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Effect of Anatomical Distribution of Mast Cells on Their Defense Function against Bacterial Infections: Demonstration Using Partially Mast Cell–deficient *tg/tg* Mice

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Abstract

Mast cells were depleted in the peritoneal cavity of WBB6F$_1$-*tg/tg* mice that did not express a transcription factor, MITF. When acute bacterial peritonitis was induced in WBB6F$_1$-+/+, WBB6F$_1$-W/W^v, and WBB6F$_1$-*tg/tg* mice, the proportion of surviving WBB6F$_1$-+/+ mice was significantly higher than that of surviving WBB6F$_1$-W/W^v or WBB6F$_1$-*tg/tg* mice. The poor survival of WBB6F$_1$-W/W^v and WBB6F$_1$-*tg/tg* mice was attributed to the deficient influx of neutrophils into the peritoneal cavity. The injection of cultured mast cells (CMCs) derived from WBB6F$_1$-+/+ mice normalized the neutrophil influx and reduced survival rate in WBB6F$_1$-W/W^v mice, but not in WBB6F$_1$-*tg/tg* mice. This was not attributable to a defect of neutrophils because injection of TNF-α increased the neutrophil influx and survival rate in both WBB6F$_1$-W/W^v and WBB6F$_1$-*tg/tg* mice. Although WBB6F$_1$-+/+ CMCs injection normalized the number of mast cells in both the peritoneal cavity and mesentery of WBB6F$_1$-W/W^v mice, it normalized the number of mast cells only in the peritoneal cavity of WBB6F$_1$-*tg/tg* mice. Mast cells within the mesentery or mast cells in the vicinity of blood vessels appeared to play an important role against the acute bacterial peritonitis. WBB6F$_1$-*tg/tg* mice may be useful for studying the effect of anatomical distribution of mast cells on their antiseptic function.

Key words: mast cell • innate immunity • MITF • acute bacterial peritonitis • TNF-α

Introduction

The mouse *mi* locus encodes a transcription factor belonging to the basic-helix-loop-helix-leucine zipper family (hereafter, microphthalmia transcription factor [MITF]*) (1, 2). The mutant *mi* allele produces an abnormal MITF, in which 1 out of 4 consecutive arginines is deleted in the basic domain (hereafter, *mi*-MITF) (1, 3, 4). The *mi*-MITF is defective in DNA binding, nuclear translocation and transactivation of target genes (5–13). On the other hand, the mutant *tg* allele is a transgene insertion mutation in the 5′ flanking region of the MITF gene (1, 14). Although the coding region of the MITF gene was normal in C57BL/6 (B6)-*tg/tg* mice, no significant amount of MITF was detected in cultured mast cells (CMCs) derived from the spleen of B6-*tg/tg* mice (15).

Both B6-*mi/mi* and B6-*tg/tg* mice show microphthalmia, lack of melanocytes, and decrease of skin mast cells (16). B6-*mi/mi* mice show osteopetrosis, but B6-*tg/tg* mice do not (17). Most B6-*mi/mi* mice die on weaning due to the failure of teeth eruption caused by the osteopetrosis, whereas most B6-*tg/tg* mice survived to adulthood. The number of mast cells in skin tissues was comparable between B6-*mi/mi* and B6-*tg/tg* mice (one third that of B6-+/+ mice; reference 18). However, the decrease of heparin content in skin mast cells was observed only in B6-*mi/mi* mice (19).

Although mast cells develop before birth in the skin tissue of normal B6-+/+ mice, they develop after weaning in tissues other than the skin of B6-+/+ mice (20). As adult B6-*tg/tg* mice were easily obtained, we attempted to investigate development of mast cells in tissues other than the skin of B6-*tg/tg* mice. We found the lack of mast cells in the peritoneal cavity of adult B6-*tg/tg* mice.

Involvement of mast cells in the innate immunity has been studied using WBB6F$_1$-W/W^v mice (21, 22). When acute bacterial peritonitis was induced, the proportion of

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Abbreviations used in this paper: B6, C57BL/6; CLP, caecal ligation and puncture; CMC, cultured mast cell; MITF, microphthalmia transcription factor; SCF, stem cell factor.

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surviving WBB6F$_1$-W/W^v mice was significantly lower than that of WBB6F$_1$-+/+ mice (21, 22). The prior intraperitoneal transplantation of CMCs derived from WBB6F$_1$-+/+ mice normalized the reduced proportion of surviving WBB6F$_1$-W/W^v mice (21, 22). In the present study, we examined whether comparable results were obtained in WBB6F$_1$-*tg/tg* mice, which lacked peritoneal mast cells as WBB6F$_1$-W/W^v mice. Although the proportion of surviving WBB6F$_1$-*tg/tg* mice was reduced as WBB6F$_1$-W/W^v mice, the reduced survival rate was not normalized by the prior transplantation of WBB6F$_1$-+/+ CMCs. We investigated the mechanism of this unexpected result and found that the anatomical distribution of the transplanted CMCs was different between WBB6F$_1$-*tg/tg* and WBB6F$_1$-W/W^v mice.

Materials and Methods

Mice. The original stock of B6-Mi^{wh}/+ mice was purchased from The Jackson Laboratory. The B6-mi^{ew}/+ and B6-mi^{ce}/+ mice were given by Dr. M.L. Lamoreux (Texas A&M University, College Station, TX). The original stock of VGA-9-*tg/tg* mice, in which the mouse vasopressin-*Escherichia coli* β-galactosidase transgene was integrated at the 5′ flanking region of the MITF gene (1), was given by H. Arnheiter (National Institutes of Health, Bethesda, MD). B6-Mi^{wh}/+, B6-mi^{ew}/+, and B6-mi^{ce}/+ mice were maintained by consecutive backcrosses to our own inbred B6 colony (more than 18 generations at the time of the present experiment). Homozygous mice were produced by crosses between female and male heterozygotes of each genotype, and selected by their white coat color. The VGA-9-*tg/tg* mice were maintained by repeated backcrosses to our own inbred B6 and WB colonies more than 12 generations. B6-*tg*/+ mice were crossed together, and WB-*tg*/+ mice were crossed to B6-*tg*/+ mice. The resulting B6-*tg/tg* and WBB6F$_1$-*tg/tg* mice were selected by their white coat color. WBB6F$_1$- W/W^v and WBB6F$_1$-+/+ mice were purchased from the Japan SLC.

