Control of *Pseudomonas aeruginosa* Skin Infections in Mice Is Mast Cell-Dependent

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Mast cells (MCs) have recently been shown to be essential for the elicitation of efficient immune responses in murine sepsis. To explore whether MCs also contribute to the control of bacterial skin infections, we studied skin lesions induced by *Pseudomonas aeruginosa* (PA) in genetically MC-deficient *Kit^W/Kit^{W-v}* mice, normal *Kit^{+/+}* mice, and MC-reconstituted *Kit^W/Kit^{W-v}* mice. PA injections resulted in strikingly (>2-fold) larger skin lesions in *Kit^W/Kit^{W-v}* mice than in *Kit^{+/+}* mice, which exhibited pronounced MC degranulation at infection sites. In addition, neutrophil recruitment following PA injections and bacterial clearance from sites of infection was significantly impaired in *Kit^W/Kit^{W-v}* mice compared with *Kit^{+/+}* mice. Notably, the adoptive transfer of MCs to the skin of *Kit^W/Kit^{W-v}* mice before PA infection resulted in normal neutrophil accumulation as well as skin lesions comparable with those in *Kit^{+/+}* mice in both bacterial burden and size. These findings demonstrate for the first time that activated MCs are crucial for the induction of protective innate immune responses to bacterial skin infections. (Am J Pathol 2007, 170:1910–1916; DOI: 10.2353/ajpath.2007.060770)

Skin mast cells (MCs) are known to contribute to the pathology of various skin disorders including allergic and autoimmune dermatoses.^{1,2} In contrast, physiological and health-promoting functions of skin MCs have not been investigated in detail and remain largely unknown.^{3–5} Recently, peritoneal MCs, which share many characteristic features with skin MCs, have been described as important contributors to the control of mor-

bidity and mortality in murine bacterial sepsis.^{6–9} Using the cecal ligation and puncture model of septic peritonitis, we and others have demonstrated that peritoneal MCs are activated via Toll-like receptors as well as complement and endothelin-1 (ET-1) receptors. In addition, we and others have shown that MC-dependent innate immunity to bacteria involves tumor necrosis factor-mediated neutrophil recruitment, which results in bacterial clearance.^{9–12}

These findings led us to speculate that MCs are also involved in the induction of host defense responses to bacterial infections of the skin. This hypothesis is supported by several observations. 1) Skin MCs are preferentially localized beneath the epidermis and at skin sites that are frequently targeted by pathogenic bacteria, ie, the face, hands, and feet.¹³ 2) Skin MCs can detect and be activated by invading bacteria via various receptors including Toll-like receptors as well as complement and ET-1 receptors.^{9,11,14} 3) Skin MCs produce and release a large variety of host defense mediators such as tumor necrosis factor, interferon γ , and leukotriene B₄.^{10,15,16} 4) Skin MC responses, eg, after allergen challenge or during foreign body granuloma formation, have been shown to involve the recruitment of neutrophils and other inflammatory cells that importantly contribute to the containment and control of pathogens during infection.^{17–19} Despite this overwhelming body of suggestive evidence, host defense functions of skin MCs in antibacterial immunity have not been previously investigated *in vivo*.

Here, we have used the well-established model of genetically MC-deficient *Kit^W/Kit^{W-v}* mice, and their reconstitution with functional MCs,¹⁸ to test whether skin MCs are critical for the control of *Pseudomonas aeruginosa* (PA) skin infections. PA, a gram-negative rod, is the

Supported in part by grants from the Deutsche Forschungsgemeinschaft (SPP1110, B10SFB548) and from European Centre for Allergy Research Foundation (to M.M.) and by the Global Allergy and Asthma European Network (GA²LEN).

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Accepted for publication March 5, 2007.

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most frequent pathogen isolated from hospitalized patients and a common and increasing cause of skin infections that can result in potentially life-threatening septicemia.²⁰ Our results provide, for the first time, direct and conclusive evidence that MCs are indeed functionally important for the induction of host defense responses to cutaneous bacterial infections. Skin MCs are critically important for the accumulation of neutrophils and the clearance of bacteria at sites of cutaneous PA infections.

