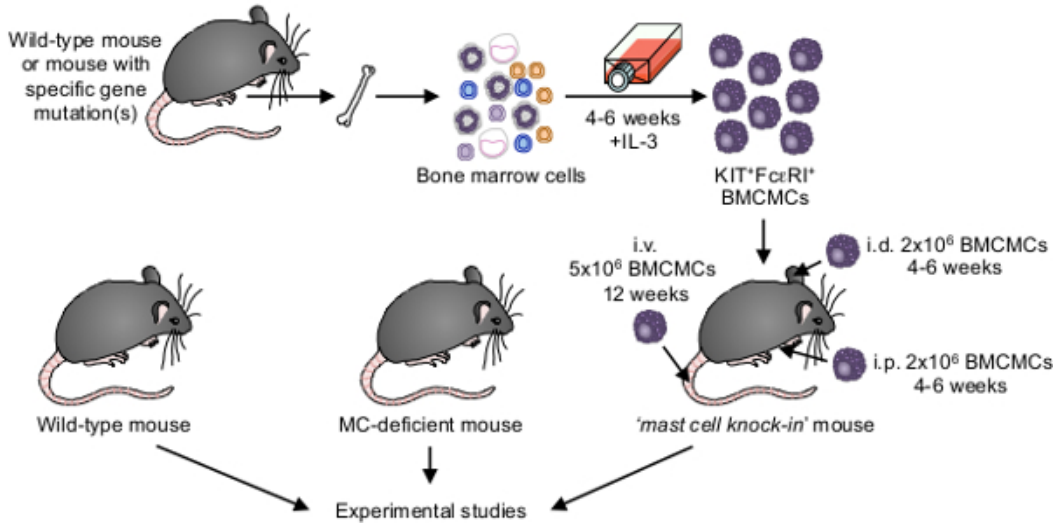


**Figure 1: '*Mast cell knock-in*' mouse model for analyses of MC functions *in vivo*.** Wild type or mutant bone marrow-derived cultured MCs (BMCMCs) are generated by culturing bone marrow cells for at least 4-6 weeks in 20% WEHI-3 cell-conditioned medium as a source of IL-3 (or alternatively in medium containing 10 ng/ml recombinant mouse IL-3). These BMCMCs can then be engrafted into MC-deficient mice to create so-called '*mast cell knock-in*' mice. BMCMCs can be injected *via* different routes (intravenous [i.v.], intraperitoneal [i.p.] or intradermal [i.d.]) for local (i.d., i.p.) or systemic (i.v.) reconstitution of various MC populations. MC function(s) in various biological responses can then be analyzed by comparing the responses in wild type mice, MC-deficient mice and '*mast cell knock-in*' mice. The contribution(s) of specific MC products can be analyzed by comparing responses of '*mast cell knock-in*' mice engrafted with either wild type BMCMCs or BMCMCs derived from mice that lack, or express genetically-altered forms of, such products. (This is a modified version of **Figure 1** from ref.[12]).