Estimation of Mast Cell Numbers in the Peritoneal Cavity and Tissues. To harvest peritoneal cells, 2 ml of Tyrode's buffer (23) containing 0.1% gelatin (Sigma-Aldrich) was injected into the peritoneal cavity, and the abdomen was massaged gently for 30 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated with a Pasteur pipette. After centrifugation, the pellet was resuspended with the Tyrode's buffer (1.0 ml), and was divided into two parts. One part (0.8 ml) was used for the direct counting of mast cells as described by Gilbert and Ornstein (24). The cell suspension was centrifuged again, suspended in the Tyrode's buffer (0.1 ml), and diluted with 0.4 ml saline containing 0.1% EDTA-Na (solution A). Then 0.45 ml of a solution containing 0.076% cetylpyridinium chloride, 0.7% lanthanum chloride-6H_2O, 0.9% NaCl, 0.21% Tween 20 (Sigma-Aldrich), and 0.143% alcian blue 8GX (Eastman Kodak Co.; solution B) were added to the cell suspension. After 1 min of gentle agitation, 0.05 ml of 1 N HCl (solution C) was added, and the mixture was gently agitated. The cell suspension was centrifuged, resuspended in 0.05 ml of the mixture of the Tyrode's buffer and solutions A, B, and C (Tyrode:A:B:C = 2:8:9:1). After 20 min, alcian blue–positive cells were counted with a standard hemocytometer. Numbers of mast cells per mouse were calculated.

Another part (0.2 ml) of the peritoneal cell suspension was centrifuged at 600 rpm for 5 min with a Cytospin 2 centrifuge (Shandon) to attach cells to a microscope slide. The cytospin preparations were fixed in Carnoy's solution, and stained with alcian blue and nuclear fast red. Proportion of alcian blue-positive cells in 1,000 nucleated peritoneal cells was determined.

Mast cell numbers in the mesentery, glandular stomach, spleen, and lung were estimated as described previously (18, 20, 25).

CMCs. PWM-stimulated spleen cell–conditioned medium (PWM-SCM) was prepared according to the method described by Nakahata et al. (26). Mice of B6-+/+, B6-*tg/tg*, and WBB6F$_1$-+/+ were used to obtain CMCs. Mice were killed by decapitation after ether anesthesia, and spleens were removed. Spleen cells were cultured in α-MEM (ICN Biomedicals) supplemented with 10% PWM-SCM and 10% FCS (Nippon Bio-supp Center). Half of the medium was replaced every 7 d. More than 95% of cells were CMCs 4 wk after the initiation of the culture.

Intraperitoneal Injection of CMCs. CMCs (1.0×10^6) derived from B6-+/+, B6-*tg/tg*, or WBB6F$_1$-+/+ mice were suspended in 0.5 ml of α-MEM, and were injected into the peritoneal cavity of the recipient mice. At the intervals indicated, peritoneal cells were harvested as described above. Proportions of mast cells in 1,000 peritoneal cells were determined as mentioned above.

Caecal Ligation and Puncture. Caecal ligation and puncture (CLP) was performed as described previously by Echtenacher et al. (21) with slight modifications. In brief, the mice were anesthetized by sevofrane (Maruishi Pharmaceuticals). A 1-cm midline incision on the anterior abdominal wall was made. The cecum was exposed and filled with feces by squeezing stool back from the ascending colon. The cecum was 50% ligated below the ileocecal valve and then punctured using a 0.65 mm needle followed by gentle squeezing of the cecum. Mice were examined every day for survival rate.

Neutrophil Counts in Peritoneal Exudates. Peritoneal exudates were collected from mice and total cell numbers were counted. The cytospin preparation of peritoneal cells was made as described previously. The cells were stained with May-Grunwald-Giemsa. Proportions of neutrophils in peritoneal cells were determined. Then the number of neutrophils was calculated.

Injection of TNF-α. Acute bacterial peritonitis was induced by CLP in WBB6F$_1$-*tg/tg* and WBB6F$_1$-W/W^v mice. Immediately after CLP, a single intraperitoneal injection of murine TNF-α (PeproTech; reference 21) or control diluents (0.5 ml PBS per mouse) was done. Numbers of neutrophils were counted 3 h after CLP. Survival rate was also determined.

Intravenous Injection. CMCs (1.0×10^6) derived from WBB6F$_1$-+/+ mice were suspended in 0.2 ml of α-MEM, and were injected into the tail vein of the recipient mice. Proportions of mast cells in 1,000 peritoneal cells and number of mast cells in the mesentery, glandular stomach, and spleen were determined 5 wk after the injection.

Mast Cell Staining with Berberine Sulfate. The cytospin preparation of peritoneal cells was made as described previously. After fixation with Carnoy's fluid, the cells were stained with berberine sulfate (Sigma-Aldrich), as described by Enerback (27).

Semiquantitative RT-PCR Analysis. 4 μg of total RNAs were extracted from the mesentery and spleen of WBB6F$_1$-+/+, WBB6F$_1$-W/W^v, and WBB6F$_1$-*tg/tg* mice. The extracted RNAs were subjected to reverse transcription by Superscript (Invitrogen Corp.), and the single strand cDNAs were obtained. 1, 0.1, or 0.01 μl of the reaction mixture was added to 25 μl of PCR mixture containing 1.25 U of Taq DNA polymerase (Roche Diagnostics GmbH) and 25 pmol of each of the primers. PCR was performed to amplify the fragment of the MITF, stem cell factor

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(SCF), and β-actin genes using the following primers; 5′-ACAGAGTCTGAAGCAAGAGCA and 5′-GGTGATGGTACCGTCCGTGAG for MITF, 5′-AAGACTCGGGCCTACAATGGACAGCCATGG and 5′-CAATGTTGATACGTCCACAATTAC for SCF, and 5′-TAAAGACCTCTATGCCAACAC and 5′-CTCCTGCTTGCTGATCCACAT for β-actin.