Materials and Methods

Animals

C57BL/6 mice, genetically MC-deficient WBB6F₁-*Kit^{WV}/Kit^{WV-v}* (*Kit^{WV}/Kit^{WV-v}*) mice, which ordinarily express <1% of the numbers of dermal MCs present in the skin of congenic normal *Kit^{+/+}* mice,^{21–23} and congenic normal WBB6F₁^{+/+} (*Kit^{+/+}*) mice were bred under specific pathogen-free conditions. Mice were kept in community cages (5–10 mice per cage) at light periods of 12 hours and were fed water and mouse chow (ssniff R/M-H, 10 mm; ssniff Spezialdiäten, Soest, Germany) *ad libitum*. All animal care and experimentation were conducted in accordance with current federal, state, and institutional guidelines.

P. aeruginosa Culture and Skin Infection

PA strain M2, which produces exotoxin A and extracellular proteases, was originally isolated from the intestinal tract of normal CF-1 mice^{24,25} and kindly provided by I.A. Holder (Shriners Burns Institute, Cincinnati, OH). Bacterial cultures were routinely grown in brain-heart broth (Heipha, Heidelberg, Germany) at 37°C for 24 hours, washed twice, and diluted in 0.9% NaCl. Defined numbers of diluted colony forming units (CFU) were injected subcutaneously in a total volume of 100 μ l into the previously shaved lower back skin of mice.

Selective MC Reconstitution of *Kit^{WV}/Kit^{WV-v}* Mice

Kit^{WV}/Kit^{WV-v} mice (4 to 6 weeks old) were repaired of their cutaneous MC deficiency by the injection of interleukin-3-dependent bone marrow-derived cultured MCs into the previously shaved lower back skin. Briefly, femoral bone marrow cells from *Kit^{+/+}* mice were maintained *in vitro* for ~4 weeks in interleukin-3-containing medium until MCs represented >95% of total cells according to staining by May Grünwald-Giemsa. MCs (10^6 in 200 μ l of Dulbecco's modified Eagle's medium per cm², 20 injections of 10 μ l each) were injected intracutaneously into an area of 4 cm². Mice were used for experiments, together with gender- and age-matched MC-deficient *Kit^{WV}/Kit^{WV-v}* mice and *Kit^{+/+}* mice, 4 weeks after adoptive transfer of bone marrow-derived cultured MCs. The success of MC reconstitution in *Kit^{WV}/Kit^{WV-v}* mice was confirmed using Giemsa-stained skin sections.

Analysis of Skin Lesion Size

After subcutaneous injection of PA (6.5×10^5 CFU in 100 μ l) into the lower back skin of mice, developing skin lesions characterized by early swelling and subsequent infiltration and, in some cases, ulceration and necrosis were assessed by planimetric analysis at certain time points, ie, 2, 4, 6, 8, 12, 18, 24, 36, 48, and 72 hours after infection. Skin lesion size was assessed by measuring vertical and perpendicular diameters and calculating the area by using the formula for an ellipse: (vertical diameter/2) $\times$ (perpendicular diameter/2) $\times \pi$.

Analysis of MC Degranulation in Vivo and ex Vivo

Mice were sacrificed 1 hour after subcutaneous injection of PA (6.5×10^5 CFU in 100 μ l, 6.5×10^6 CFU in 100 μ l, or 6.5×10^7 CFU in 100 μ l) or vehicle, and skin was harvested, fixed in 0.1 mol/L cacodylate buffer for 24 hours, and embedded in Epon. Sections (1 μ m) were stained with alkaline Giemsa, and MC degranulation was assessed histomorphometrically as described previously.^{9,26} In brief, MCs were classified at $\times 400$ as extensively degranulated (>50% of cytoplasmic granules exhibiting staining alterations, fusion and/or exteriorization), moderately degranulated (10 to 50% of granules affected), or not degranulated (<10% granules affected).