# Figure 2

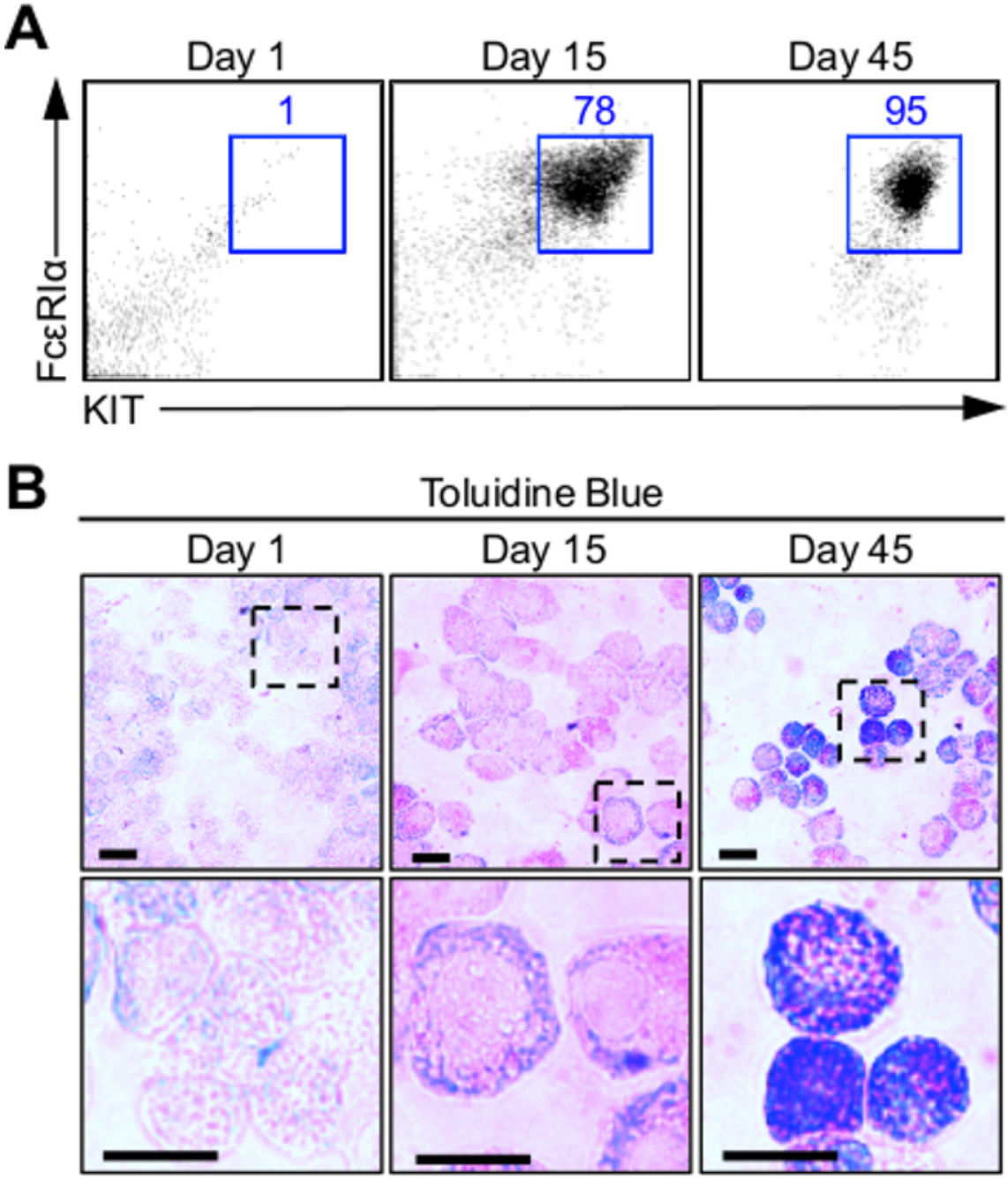


**Figure 2: Evaluation of the purity of BMCMC preparations by flow cytometry and microscopy.** (**A**) Representative flow cytometry analyses of FcεRIα and KIT (CD117) expression on the surface of 1 day (left panel), 15 days (middle panel) and 45 days (right panel) old BMCMCs. Propidium iodide (PI)-positive dead cells were excluded from the analysis (not shown). Numbers indicate the percentage of $Fc\varepsilon RI\alpha^{+}KIT^{+}$ BMCMCs gated in the blue square. (**B**) Representative pictures of BMCMCs stained with toluidine blue, lower panel is a magnification of the regions of the upper panel defined in black dotted lines. Bars = 10 µm.