Statistical Analysis. Statistical analysis of most data was performed using the Student's *t* test. Statistical analysis of the survival rate was done using the log rank test.

Results

Mast Cell Deficiency of tg/tg Mice. The number of mast cells was examined at various ages in the peritoneal cavity and glandular stomach of B6-+/+ and B6-*tg/tg* mice. In B6-+/+ mice, mast cells appeared in the peritoneal cavity 6 wk after birth and in the glandular stomach 4 wk after birth (Fig. 1). The number of mast cells increased thereafter. On the other hand, in B6-*tg/tg* mice, no detectable number of mast cells appeared in either peritoneal cavity or glandular stomach at any ages examined (Fig. 1). We also examined whether mast cells appeared in spleens and lungs of B6-*tg/tg* mice at 10 wk of age. Mast cells were not detectable in histological sections of lungs and spleens of B6-*tg/tg* mice (unpublished data).

We further investigated the mast cell deficiency in the peritoneal cavity of other MITF mutants. The number of peritoneal mast cells shown in Fig. 1 was obtained by the direct counting of peritoneal cells with the hemocytometer. In addition to the direct counting, another method was used to identify mast cells more precisely. Cytospin preparations of peritoneal cell suspensions were made, and proportions of mast cells in 1,000 nucleated peritoneal cells were counted. A few mast cells were recognized in the cytospin preparations of peritoneal cells obtained from B6-*tg/tg*, B6-mi^{ew}/mi^{ew}, B6-mi^{ce}/mi^{ce}, and B6-Mi^{wh}/Mi^{wh} mice, but the mean number of mast cells did not exceed 0.1 per 1,000 nucleated peritoneal cells in each mutant mouse (Table I). The results obtained by these two methods were consistent and showed the apparent deficiency of mast cells in the peritoneal cavity of B6-mi^{ew}/mi^{ew}, B6-mi^{ce}/mi^{ce}, and B6-Mi^{wh}/Mi^{wh} mice as in the case of B6-*tg/tg* mice (Table I).

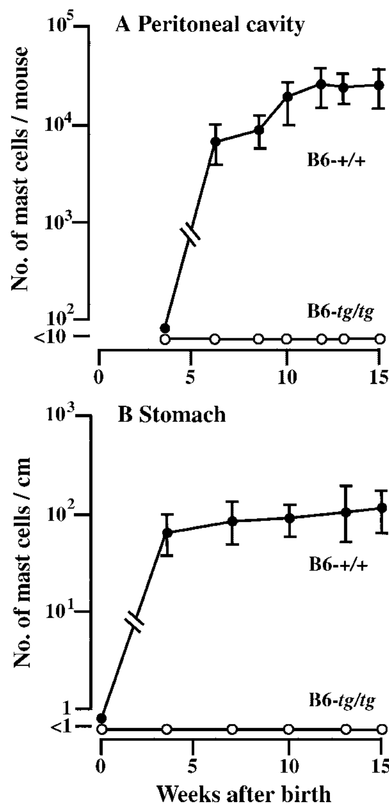

Figure 1. The number of mast cells in the peritoneal cavity (A) and glandular stomach (B) of B6-+/+ and B6-*tg/tg* mice. The number of mast cells was examined at various times after birth. At each time point, the mean values of 6 to 8 mice were plotted with bars indicating SE.

We injected B6-+/+ or B6-*tg/tg* CMCs into the peritoneal cavity of B6-*tg/tg* mice. At various weeks after injection, we examined the proportions of mast cells in 1,000 nucleated peritoneal cells. The B6-+/+ CMCs survived 5 wk after the injection, but B6-*tg/tg* CMCs did not (Fig. 2). We also used WBB6F$_1$-W/W^v mice as recipients because they are used as a standard of mast cell–deficient animals (28, 29). B6-*tg/tg* CMCs did not survive in the peritoneal cavity of WBB6F$_1$-W/W^v mice, either (Fig. 2).

Mortality from Acute Bacterial Peritonitis. As mast cell deficiency was observed in the peritoneal cavity of B6-*tg/tg* mice, we induced the acute bacterial peritonitis by CLP and compared the survival rate between B6-*tg/tg* and B6-+/+ mice. The proportion of surviving B6-*tg/tg* mice was significantly lower than that of surviving B6-+/+ mice

Table I. *Mast Cell Deficiency in the Peritoneal Cavity of Various MITF Mutant Mice of B6 Strain Estimated by Two Methods*

		Direct count	Proportion in cytospin preparation
Mice[a]	No. of mice	No. of mast cells/ mouse (× 10^3)[b]	No. of mast cells/ 10^3 peritoneal cells[c]
B6-+/+	8	20.0 ± 2.5	29.5 ± 2.8
B6-*tg/tg*	9	ND[d]	<0.1
B6-mi^{ew}/mi^{ew}	8	ND[d]	<0.1
B6-mi^{ce}/mi^{ce}	10	ND[d]	<0.1
B6-Mi^{wh}/Mi^{wh}	8	ND[d]	<0.1

[a]The number of mast cells was examined at 10 wk of age.
[b]After harvesting peritoneal cells, cells were divided into two parts. One part was stained with alcian blue in suspension, and the positive cells were counted with a hemocytometer. Mean and SE are shown.
[c]Cytospin preparation was made from another part and was stained with alcian blue and nuclear fast red. Proportion of alcian blue-positive cells to 10^3 nucleated peritoneal cells was determined. Mean and SE are shown.
[d]Not detectable.