To assess MC degranulation after stimulation with PA *ex vivo*, skin MCs were isolated from the ears of C57BL/6 mice. MCs (5000 to 15,000) were resuspended in 2 ml of complete medium (Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, and 50 μ mol/L β -mercaptoethanol) and incubated with 2 μ Ci/ml tritium-marked serotonin for 2 hours at 37°C and 5% CO₂. To remove extracellular radioactivity, cell suspensions were washed three times in complete medium. MCs were coincubated with different dilutions of PA, prepared as described above, for 15 minutes or stimulated with 0.9% NaCl as a negative control or calciumionophore A23187 (2×10^{-5} mol/L) or ET-1²⁷ as positive controls. Specific serotonin release was assessed by scintillation counting of the supernatant after centrifugation (3 minutes at $350 \times g$ at room temperature) and the remaining pellet as described previously.^{27–29}

Quantification of Myeloperoxidase and Bacteria at Infection Sites

Mice were sacrificed 3 hours after PA subcutaneous injections (6.5×10^5 CFU in 100 μ l) into the lower back, and skin from infection sites was harvested using a 6-mm biopsy punch. To assess skin myeloperoxidase (MPO) levels, specimens were homogenized in 0.5 ml of 0.5% hexadecyltrimethylammonium bromide in 50 mmol/L potassium phosphate buffer, pH 6.0, diluted in the same buffer, and sonicated for 2 minutes in ice water. Diluents were centrifuged ($3000 \times g$ for 30 minutes at 4°C), and 50 μ l of the supernatant were incubated with 1450 μ l of

MPO reaction diluent (0.5% hexadecyltrimethylammonium bromide in 50 mmol/L potassium phosphate buffer, pH 6, 0.167 mg/ml *o*-dianisidine dihydrochloride, and 0.0005% H_2O_2) for 30 minutes. Absorption was assessed by measuring optical density at 460 nm.^{30,31}

To assess bacterial burden at infection sites, mice were sacrificed 24 hours after infection. After washing with 70% ethanol, skin was harvested, homogenized, and plated. Plates were then incubated at 37°C, and numbers of CFU were counted after 24 hours.^{10,32}

Endothelin-1 Enzyme-Linked Immunosorbent Assay

PA (6.5×10^5 CFU in 100 μ l) was injected subcutaneously into the shaved lower back of C57BL/6 mice. At defined time points (0, 1, 3, 6, 12, 24, 48, and 72 hours after infection) mice were sacrificed, and skin was harvested by using a 6-mm biopsy punch, which yielded skin biopsies of virtually identical size and very similar weight. Skin was homogenized in liquid nitrogen and dissolved in lysis buffer containing 50 mmol/L Tris/HCl, pH 8.0, 150 mmol/L NaCl, 1 mmol/L ethylenediamine tetraacetic acid, 1 mmol/L phenylmethylsulfonylfluoride, 5 mmol/L iodoacetamide, 10 μ g/ml aprotinin, 0.2% sodium dodecyl sulfate, 1% Igepal, and 1% Triton X-100 followed by sonication in ice water. After centrifugation (30 minutes at $13,000 \times g$ and 4°C), 200 μ l of supernatant were assessed for ET-1 concentrations by enzyme-linked immunosorbent assay following the protocol provided by the manufacturer (Biomedica, Vienna, Austria). Extinction was assessed spectrophotometrically at 450 nm,⁹ and ET-1 levels were calculated per mm² skin surface.

Routine Histology

Skin was harvested 24 hours after subcutaneous infection with (6.5×10^5 CFU in 100 μ l) PA and fixed in 4% formalin, dehydrated, and embedded in paraffin. Sections were processed for hematoxylin/eosin staining.

Statistics

Quantification of bacteria and histomorphometrical analysis of MC degranulation were compared by χ^2 test. All other data were tested for statistical significance using either the unpaired two-tailed Student's *t*-test for single point analysis or multiple analysis of variance for repeated measurements for the analysis of time course experiments (n.s. not significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.005).