# Figure 3

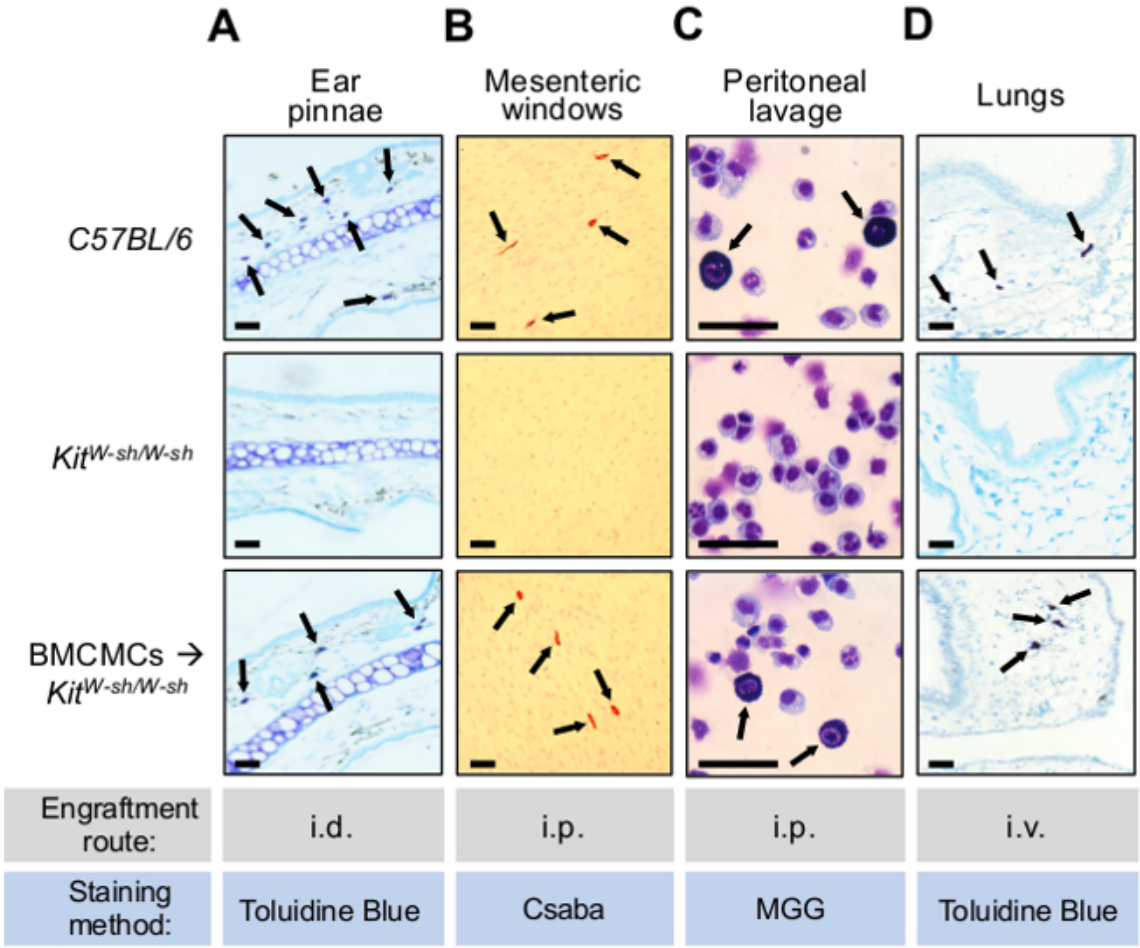


**Figure 3: Evaluation of the efficiency of MC engraftment at various anatomical sites.** (**A**) Representative pictures of 4 µm ear pinnae sections stained with toluidine blue. (**B**) Representative pictures of mesenteric windows stained with Safranin/Acian Blue ('Csaba' stain). (**C**) Representative pictures of cells present in peritoneal lavage fluids and stained with May-Grünwald Giemsa (MGG). (**D**) Representative pictures of 4 µm lung sections stained with toluidine blue. Bars = 50 µm (**A**, **B** and **D**) or 20 µm (**C**). Pictures are from C57BL/6 wild type mice (upper panel), MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice (middle panel) and MC-deficient mice engrafted with wild type BMCMCs: BMCMCs *Kit*$^{W\text{-}sh/W\text{-}sh}$ (lower panel). MCs are indicated with arrows.

| **Mice** | **Available Backgrounds** | **MC numbers** (steady state) | **Other phenotypes** (reviewed in[9,10,13]) | **Reported engraftments with BMCMCs** |
|---|---|---|---|---|
| ***Kit*$^{W/W\text{-}v}$** | **(WB/Re x C57BL/6)F$_1$** (Jackson Laboratories – WBB6F$_1$/J-*KitW*/*KitW-v*/J) | Absence of connective-tissue and mucosal MCs | Anemia, reduced basophil and neutrophil numbers, deficiencies in melanocytes and interstitial cells of Cajal, sterile, etc. | i.v.[35,37]; i.p.[31,62]; i.d.[28,29]; i.c.[50,51]; i.a.[49]; fp.i.[63] |
| ***Kit*$^{W\text{-}sh/W\text{-}sh}$** | **C57BL/6** (Jackson Laboratories – B6.Cg-*KitW-sh*/HNihrJaeBsmJ) Single nucleotide polymorphism analysis performed at Jackson laboratories shows that these mice are only ~87% C57BL/6-genetic background. | Absence of connective-tissue and mucosal MCs | Moderate increase in basophil and neutrophil numbers, increased numbers of myeloid-derived suppressor cells[64], deficiencies in melanocytes and interstitial cells of Cajal | i.v.[23,34-36]; i.p.[31,62]; i.d.[28,29]; i.a.[49] |
| | **C57BL/6J**[62] | | | |