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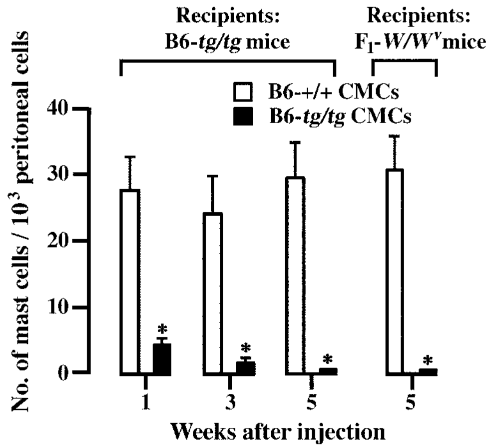

Figure 2. Survival of transplanted CMCs in the peritoneal cavity of B6-*tg/tg* and WBB6F$_1$-W/W^v mice. B6-+/+ or B6-*tg/tg* CMCs (1.0×10^6) were injected into the peritoneal cavity of the recipient mice. At 1, 3, or 5 wk after the injection, the proportion of mast cells in 1,000 nucleated peritoneal cells was determined. The mean values of five mice were shown with bars indicating SE. In some cases, SE was too small to be shown by bars. *$P < 0.01$ by *t* test when compared with the value obtained after the injection of B6-+/+ CMCs.

(Fig. 3). Then we investigated the effect of the prior transplantation of B6-+/+ CMCs because Echtenacher et al. (21) reported that the reduced survival rate of WBB6F$_1$-W/W^v mice after CLP is normalized by the prior transplantation of WBB6F$_1$-+/+ CMCs. Unexpectedly, the prior transplantation of B6-+/+ CMCs (2.0×10^6) did not increase the survival rate of B6-*tg/tg* mice after CLP (Fig. 3).

There was a possibility that the difference of mouse genetic background (B6 versus WBB6F$_1$) may cause this unexpected result. We produced WBB6F$_1$-*tg/tg* mice, and induced the acute bacterial peritonitis in intact WBB6F$_1$-*tg/tg* mice and WBB6F$_1$-*tg/tg* mice which had received the prior transplantation of WBB6F$_1$-+/+ CMCs. As a control, we also induced the bacterial peritonitis in intact WBB6F$_1$-W/W^v mice and WBB6F$_1$-W/W^v mice which had received the prior tansplantation of WBB6F$_1$-+/+ CMCs. WBB6F$_1$-W/W^v mice gave a comparable result that had been reported by Echtenacher et al. (21; Fig. 4 A). In contrast, the result obtained by WBB6F$_1$-*tg/tg* mice was consistent with the result of B6-*tg/tg* mice (compare Figs. 3 and 4 B).

The number of neutrophils was counted in the peritoneal cavity of WBB6F$_1$-+/+, WBB6F$_1$-W/W^v, and WBB6F$_1$-*tg/tg* mice 3 h after CLP. The number of neutrophils was significantly lower in the peritoneal cavity of WBB6F$_1$-W/W^v mice and WBB6F$_1$-*tg/tg* mice than in the peritoneal cavity of WBB6F$_1$-+/+ mice (Fig. 5). The prior transplantation of WBB6F$_1$-+/+ CMCs normalized the neutrophil response in WBB6F$_1$-W/W^v mice but not in WBB6F$_1$-*tg/tg* mice (Fig. 5).

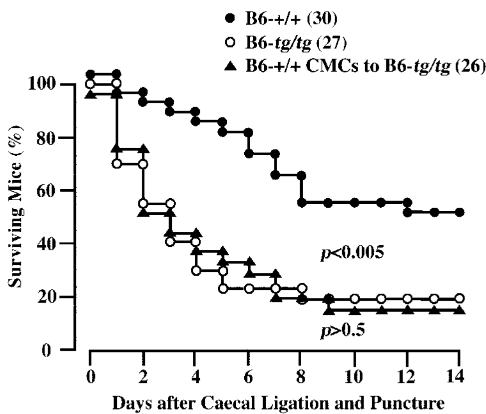

Figure 3. Effect of +/+ CMC injection on the survival of B6-*tg/tg* mice. Peritonitis was induced by CLP in B6-+/+ mice, B6-*tg/tg* mice, and B6-*tg/tg* mice which received the prior intraperitoneal injection of B6-+/+ CMCs 5 wk before CLP. Number of mice is shown in parentheses. The proportion of survival was compared by log rank test.