Results

P. aeruginosa Infections Cause Markedly Larger Lesions in the Absence of Mast Cells

Cutaneous infection with PA resulted in the development of skin lesions that were found to increase until 12 to 24

Figure 1. *P. aeruginosa* infections cause markedly larger lesions in the absence of MCs. Skin lesion size in MC-deficient *Kit^W/Kit^{W-v}* mice (*n* = 27) and normal *Kit^{+/+}* mice (*n* = 32) after subcutaneous injection of PA (6.5×10^5 CFU) in 100 μ l. Data were pooled from six independent experiments and expressed as means $\pm$ SEM. Statistical analyses were performed by using the unpaired two-tailed Student's *t*-test for single point comparison; time course data were tested by multiple analysis of variance for repeated measurements. **P* < 0.05, ***P* < 0.01, ****P* < 0.005.

hours after infection followed by continuous reduction in lesion size in both groups. Interestingly, skin lesions in genetically MC-deficient *Kit^W/Kit^{W-v}* mice were significantly larger (>2-fold) and exhibited a prolonged progression at infection sites compared with normal *Kit^{+/+}* mice (Figure 1).

Mast Cells Are Activated at Sites of *P. aeruginosa* Skin Infections

Skin MCs exhibited pronounced and dose-dependent degranulation at sites of cutaneous PA infection as assessed by quantitative histomorphometry. After subcutaneous injection of 6.5×10^7 CFU of PA (100 μ l) in the back skin of normal *Kit^{+/+}* mice, 54% of skin MCs showed signs of moderate or extensive degranulation, whereas only 21.6% of MCs exhibited degranulation in vehicle treated control mice (Figure 2A). To test whether the activation of MCs is due to a direct interaction with PA, skin MCs were coincubated with different concentrations of PA, which resulted in a low, albeit statistically significant and dose-dependent, MC degranulation (Figure 2B). The fact that MC degranulation was much more prominent *in vivo* compared with *in vitro* prompted us to hypothesize that MC activation at sites of PA infection might be a result of the interaction with a soluble factor released after cutaneous bacterial challenge, eg, complement factors or ET-1. ET-1 is one of the most potent activators of mouse MCs and is known to be up-regulated during bacterial infection.⁵ In fact, ET-1 levels were found to be rapidly and markedly (over twofold) increased in PA-infected skin with a maximum at 6 hours after infection (Figure 2C).

Figure 2. MCs are activated at sites of *P. aeruginosa* skin infections. **A:** Degranulation of skin MCs *in vivo* after subcutaneous injection of defined amounts of PA. Data were pooled from two independent experiments ($n = 5-12$). $*P < 0.01$, $***P < 0.005$. **B:** Activation/serotonin release of skin MCs *ex vivo* after incubation with defined amounts of PA. Data were obtained from three to six independent experiments and expressed as means $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$. **C:** ET-1 skin levels were assessed from 6-mm punch biopsies, virtually identical in size and weight, harvested at defined time points after subcutaneous injections of PA and calculated as fmol/mm² skin surface. Data were pooled from two independent experiments ($n = 6-11$) and expressed as means $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.005$.

Neutrophil Accumulation and Bacterial Clearance at Sites of *P. aeruginosa* Infection Are Impaired in the Absence of Mast Cells

Skin levels of the neutrophil protease MPO closely reflect the total amount of neutrophils recruited to sites of inflammation.^{30,31} MPO levels in PA-infected skin areas of normal *Kit*^{+/+} mice were found to be dramatically increased (>7-fold) 3 hours after subcutaneous injection of PA compared with baseline levels (Figure 3A). In contrast, MC-deficient *Kit*^{W/Kit}^{W-v} mice exhibited much lower levels of MPO at infection sites, ie, 22.4% of MPO levels found in *Kit*^{+/+} mice. *Kit*^{W/Kit}^{W-v} mice also showed markedly impaired clearance of PA from sites of infection compared with *Kit*^{+/+} mice: 24 hours after injection, more than 90% of *Kit*^{W/Kit}^{W-v} mice showed significant amounts of PA at infection sites (up to 2.5×10^6 CFU/g skin), whereas approximately 50% of normal *Kit*^{+/+} mice had the infected site completely cleared from PA (Figure 3B).