| | | | | |
|---|---|---|---|---|
| | (Jackson Laboratories – B6.Cg-*KitWsh*/HNihrJaeBsmGlliJ) These mice have been backcrossed >11 times on the C57BL/6J background. | | | |
| | **BALB/c**[65] (C.B6-*Kit*$^{W\text{-}sh}$) | | | |
| ***Mcpt5-Cre; R-DTA*** | **C57BL/6**[25] (Tg(Cma1-cre) ARoer; B6.129P2-*Gt(ROSA)26Sortm1(DTA)Lky*/J) | Marked reductions in peritoneal (98%) and skin (89-96.5%) MCs, mucosal MCs unlikely to be depleted | Probable presence of mucosal MCs; reporter mice reveals Cre-mediated deletion of 'floxed' YFP transgene in ~30% spleen NK cells[66] | none |
| ***Cpa3*$^{Cre/+}$** | **C57BL/6**[26] (B6.129P2-*Cpa3tm3(icre)*Hrr) | Absence of connective-tissue and mucosal MCs | *Cpa3* expressed in other cell types; reduced basophil numbers | i.v.[26] |
| | **BALB/c**[26] (B6.129P2/OlaHsd.BALB/c-*Cpa3tm3(icre)*Hrr) | | | |
| ***Cpa3-Cre; Mcl-1*$^{fl/fl}$** | **C57BL/6**[24] (Tg(Cpa3-cre)3Glli; B6;129-*Mcl1*$^{tm3sjk}$J) | Absence of connective-tissue and mucosal MCs | *Cpa3* expressed in other cell types; increased spleen neutrophils & mild anemia; reduced basophil numbers | i.d.[24]; i.a.[49] i.v. (our unpublished data) |

**Table 1: Strains of mice with constitutive MC deficiencies.** Several strains of mice with c-*kit*-dependent or c-*kit*-independent constitutive MC deficiency are available. In principle, all of these strains can be used to generate '*mast cell knock-in*' mice (although, to the best of our knowledge, this has not yet been reported for *Mcpt5-Cre;R-DTA* mice). However, each of these strains has other phenotypic abnormalities and limitations that should be kept in mind when interpreting results obtained with these mice. Some examples of successful MC engraftment can be found in the references. (This is a modified and updated version of **Table 1** from ref.[9]).

## Discussion

Almost 30 years after its initial description[38], the '*mast cell knock-in*' approach continues to provide valuable information about what MCs can do or can't do *in vivo*. The functions of MCs were long thought to be limited to their role in allergy. Data generated using the '*mast cell knock-in*' approach have changed this view, by providing evidence that MCs can, among other functions, play critical roles in host defense against certain pathogens[4,39] or venoms[28,31], or can even suppress certain immune responses[29,34,40].

In our protocol description, we decided to focus on the generation and engraftment of bone marrow-derived cultured MCs (BMCMCs), because large numbers of these cells can be generated *in vitro* from the bone marrow of wild type or mutant mice. However, MCs can also be cultured directly from embryonic stem cells (embryonic stem cell-derived cultured MCs [ESCMCs])[41] and, when genetically compatible, these cells can also be used for engraftment into MC-deficient mice. This alternative approach is particularly interesting for studying the role of a protein whose deficiency induces embryonic lethality in mice, and therefore for which BMCMCs deficient for this protein cannot be generated. Both BMCMCs and ESCMCs can also be transduced *in vitro* with lentiviruses encoding genes of interest or shRNA to silence genes of interest, before engraftment of these cells into MC-deficient mice[31,33].

We usually use 20% WEHI-3-conditioned medium as a source of IL-3 for the culture of BMCMCs. However, recombinant IL-3 (10 ng/ml, as described in step 1.2.1) can also be used, and addition of recombinant stem cell factor (SCF) to the culture medium can substantially enhance the numbers of BMCMCs generated[42,43]. Depending on the study, 10 to 100 ng/ml of recombinant SCF have been used, in addition to IL-3, to generate BMCMCs[36,44,45]. One should keep in mind that commercially available murine recombinant SCF preparations from different suppliers may differ in their potency in influencing the development of BMCMCs. It is also important to recognize that details of approach used to generate BMCMCs (such as whether one adds recombinant SCF to IL-3-containing medium, the duration of the culture period, *etc.*) may influence the phenotype and function of such cells. For example, it has been reported that BMCMCs chronically exposed to SCF have increased levels of histamine and certain proteases[44,46], but display a marked attenuation of FcεRI-mediated degranulation and cytokine production *in vitro*[45]. Finally, in addition to IL-3, WEHI-3-conditioned medium contains many biologically active molecules that may affect MC functions. BMCMCs obtained with WEHI-3-conditioned medium are therefore likely to differ from BMCMCs obtained with recombinant IL-3 (or with recombinant IL-3 plus SCF). There have been few studies of whether or for how long any such differences in the phenotypes of BMCMCs generated in different types of culture medium are retained after the cells' engraftment into different anatomical sites *in vivo*, and additional studies of this type may be of interest. However, regardless of the chosen culture conditions, the same culture medium recipe should be used to generate all of the BMCMCs to be used for engraftment in experiments from which results will be pooled for analysis. Moreover, MCs should be cultured for at least 4 to 6 weeks before their engraftment into MC-deficient mice, in order to reach a purity of 95-98% (**Figure 2**). This is to reduce the possibility that the presence, in the "BMCMC populations", of hematopoietic cells other than those committed to the mast cell lineage (which are present in the cultures at early intervals after placing the bone marrow cells *in vitro*) might result in the appearance of donor-derived cells in addition to MCs in the '*mast cell knock-in*' mice.