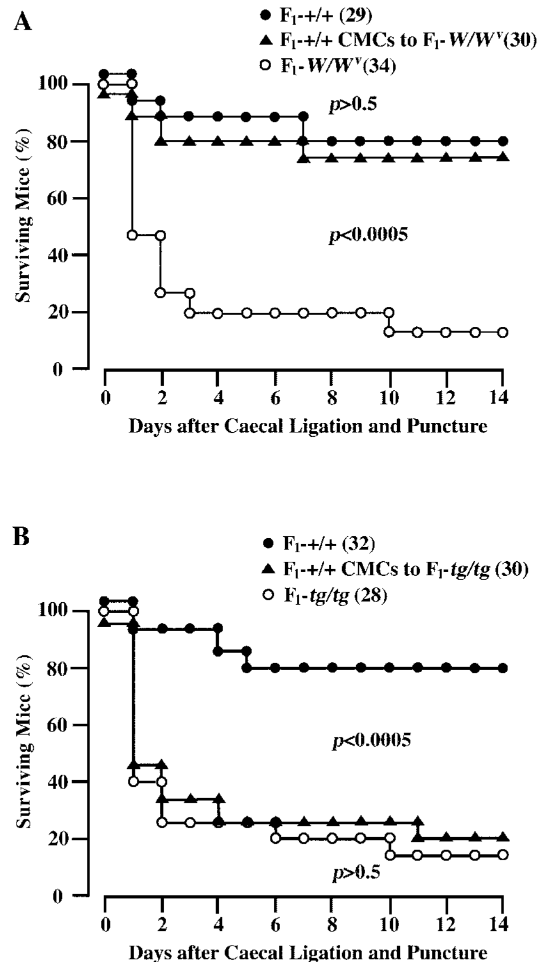

Figure 4. Effect of injection of WBB6F$_1$-+/+ CMCs on survival of WBB6F$_1$-W/W^v and WBB6F$_1$-*tg/tg* mice. Peritonitis was induced by CLP. (A) Proportions of surviving WBB6F$_1$-+/+ mice, WBB6F$_1$-W/W^v mice, and WBB6F$_1$-W/W^v mice that had received the prior intraperitoneal injection of WBB6F$_1$-+/+ CMCs 5 wk before CLP. (B) Proportions of surviving WBB6F$_1$-+/+ mice, WBB6F$_1$-*tg/tg* mice, and WBB6F$_1$-*tg/tg* mice that had received the prior intraperitoneal injection of WBB6F$_1$-+/+ CMCs 5 wk before CLP. The number of mice is shown in parentheses. The proportion of survival was compared by log rank test.

There was a possibility that the migration activity of neutrophils of WBB6F$_1$-*tg/tg* mice was defective. The effect of intraperitoneal injection of TNF-α on the number of infiltrating neutrophils was compared between WBB6F$_1$-*W/W*^{v} and WBB6F$_1$-*tg/tg* mice. TNF-α was injected intraperitoneally immediately after CLP. The number of infiltrating neutrophils after CLP was increased by the intraperitoneal injection of TNF-α not only in WBB6F$_1$-*W/W*^{v} mice but also in WBB6F$_1$-*tg/tg* mice (Fig. 6). The values of both mice were comparable. The survival rate was also increased by the injection of TNF-α in both WBB6F$_1$-*W/W*^{v} (Fig. 7 A) and WBB6F$_1$-*tg/tg* mice (Fig. 7 B).

Different Effect of CMC Transplantation between tg/tg and W/W^v *Mice.* Presence of mast cells in the peritoneal cavity of B6-*tg/tg* mice 5 wk after intraperitoneal transplantation of B6-+/+ CMCs was shown in Fig. 2.

The prior intraperitoneal transplantation of WBB6F$_1$-+/+ CMCs normalized the neutrophil response and survival rate of WBB6F$_1$-*W/W*^{v} mice, but did not affect the neutrophil response and survival rate of WBB6F$_1$-*tg/tg* mice. There was a possibility that the intraperitoneal transplantation of WBB6F$_1$-+/+ CMCs had different effects between WBB6F$_1$-*W/W*^{v} and WBB6F$_1$-*tg/tg* recipients. We compared the effect of the intraperitoneal transplantation between WBB6F$_1$-*W/W*^{v} and WBB6F$_1$-*tg/tg* mice. The number of mast cells in the peritoneal cavity was comparable between WBB6F$_1$-*W/W*^{v} and WBB6F$_1$-*tg/tg* mice (Table II). Although WBB6F$_1$-+/+ CMCs were not stained with berberine sulfate before the transplantation, mast cells recovered from the peritoneal cavity of either WBB6F$_1$-*W/W*^{v} or WBB6F$_1$-*tg/tg* mice were stained with berberine sulfate (unpublished data), suggesting the content of heparin (27). The number of mast cells in the stretch preparation of the mesentery of WBB6F$_1$-*W/W*^{v} mice was normalized by the intraperitoneal transplantation of WBB6F$_1$-+/+ CMCs (Table II and Fig. 8). However, the same procedure did not result in development of mast cells in the mesentery of WBB6F$_1$-*tg/tg* mice (Table II and Fig. 8).

Intraperitoneal transplantation of WBB6F$_1$-+/+ CMCs resulted in development of mast cells in the mesentery of

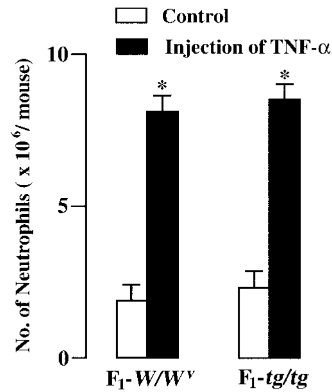

Figure 6. Effect of TNF-α injection on the number of infiltrating neutrophils in the peritoneal cavity of WBB6F$_1$-*W/W*^{v} and WBB6F$_1$-*tg/tg* mice. Mice received TNF-α injection immediately after CLP and the number of neutrophils was counted 3 h after CLP. Mean ± SE of 5 to 7 mice are shown. *P < 0.01 by *t* test when compared with the value of control mice of the same genotype.

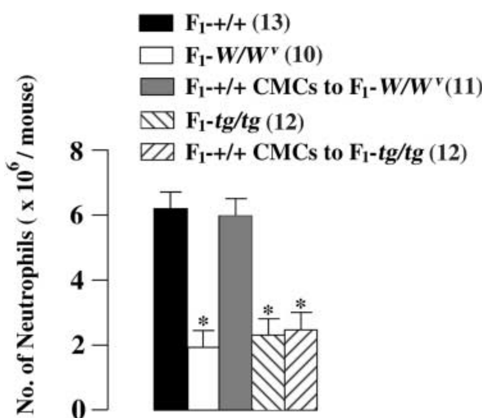

Figure 5. Number of infiltrating neutrophils in the peritoneal cavity of WBB6F$_1$-+/+ mice, WBB6F$_1$-*W/W*^{v}, mice, and WBB6F$_1$-*W/W*^{v} mice that had received the prior intraperitoneal injection of WBB6F$_1$-+/+ CMCs 5 wk before CLP, WBB6F$_1$-*tg/tg* mice and WBB6F$_1$-*tg/tg* mice that had received the prior intraperitoneal injection of WBB6F$_1$-+/+ CMCs 5 wk before CLP. Numbers of mice are shown in parentheses. *P < 0.01 by *t* test, when compared with the value observed in WBB6F$_1$-+/+ mice.