Control of *P. aeruginosa* Skin Infections Including Neutrophil Influx and Bacterial Clearance Is MC-Dependent

To test whether the increased lesion size as well as the impaired neutrophil recruitment and bacterial clearance

Figure 3. Neutrophil accumulation and bacterial clearance at sites of *P. aeruginosa* infection are impaired in the absence of MCs. **A:** MPO skin levels (reflecting cutaneous neutrophil accumulation) after PA infection of MC-deficient *Kit*^{W/Kit}^{W-v} mice and normal *Kit*^{+/+} mice. Data were derived from three independent experiments ($n = 9-14$) and expressed as means $\pm$ SEM. $*P < 0.05$, $***P < 0.005$ versus vehicle; $***P < 0.005$ versus *Kit*^{W/Kit}^{W-v} mice. **B:** Bacterial clearance from sites of PA infections in *Kit*^{W/Kit}^{W-v} mice. Data were pooled from three independent experiments ($n = 14-15$). $***P < 0.005$.

observed in *Kit*^{W/Kit}^{W-v} mice is due to the lack of MCs in these mice, *Kit*^{W/Kit}^{W-v} mice that had been repaired of their cutaneous MC deficiency by selective adoptive transfer of bone marrow-derived cultured MCs obtained from normal *Kit*^{+/+} mice (*Kit*^{W/Kit}^{W-v} + MC mice) were subjected to PA skin infection. Notably, skin lesion sizes of infected *Kit*^{W/Kit}^{W-v} + MC mice were comparable with those in normal *Kit*^{+/+} mice (Figure 4A). In addition, MPO levels in these mice were found to be significantly increased compared with MC-deficient *Kit*^{W/Kit}^{W-v} mice (Figure 4B). Impaired recruitment of neutrophils to sites of PA skin infection in *Kit*^{W/Kit}^{W-v} mice was also observed in histopathological analyses of the lesions 24 hours after subcutaneous infection (Figure 4, D-F). At this time point, neither ulcerative dermatitis nor necrosis, both of which were seen macroscopically at later stages, could be detected. Interestingly, *Kit*^{W/Kit}^{W-v} + MC mice also exhibited markedly improved bacterial clearance after PA infection, which resulted in complete clearance of PA in 64% of infected *Kit*^{W/Kit}^{W-v} + MC mice compared with 7% in *Kit*^{W/Kit}^{W-v} mice (Figure 4C).

Discussion

Recently, MCs have been reported to importantly contribute to innate immune responses against bacterial pathogens in a model of septic peritonitis. However, whether MCs also contribute to host defense and protection in other models of bacterial infection or in different environments where MCs are also preferentially located, eg, in the skin, has not yet been clarified. Therefore, the findings presented here demonstrate, for the first time to our knowledge, that the clearance of bacterial skin infections in mice is, at least in part, MC-dependent. Protective immune responses to PA skin infections are characterized by early pronounced MC degranulation followed by MC-dependent recruitment of neutrophils and clearance of PA from sites of infection.

These observations complement and extend earlier reports showing that MCs are activated after bacterial challenge at noncutaneous sites.^{6,16} In some of these settings, MCs have been shown to be activated by bac-

Figure 4. Control of *P. aeruginosa* skin infections including polymorphonuclear neutrophil influx and bacterial clearance are MC-dependent. Skin lesion size (**A**), neutrophil recruitment (**B**), and bacterial clearance from sites of infection (**C**) in MC-reconstituted *Kit^W/Kit^{W-v}* mice (*Kit^W/Kit^{W-v}* mice + MC; *n* = 9–14), *Kit^{+/+}* mice (*n* = 9–32), and *Kit^W/Kit^{W-v}* mice (*n* = 9–27) injected subcutaneously with PA. Skin was harvested 24 hours after subcutaneous infection with PA. Histological sections were processed for H&E staining and imaged at $\times 50$ (**D–F**) and $\times 400$ (**G–I**) magnification. Data were pooled from three to six independent experiments and expressed as means $\pm$ SEM. Statistical analyses were performed by using the unpaired two-tailed Student's *t*-test for single point comparison; time course data were tested by multiple analysis of variance for repeated measurements. **P* < 0.05, ***P* < 0.01, ****P* < 0.005 versus *Kit^{+/+}* mice; n.s., not significant.