We present here a detailed protocol for engrafting MC-deficient mice with wild type or mutant BMCMCs intraperitoneally (i.p.), intravenously (i.v.) or intradermally (i.d.) in the ear pinna, since these routes of injection have been used by many investigators. However, BMCMCs have also been

successfully engrafted into the back skin[47], in the footpad[48], intra-articularly[49] or intra-cranially[50,51]. The number of BMCMCs to engraft, as well as the interval between engraftment and experiment, can vary depending on the route of injection and the targeted organ to engraft (**Figure 1**). It is very important to respect such intervals after engrafting the BMCMCs before starting the experiment in order to allow sufficient time for BMCMCs (which are not fully mature MCs) to become more mature *in vivo*. Because the content of mediators stored in the MC's cytoplasmic granules can continue to increase during the course of the cell's lifetime[38,52], for certain experiments one may wish to increase the interval between MC engraftment and the initiation of the experiment to assess MC function.

Depending on the route of injection and/or the numbers of BMCMCs injected, the numbers and/or anatomical distribution of the adoptively transferred MCs can differ from those of the corresponding native MC populations in wild type mice[12,23,53,54]. MC-deficient mice engrafted i.p. or i.d. with BMCMCs can have about the same numbers and distribution of MCs than the native MC population in wild type mice, in the peritoneal cavity and mesentery and in the dermis, respectively when assessed 4 to 8 weeks after MC transfer[12,23]. Intravenous transfer of BMCMCs does not lead to normal MC numbers and/or distribution in most tissues. For example, no or very few MCs are found in the skin of such i.v.-engrafted '*mast cell knock-in*' mice. At 4-28 weeks after i.v. injection of BMCMCs into MC-deficient mice, numbers of MCs in the trachea are substantially lower than those in the corresponding wild type mice. By contrast, the numbers of MCs in the periphery of the lung are typically greater than those in the corresponding wild type mice[12,23,53,55]. I.v. transfer of BMCMCs also results in high levels of MCs in the spleen, whereas very few native MCs are typically found in this organ in wild type mice[23,56]. Importantly, previous reports demonstrated that i.v. injection of BMCMCs into MC-deficient mice fails to result in engraftment of the MC populations in specific anatomical sites such as the spinal cord, lymph nodes or heart[54,57]. Several groups have also noted that such i.v. engraftment does not result in engraftment of the intestinal mucosal MC (MMC) population[23,58-60]. Such differences in MC numbers and/or distribution of adoptively-transferred MCs *versus* native MCs must be taken into account when interpreting data obtained using the MC '*mast cell knock-in*' model[9].

Several strains of MC-deficient mice exist, and choosing which one(s) to use in a particular project is important. c-*kit* mutant MC-deficient mice, such as $Kit^{W/W\text{-}v}$ or $Kit^{W\text{-}sh/W\text{-}sh}$ mice, have been traditionally used by many investigators. However, such mice suffer from many c-*kit*-related phenotypic abnormalities beside their profound MC deficiency (**Table 1**). In recent years, several strains of mice with c-*kit*-independent constitutive MC deficiency have been reported[24-26]. Some of these mice also exhibit other phenotypic abnormalities beside their MC deficiency (**Table 1**), and additional abnormalities might also be discovered as the phenotype of these newly described strains is still under investigation. All these mice and some additional new types of MC-deficient mice have been recently reviewed in detail[9,10,13].