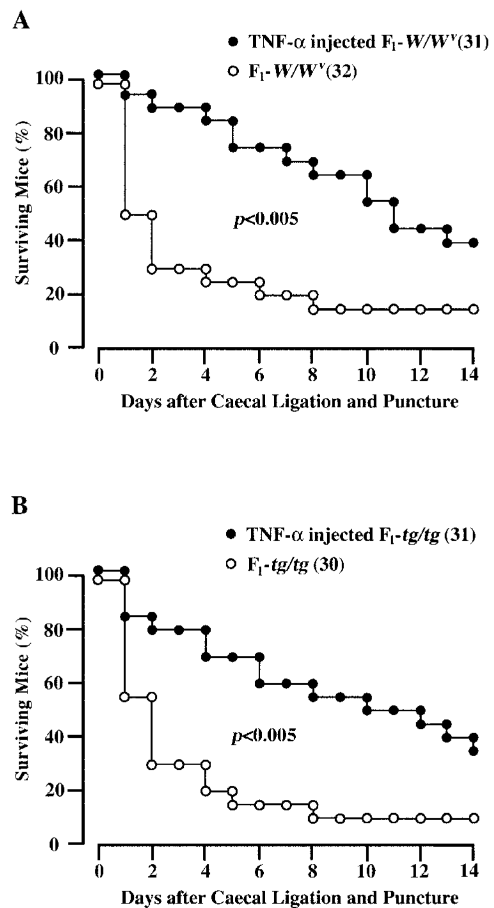

Figure 7. Effect of TNF-α injection on the proportions of surviving WBB6F$_1$-*W/W*^{v} (A) and WBB6F$_1$-*tg/tg* mice (B). The experimental condition was same as the experiment shown in Fig. 6. Numbers of mice are shown in parentheses. The proportion of survival was compared by log rank test.

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Table II. *Number of Mast Cells in the Peritoneal Cavity and Mesentery of WBB6F$_1$-W/W^v and WBB6F$_1$-tg/tg Mice 5 wk after Intraperitoneal Injection of 10^6 WBB6F$_1$-+/+ CMCs*

Mice[b]	No. of mast cells[a]	
	Peritoneal cavity (no./10^3 peritoneal cells)[c]	Mesentery (no./mm^2)[d]
F$_1$-+/+ mice (8)	31.0 ± 4.4	10.4 ± 2.8
F$_1$-*W/W^v* mice (8)	<0.1	ND[e]
F$_1$-*W/W^v* mice that received F$_1$-+/+ CMCs (8)	35.8 ± 4.2	11.4 ± 1.8
F$_1$-*tg/tg* mice (11)	<0.1	ND[e]
F$_1$-*tg/tg* mice that received F$_1$-+/+ CMCs (8)	36.1 ± 5.6	ND[e]

[a]Mean and SE are shown.
[b]Number of mice is shown in parentheses.
[c]Cytospin preparation was made from harvested peritoneal cells and was stained with alcian blue and nuclear fast red. Proportion of alcian blue-positive cells to 10^3 nucleated peritoneal cells was determined.
[d]Number of mast cells per mm^2 of stretched mesentery.
[e]Not detectable.

WBB6F$_1$-*W/W^v* mice but not in the mesentery of WBB6F$_1$-*tg/tg* mice. Next, WBB6F$_1$-+/+ CMCs were transplanted intravenously into WBB6F$_1$-*W/W^v* and WBB6F$_1$-*tg/tg* mice. As already reported, mast cells appeared in the peritoneal cavity, mesentery, stomach, and spleen of WBB6F$_1$-*W/W^v* mice (25), but did not in the corresponding tissues of WBB6F$_1$-*tg/tg* mice (Table III and Fig. 8).

The deficient development of mast cells in the mesentery was confirmed in B6-*tg/tg* mice after intraperitoneal transplantation of B6-+/+ CMCs (Table IV). Since there is a possibility that the transgene insertion in the *tg* allele affects the *mi* gene as well as neighboring genes which may function in the mesentery, we also transplanted B6-+/+ CMCs into the peritoneal cavity of B6-*mi^{ew}/mi^{ew}* mice. Although the phenotype of B6-*mi^{ew}/mi^{ew}* mice is not distinguishable from that of B6-*tg/tg* mice, the *mi^{ew}* allele encodes mutant MITF with a large deletion of the basic domain (30). In spite of the difference, the intraperitoneal injection of B6-+/+ CMCs gave similar results in both B6-*tg/tg* and B6-*mi^{ew}/mi^{ew}* mice. Mast cells appeared in the peritoneal cavity but did not in the mesentery (Table IV).

There is a possibility that tissues of mice of *tg/tg* genotype have a defect in microenvironment that is necessary for the migration and settlement of normal CMCs. First, we examined whether MITF was expressed in the mesentery and spleen of WBB6F$_1$-+/+, WBB6F$_1$-*W/W^v*, and WBB6F$_1$-*tg/tg* mice. The expression of MITF mRNA was detectable in the mesentery and spleen of intact WBB6F$_1$-+/+ and WBB6F$_1$-*W/W^v* mice, but did not in those tissues of intact WBB6F$_1$-*tg/tg* mice (Fig. 9). As the defect of WBB6F$_1$-*tg/tg* mice was somewhat reminiscent of the defect of WBB6F$_1$-*Sl/Sl^d* mice that do not produce a ligand of c-*kit* receptor tyrosine kinase (SCF; references 31–34), we next examined the expression of SCF in the mesentery and spleen of intact WBB6F$_1$-+/+, WBB6F$_1$-*W/W^v*, and WBB6F$_1$-*tg/tg* mice. In contrast to our expectation, the amount of SCF mRNA was comparable among tissues of all the WBB6F$_1$-+/+, WBB6F$_1$-*W/W^v*, and WBB6F$_1$-*tg/tg* mice (Fig. 9).