terial signals, eg, CD48 and Toll-like receptors, or via complement or ET-1 receptors. Our findings indicate that direct, ie, bacteria-derived signals, also contribute to MC activation in skin PA infections as reflected by a dose-dependent and significant increase in the activation of murine skin MCs after coinubation with PA *ex vivo*. However, MC degranulation was found to be much more pronounced *in vivo*, suggesting that additional mechanisms, ie, host-derived factors, may contribute to MC activation in this model. One such signal may be ET-1, which we found to be rapidly and markedly up-regulated in PA-infected skin. ET-1 is one of the most potent acti-

vating signals for connective tissue-type MCs acting via its ET_A receptor expressed on MC surfaces. As we have reported previously, ET-1-mediated MC activation significantly contributes to the control of morbidity and mortality in septic peritonitis.⁹ In addition, we have recently shown that nanomolar concentrations of ET-1 are sufficient to induce significant skin MC degranulation after UV light exposure. Moreover, the subsequent skin inflammation requires the activation by ET-1, which is increased to amounts comparable with those seen after PA infection.³³ ET-1 is only one of many candidate signals—which also include other neuropeptides and complement

components—that could promote MC degranulation in PA-infected skin. Thus, experiments using highly selective and specific antagonists or *Kit^W/Kit^{W-v}* mice reconstituted with receptor-deficient MCs will have to clarify to what extent MCs at sites of PA infection are activated by ET-1 and/or other host-derived signals. In addition, there is increasing evidence that MCs can release mediators such as cytokines in the absence of extensive degranulation. For example, MCs are known to rapidly release multiple proinflammatory mediators including tumor necrosis factor after activation by lipopeptides, lipopolysaccharide, and other bacterial signals that do not induce MC degranulation.

The number of immigrating neutrophils to sites of cutaneous infection was found to be markedly reduced in the absence of MCs. This observation is supported by previous reports showing that skin MCs are critically involved in neutrophil recruitment under various pathological conditions, including allergic and nonspecific inflammatory responses, cutaneous granuloma formation, and wound healing.^{18,26,34} Furthermore, MC-derived tumor necrosis factor has been shown to contribute to MC-dependent host protection by promoting neutrophil influx and subsequent bacterial clearance in the context of noncutaneous bacterial infections such as acute septic peritonitis.⁶ Notably, we also found neutrophil levels to closely correlate with the extent of bacterial clearance and the control of the pathology at sites of cutaneous PA infection. In MC-deficient skin, PA injections resulted in markedly larger and more persistent skin lesions associated with a 78% reduction of lesional neutrophil numbers and an 86% decrease in bacterial clearance. Taken together, these results demonstrate that skin MCs control PA-induced skin infections by the augmentation of neutrophil recruitment and bacterial clearance.

Most importantly, our findings provide formal evidence that skin MCs are significant sentinels of cutaneous innate immunity against bacteria. We demonstrate, to our knowledge for the first time, that the control of bacterial skin infections (lesion size and bacterial clearance) and its underlying mechanism (neutrophil recruitment) are MC-dependent. The fact that *Kit^W/Kit^{W-v}* mice repaired for their skin MC deficiency show essentially normal control of PA skin infections, ie, *Kit^{+/+}* levels of skin lesion sizes and bacterial clearance, demonstrates that these defects in *Kit^W/Kit^{W-v}* mice are due to their lack of MCs. Peritoneal MCs are found in mice but not in humans. In contrast, murine and human skin MC populations share many characteristics including size, distribution, expression of receptors, and production of mediators, suggesting that skin MCs may also contribute to antibacterial host defense in humans. This hypothesis is supported by *in vitro* observations showing that PA-activated human MCs can induce transendothelial neutrophil migration by releasing interleukin-1.³⁵

Taken together, our data demonstrate that cutaneous PA infections are critically controlled by activated MCs. In addition to our ongoing attempts to characterize better the role of individual MC-activating signals including ET-1, the evident next step is to explore the relevance of our findings in the context of PA infections of human skin.

If MCs also prove to be key effector cells in human anti-PA host defense, we may ultimately succeed in developing novel and better strategies for the prevention and/or treatment of PA infections using MCs as targets of intervention.

Acknowledgments

We thank D. Benner, S. Dinges, and A. Bolch for excellent technical support, I. A. Holder for providing PA, and J. Urcioli for proofreading the manuscript.

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