Given the limitations of each of the MC-deficient strains currently available, we recommend attempting to test hypotheses about MC function using more than one model of MC deficiency[9]. In our laboratory, we generally perform pilot experiments in $Kit^{W\text{-}sh/W\text{-}sh}$ and *Cpa3-Cre;* $Mcl\text{-}1^{fl/fl}$ mice. If we obtain concordant results in both types of MC-deficient mice, we then proceed to engraftment experiments to ascertain the role of MCs and assess the potential roles of certain MC-derived products. Finally, it should be noted that several strains allowing inducible depletion of MCs or Cre recombinase-mediated deletion of "floxed" genes in MCs have also been recently described[25,49,61]. These strains have been reviewed in detail elsewhere[9,10,13] and represent promising alternative - or complementary - approaches to study MC functions *in vivo*.

## Disclosures

The authors have nothing to disclose.

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## References

1. Kitamura, Y. Heterogeneity of mast cells and phenotypic change between subpopulations. *Annu. Rev. Immunol.* **7**, 59-76 (1989).
2. Galli, S. J., Borregaard, N., Wynn, T. A. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat. Immunol.* **12**, 1035-1044 (2011).
3. Gurish, M. F., Austen, K. F. Developmental origin and functional specialization of mast cell subsets. *Immunity.* **37**, 25-33 (2012).
4. Abraham, S. N., St John, A. L. Mast cell-orchestrated immunity to pathogens. *Nat. Rev. Immunol.* **10**, 440-452 (2010).
5. Galli, S. J., Grimbaldeston, M., Tsai, M. Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nat. Rev. Immunol.* **8**, 478-486 (2008).
6. Reber, L. L., Frossard, N. Targeting mast cells in inflammatory diseases. *Pharmacol. Ther.* **142**, 416-435 (2014).
7. Galli, S. J. Mast cells as 'tunable' effector and immunoregulatory cells: recent advances. *Ann. Rev. Immunol.* **23**, 749-786 (2005).
8. Moon, T. C. Advances in mast cell biology: new understanding of heterogeneity and function. *Mucosal Immunol.* **3**, 111-128 (2010).
9. Reber, L. L., Marichal, T., Galli, S. J. New models for analyzing mast cell functions in vivo. *Trends Immunol.* **33**, 613-625 (2012).
10. Rodewald, H. R., Feyerabend, T. B. Widespread immunological functions of mast cells: fact or fiction. *Immunity.* **37**, 13-24 (2012).
11. Siebenhaar, F. The search for Mast Cell and Basophil models - Are we getting closer to pathophysiological relevance. *Allergy.* (2014).
12. Tsai, M., Grimbaldeston, M. A., Yu, M., Tam, S. Y., Galli, S. J. Using mast cell knock-in mice to analyze the roles of mast cells in allergic responses in vivo. *Chem. Immunol. Allergy.* **87**, 179-197 (2005).
13. Galli, S. J., *et al.* Approaches for analyzing the roles of mast cells and their proteases in vivo. *Adv. Immunol.* (2015).