Discussion

Mast cells were practically absent in the peritoneal cavity of B6-*tg/tg*, B6-*mi^{ew}/mi^{ew}*, B6-*mi^{ce}/mi^{ce}*, and B6-*Mi^{wh}/Mi^{wh}* mice. Since mast cells develop in the peritoneal cavity 6 wk after birth even in B6-+/+ mice and since most of B6-*mi/mi* mice die on weaning (4 wk after birth), it is rather diffi-

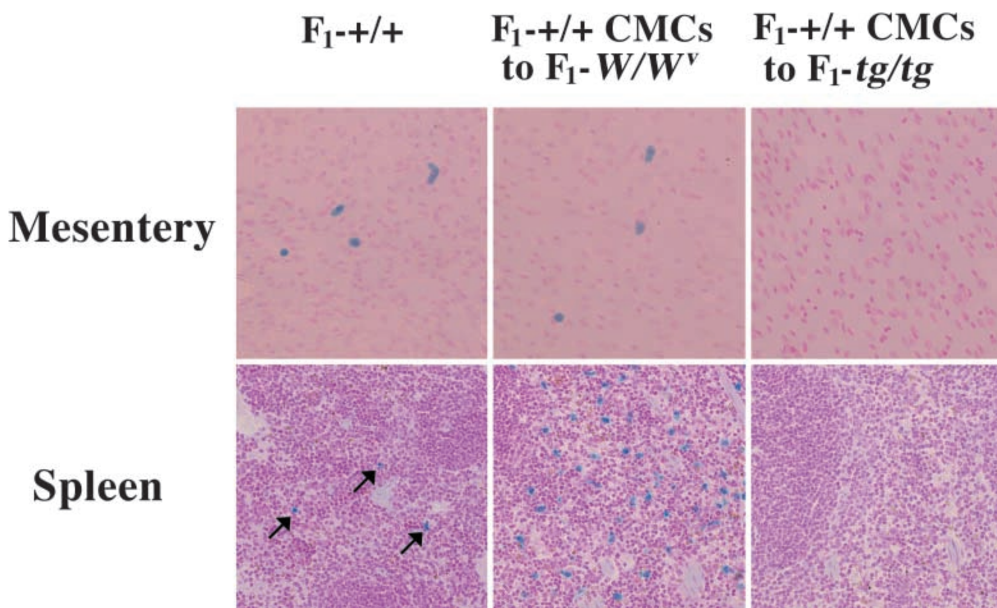


Figure 8. Nonappearance of mast cells in the mesentery and spleen of a WBB6F$_1$-*tg/tg* mouse 5 wk after intraperitoneal transplantation of WBB6F$_1$-+/+ CMCs. The mesentery and spleen of an intact WBB6F$_1$-+/+ mouse and those of a WBB6F$_1$-*W/W^v* mouse which had received the intraperitoneal transplantation of WBB6F$_1$-+/+ CMCs are also shown. Arrows in the spleen section of the WBB6F$_1$-+/+ mouse show mast cells. The number of mast cells in the spleen of the WBB6F$_1$-*W/W^v* mouse was remarkably larger than that observed in the intact WBB6F$_1$-+/+ mouse. Mast cells were stained with alcian blue and nuclear fast red. Original magnification, ×200.

Table III. *Number of Mast Cells 5 wk after Intravenous Injection of 10^6 WBB6F$_1$-+/+ CMCs*

Donors	Recipients[b]	No. of mast cells[a]			
		Peritoneal cavity[c]	Mesentery[d]	Stomach[e]	Spleen[f]
F$_1$-+/+	F$_1$-*W/W^v* (9)	5.0 ± 1.0	1.9 ± 0.4	178 ± 10	41 ± 3
F$_1$-+/+	F$_1$-*tg/tg* (9)	<0.1[g]	ND[h]	ND[h]	ND[h]

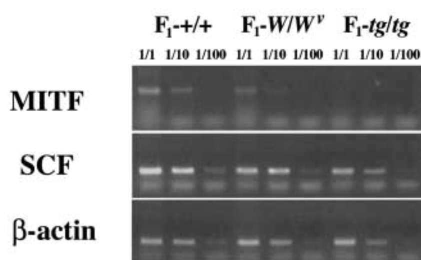
[a]Mean and SE are shown.
[b]Number of recipients is shown in parentheses.
[c]Cytospin preparation was made from harvested peritoneal cells and was stained with alcian blue and nuclear fast red. Proportion of alcian blue-positive cells to 10^3 nucleated peritoneal cells was determined.
[d]Number of mast cells per mm^2 of stretched mesentery.
[e]Number of mast cells per cm of stomach section.
[f]Number of mast cells per mm^2 of spleen section.
[g]$P < 0.01$ by *t* test when compared to the value of WBB6F$_1$-*W/W^v* mice which received WBB6F$_1$-+/+ CMCs.
[h]Not detectable.

cult to evaluate mast cell number in the peritoneal cavity of B6-*mi/mi* mice. However, mast cells are probably absent in the peritoneal cavity of B6-*mi/mi* mice as well, because all examined phenotypes of B6-*mi/mi* mice were the most severe among all MITF mutant mice (11, 17, 30, 35, 36). The *mi* and *Mi^{wh}* mutant alleles encode MITFs with deletion or alteration of a single amino acid at the basic domain (4). The *mi^{ew}* and *mi^{ce}* mutant alleles encode MITFs with large deletion in the basic and zipper domains, respectively (4, 36). All *tg*, *mi^{ew}*, and *mi^{ce}* are null mutations, whereas the

Table IV. *Number of Mast Cells in the Peritoneal Cavity and Mesentery of B6-tg/tg and B6-mi^{ew}/mi^{ew} Mice 5 wk after Intraperitoneal Injection of 10^6 B6-+/+ CMCs*

Mice[b]	No. of mast cells[a]	
	Peritoneal cavity (no./10^3 peritoneal cells)[c]	Mesentery (no./mm^2)[d]
B6-+/+ Mice (8)[e]	29.5 ± 2.8	8.4 ± 1.4
B6-*tg/tg* mice (9)[e]	<0.1	ND[f]
B6-*tg/tg* mice that received B6-+/+ CMCs (12)[g]	29.7 ± 2.9	ND[f]
B6-*mi^{ew}/mi^{ew}* mice (8)[e]	<0.1	ND[f]
B6-*mi^{ew}/mi^{ew}* mice that received B6-+/+ CMCs (8)	30.5 ± 2.8	ND[f]

[a]Mean and SE are shown.
[b]Number of mice is shown in parentheses.
[c]Cytospin preparation was made from harvested peritoneal cells and was stained with alcian blue and nuclear fast red. Proportion of alcian blue-positive cells to 10^3 nucleated peritoneal cells was determined.
[d]Number of mast cells per mm^2 of stretched mesentery.
[e]Data of the same mice are shown in Table I.
[f]Not detectable.
[g]Data of the same mice are shown in Fig. 2.



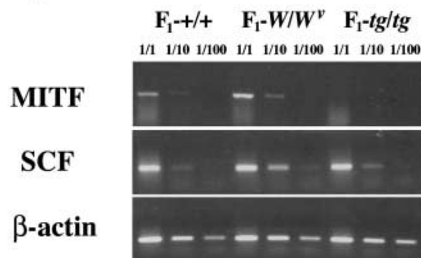


Figure 9. Expression levels of MITF and SCF mRNAs in the mesentery and spleen of intact WBB6F$_1$-+/+, WBB6F1-*W/W^v*, and WBB6F$_1$-*tg/tg* mice. Semiquantitative RT-PCR was done to compare the expression levels of MITF, SCF, and β-actin mRNAs. RNA extracted from the mesentery and spleen of intact WBB6F$_1$-+/+, WBB6F1-*W/W^v*, and WBB6F$_1$-*tg/tg* mice were subjected to RT-PCR.

mi is an inhibitory mutation (11, 30, 36). The *Mi^{wh}* showed decreased but detectable transcription activities on some genes and also significant inhibitory effects on transcription of other genes (35, 37). In spite of different structural and functional abnormalities of each mutant MITF, depletion of peritoneal mast cells was common among all MITF homozygous mutants examined. Tissues other than the skin of all MITF mutant mice also lacked mast cells. In contrast, mast cells were present in the skin tissue of all MITF mutants (30, 35–37). Probably, the skin is an exceptional tissue for development of mast cells. In fact, mast cells develop before birth only in the skin tissue (20). Depletion of mast cells in the peritoneal cavity appeared suitable for investigation of the mechanisms of development of mast cells.

The peritoneal cavity is also suitable for examining the involvement of mast cells in the innate immunity (21, 22). The reduced survival rate of WBB6F$_1$-*W/W^v* mice after CLP was normalized by the intraperitoneal transplantation of WBB6F$_1$-+/+ CMCs. WBB6F$_1$-*tg/tg* mice also lacked peritoneal mast cells and showed the reduced survival rate after CLP. In contrast to WBB6F$_1$-*W/W^v* mice, however, the survival rate of WBB6F$_1$-*tg/tg* mice was not normalized by the prior transplantation of WBB6F$_1$-+/+ CMCs. We examined the effect of the transplantation between WBB6F$_1$-*W/W^v* and WBB6F$_1$-*tg/tg* mice, and found that anatomical distribution of mast cells was different between them. Mast cell number was normalized in the peritoneal cavity of both WBB6F$_1$-*W/W^v* and WBB6F$_1$-*tg/tg* mice.

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Mast cells did appear in the mesentery of WBB6F$_1$-W/W^v mice, but did not in the mesentery of WBB6F$_1$-*tg/tg* mice. Mast cells within tissues or mast cells in the vicinity of blood vessels appeared to play an important role against the acute bacterial peritonitis induced by CLP.

Intraperitoneally transplanted CMCs of WBB6F$_1$-+/+ mouse origin invaded from the peritoneal cavity to the mesentery of WBB6F$_1$-W/W^v mice but did not invade to the mesentery of WBB6F$_1$-*tg/tg* mice. Intravenously transplanted WBB6F$_1$-+/+ CMCs invaded and settled in tissues of WBB6F$_1$-W/W^v mice, but did not in tissues of WBB6F$_1$-*tg/tg* mice. There is a possibility that the tissues of WBB6F$_1$-*tg/tg* mice have a defect in microenvironment that is necessary for the migration and settlement of normal CMCs. MITF was expressed in the mesentery and spleen of WBB6F$_1$-+/+ and WBB6F$_1$-W/W^v mice but did not in those of WBB6F$_1$-*tg/tg* mice. If MITF is involved in the transcription of microenvironmental factor(s) that play significant roles for the migration and settlement of normal CMCs, the absence of MITF may result in such a microenvironmental defect in tissues of WBB6F$_1$-*tg/tg* mice. We expected the deficient expression of SCF in the mesentery and spleen of WBB6F$_1$-*tg/tg* mice, but the amount of SCF mRNA was comparable among the tissues of WBB6F$_1$-+/+, WBB6F$_1$-W/W^v, and WBB6F$_1$-*tg/tg* mice. The microenvironmental defect of WBB6F$_1$-*tg/tg* mice might be attributable to the deficient transcription of factor(s) other than SCF. Such factor(s) remain to be identified.

Taken together, WBB6F$_1$-*tg/tg* mice appeared useful for studying the effect of anatomical distribution of mast cells on their antiseptic function.

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