14. Butterfield, J. H., Weiler, D., Dewald, G., Gleich, G. J. Establishment of an immature mast cell line from a patient with mast cell leukemia. *Leuk. Res.* **12**, 345-355 (1988).
15. Kirshenbaum, A. S. Characterization of novel stem cell factor responsive human mast cell lines LAD 1 and 2 established from a patient with mast cell sarcoma/leukemia; activation following aggregation of FcepsilonRI or FcgammaRI. *Leuk. Res.* **27**, 677-682 (2003).
16. Sibilano, R. The aryl hydrocarbon receptor modulates acute and late mast cell responses. *J. Immunol.* **189**, 120-127 (2012).
17. Gaudenzio, N., Laurent, C., Valitutti, S., Espinosa, E. Human mast cells drive memory CD4+ T cells toward an inflammatory IL-22+ phenotype. *J. Allergy Clin. Immunol.* **131**, 1400-1407 (2013).
18. Tertian, G., Yung, Y. P., Guy-Grand, D., Moore, M. A. Long-term in vitro. culture of murine mast cells. I. Description of a growth factor-dependent culture technique. *J. Immunol.* **127**, 788-794 (1981).
19. Yamada, N., Matsushima, H., Tagaya, Y., Shimada, S., Katz, S. I. Generation of a large number of connective tissue type mast cells by culture of murine fetal skin cells. *J. Invest. Dermatol.* **121**, 1425-1432 (2003).
20. Malbec, O. Peritoneal cell-derived mast cells: an in vitro. model of mature serosal-type mouse mast cells. *J. Immunol.* **178**, 6465-6475 (2007).
21. Galli, S. J., Zsebo, K. M., Geissler, E. N. The Kit ligand, stem cell factor. *Adv. Immunol.* **55**, 1-96 (1994).
22. Reber, L., Da Silva, C. A., Frossard, N. Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. *Eur. J. Pharmacol.* **533**, 327-340 (2006).
23. Grimbaldeston, M. A. Mast cell-deficient W.-sash. c-kit. mutant KitW.-sh./W.-sh. mice as a model for investigating mast cell biology in vivo. *Am. J. Pathol.* **167**, 835-848 (2005).
24. Lilla, J. N. Reduced mast cell and basophil numbers and function in Cpa3-Cre Mcl-1.fl/fl. mice. *Blood.* **118**, 6930-6938 (2011).
25. Dudeck, A. Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. *Immunity.* **34**, 973-984 (2011).
26. Feyerabend, T. B. Cre-Mediated Cell Ablation Contests Mast Cell Contribution in Models of Antibody and T Cell-Mediated Autoimmunity. *Immunity.* **35**, 832-844 (2011).
27. Schafer, B. Mast cell anaphylatoxin receptor expression can enhance IgE-dependent skin inflammation in mice. *J. Allergy Clin. Immunol.* **131**, 541-548 (2013).
28. Akahoshi, M. Mast cell chymase reduces the toxicity of Gila monster venom, scorpion venom, and vasoactive intestinal polypeptide in mice. *J. Clin. Invest.* **121**, 4180-4191 (2011).
29. Grimbaldeston, M. A., Nakae, S., Kalesnikoff, J., Tsai, M., Galli, S. J. Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. *Nat. Immunol.* **8**, 1095-1104 (2007).
30. Hershko, A. Y. Mast cell interleukin-2 production contributes to suppression of chronic allergic dermatitis. *Immunity.* **35**, 562-571 (2011).
31. Metz, M. Mast cells can enhance resistance to snake and honeybee venoms. *Science.* **313**, 526-530 (2006).
32. Nakahashi-Oda, C. Apoptotic cells suppress mast cell inflammatory responses via the CD300a immunoreceptor. *J. Exp. Med.* **209**, 1493-1503 (2012).
33. Piliponsky, A. M. Neurotensin increases mortality and mast cells reduce neurotensin levels in a mouse model of sepsis. *Nat. Med.* **14**, 392-398 (2008).
34. Chan, C. Y., St John, A. L., Abraham, S. N. Mast cell interleukin-10 drives localized tolerance in chronic bladder infection. *Immunity.* **38**, 349-359 (2013).
35. Yu, M. Mast cells can promote the development of multiple features of chronic asthma in mice. *J. Clin. Invest.* **116**, 1633-1641 (2006).
36. Reber, L. L., Daubeuf, F., Pejler, G., Abrink, M., Frossard, N. Mast cells contribute to bleomycin-induced lung inflammation and injury in mice through a chymase/mast cell protease 4-dependent mechanism. *J. Immunol.* **192**, 1847-1854 (2014).
37. Lee, D. M. Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science.* **297**, 1689-1692 (2002).
38. Nakano, T. Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal, and intravenous transfer into genetically mast cell-deficient W/W-v. mice. Evidence that cultured mast cells can give rise to both connective tissue type and mucosal mast cells. *J. Exp. Med.* **162**, 1025-1043 (1985).
39. Malaviya, R., Ikeda, T., Ross, E., Abraham, S. N. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature.* **381**, 77-80 (1996).
40. Lu, L. F. Mast cells are essential intermediaries in regulatory T-cell tolerance. *Nature.* **442**, 997-1002 (2006).
41. Tsai, M., Tam, S. Y., Wedemeyer, J., Galli, S. J. Mast cells derived from embryonic stem cells: a model system for studying the effects of genetic manipulations on mast cell development, phenotype, and function in vitro. and in vivo. *Int. J. Hematol.* **75**, 345-349 (2002).
42. Nocka, K., Buck, J., Levi, E., Besmer, P. Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. *EMBO J.* **9**, 3287-3294 (1990).
43. Tsai, M. Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell. *Proc. Nat. Acad. Sci. U.S.A.* **88**, 6382-6386 (1991).
44. Ronnberg, E., Calounova, G., Guss, B., Lundequist, A., Pejler, G. Granzyme D is a novel murine mast cell protease that is highly induced by multiple pathways of mast cell activation. *Infect. Immun.* **81**, 2085-2094 (2013).
45. Ito, T. Stem cell factor programs the mast cell activation phenotype. *J. Immunol.* **188**, 5428-5437 (2012).
46. Furuta, G. T., Ackerman, S. J., Lu, L., Williams, R. E., Wershil, B. K. Stem cell factor influences mast cell mediator release in response to eosinophil-derived granule major basic protein. *Blood.* **92**, 1055-1061 (1998).
47. Weller, K., Foitzik, K., Paus, R., Syska, W., Maurer, M. Mast cells are required for normal healing of skin wounds in mice. *FASEB J.* **20**, 2366-2368 (2006).
48. McLachlan, J. B. Mast cell activators: a new class of highly effective vaccine adjuvants. *Nat. Med.* **14**, 536-541 (2008).
49. Reber, L. L. Contribution of mast cell-derived interleukin-1b to uric acid crystal-induced acute arthritis in mice. *Arthritis Rheumatol.* **66**, 2881-2891 (2014).
50. Arac, A. Evidence that Meningeal Mast Cells Can Worsen Stroke Pathology in Mice. *Am. J. Pathol.* **184**, 2493-2504 (2014).
51. Christy, A. L., Walker, M. E., Hessner, M. J., Brown, M. A. Mast cell activation and neutrophil recruitment promotes early and robust inflammation in the meninges in EAE. *J. autoimmun.* **42**, 50-61 (2013).
52. Hammel, I., Lagunoff, D., Galli, S. J. Regulation of secretory granule size by the precise generation and fusion of unit granules. *J. Cell. Mol. Med.* **14**, 1904-1916 (2010).
53. Martin, T. R. Mast cell activation enhances airway responsiveness to methacholine in the mouse. *J. Clin. Invest.* **91**, 1176-1182 (1993).
54. Tanzola, M. B., Robbie-Ryan, M., Gutekunst, C. A., Brown, M. A. Mast cells exert effects outside the central nervous system to influence experimental allergic encephalomyelitis disease course. *J. Immunol.* **171**, 4385-4391 (2003).


 

55. Wolters, P. J. Tissue-selective mast cell reconstitution and differential lung gene expression in mast cell-deficient Kit.W-sh/W-sh. sash mice. *Clin. Exp Allergy.* **35**, 82-88 (2005).
56. Reber, L. L. Selective ablation of mast cells or basophils reduces peanut-induced anaphylaxis in mice. *J. Allergy Clin. Immunol.* **132**, 881-888 (2013).
57. Hara, M. Evidence for a role of mast cells in the evolution to congestive heart failure. *J. Exp. Med.* **195**, 375-381 (2002).
58. Abe, T., Nawa, Y. Localization of mucosal mast cells in W/W-v. mice after reconstitution with bone marrow cells or cultured mast cells, and its relation to the protective capacity to Strongyloides ratti. infection. *Parasite Immunol.* **9**, 477-485 (1987).
59. Groschwitz, K. R. Mast cells regulate homeostatic intestinal epithelial migration and barrier function by a chymase/Mcpt4-dependent mechanism. *Proc. Nat. Acad. Sci. U.S.A.* **106**, 22381-22386 (2009).
60. Wedemeyer, J., Galli, S. J. Decreased susceptibility of mast cell-deficient Kit.W/W-v. mice to the development of 1, 2-dimethylhydrazine-induced intestinal tumors. *Lab. Invest.* **85**, 388-396 (2005).
61. Sawaguchi, M. Role of mast cells and basophils in IgE responses and in allergic airway hyperresponsiveness. *J. Immunol.* **188**, 1809-1818 (2012).
62. Piliponsky, A. M. Mast cell-derived TNF can exacerbate mortality during severe bacterial infections in C57BL/6-Kit.W-sh/W-sh. mice. *Am. J. Pathol.* **176**, 926-938 (2010).
63. Shelburne, C. P. Mast cells augment adaptive immunity by orchestrating dendritic cell trafficking through infected tissues. *Cell Host Microbe.* **6**, 331-342 (2009).
64. Michel, A. Mast cell-deficient Kit.W-sh. 'Sash' mutant mice display aberrant myelopoiesis leading to the accumulation of splenocytes that act as myeloid-derived suppressor cells. *J. Immunol.* **190**, 5534-5544 (2013).
65. Becker, M. Genetic variation determines mast cell functions in experimental asthma. *J. Immunol.* **186**, 7225-7231 (2011).
66. Abram, C. L., Roberge, G. L., Hu, Y., Lowell, C. A. Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. *J. Immunol. Methods.* **408**, 89-100 (2014).


 