Dermal mast cells reduce progressive tissue necrosis caused by subcutaneous infection with *Streptococcus pyogenes* in mice

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A single subcutaneous (s.c.) infection with 1×10^7 c.f.u. GAS472, a group A streptococcus (GAS) serotype M1 strain isolated from the blood of a patient suffering from streptococcal toxic shock syndrome, led to severe damage of striated muscle layers in the feet of mast cell (MC)-deficient WBB6F1−KitW/KitW− (W/Wv) mice 72 h after infection. In contrast, no damage was recognized in striated muscle layers in the feet of the control WBB6F1−Kit+/Kit+ (+/+) mice 72 h after infection. In addition, adoptively transferred MCs reduced progressive tissue necrosis of the feet of W/Wv mice after infection. However, there was no significant difference in the mortality rates between the W/Wv and +/+ mice, or between the human CD46-expressing transgenic (Tg) mouse bone marrow-derived cultured MC-reconstituted W/Wv and non-Tg mouse bone marrow-derived cultured MC-reconstituted W/Wv mice after infection. Consequently, although MCs can help to reduce the severity of necrosis of the feet caused by s.c. infection with GAS472, such reduction of tissue necrosis scarcely improves the mortality rates of these mice. Moreover, human CD46 does not play a crucial role in the MC-mediated innate immune defence against GAS infection.

INTRODUCTION

Group A streptococcus (GAS) is a bacterium often found in the throat and on the skin of humans, and most people infected with GAS have no symptoms of illness. Some GAS infections result in relatively mild illnesses such as ‘strep throat’ or impetigo. However, GAS can occasionally cause severe and even life-threatening diseases. Severe GAS diseases may occur when the bacteria invade parts of the body where they are usually not found, such as the blood, muscle or lungs. These infections are termed invasive GAS diseases. Two of the most severe, but least common, forms of invasive GAS disease are necrotizing fasciitis (NF) and streptococcal toxic shock syndrome. NF (occasionally described by the media as being caused by the ‘flesh-eating’ bacterium) is defined pathologically by a deep spreading infection of the subcutaneous (s.c.) tissue that results in the progressive destruction of fascia and fat, with relative sparing of the skeletal muscle (Bisno & Stevens, 1996; Filbin et al., 2009; Fustes-Morales et al., 2002; Leitch et al., 2000).

In humans, the skin is able to activate the innate immune response in reaction to GAS, which involves antimicrobial peptides functioning on the effector side of the immune system (Steintraesser et al., 2008). In a recent study, mast cell (MC) cathelicidin LL-37, a cationic antibacterial peptide, was shown to protect mice against skin infection with GAS (Di Nardo et al., 2008). Generally, cutaneous MCs are known to contribute to the pathology of various skin disorders, including allergic and autoimmune dermatoses (Leslie, 2007). Although MCs have mainly been studied in the setting of allergic disease, they can also be activated as part of the innate immune response to pathogens (Galli et al., 2005; Marshall, 2004).
Most of the in vivo work on MC function in antibacterial host defence is done using models of bacterial infections of the peritoneum; however, recent studies have shown that MCs also play this role in other anatomical sites such as the middle ear (Ebmeyer et al., 2005), the lung (Xu et al., 2006) and skin (Siebenhaar et al., 2007). The skin plays an important role as a barrier against exogenous hazards, including microbes and physical stimuli such as UV light. MCs contribute to this barrier function and can act as sentinels in the skin, helping to limit or even prevent the damage that results from these environmental threats (Metz et al., 2008). We therefore aimed to investigate whether dermal MCs can protect mice from systemic infection with GAS472 by preventing NF. In this study, we provide evidence to suggest that the innate immune defence by MCs against GAS is limited to a reduction of the severity of localized tissue necrosis.

METHODS

Mice. The human CD46-expressing transgenic (Tg) mice were donated by Dr J. P. Atkinson of Washington University. The C57BL/6 mice employed as non-Tg control mice were obtained from Charles River Japan, where they were established as described previously (Matsui et al., 2009). MC development in vivo is highly dependent on the cytokine kit ligand/stem cell factor on the surface of mesenchymal cells and its tyrosine kinase receptor c-kit/CD117 on the surface of MC-committed progenitors. Signalling through the c-kit results in the translocation of microphthalmia transcription factor into the nucleus. WBB6F1-KIT WT/Kit WT (W/W') mice are MC-deficient secondary to a point mutation in the intracellular tyrosine kinase domain, which makes their MCs and progenitors less responsive to the c-kit ligand (Kitamura et al., 2007; Thakurdas et al., 2007). C57BL/6-KIT WT/+/Kit WT/Kit WT (W/W') mice represent another MC-deficient model for studies of MC functions in vivo (Galli et al., 2005). In this study, the W/W' and control WBB6F1-KIT WT/+ (+/+ ) mice were obtained from SLC Japan. All mice were bred at the animal facility at the Kitasato Institute, and all mouse experiments were performed in accordance with institutional guidelines under an approved protocol.

Bacteria. GAS472 (β-haemolytic GAS serotype M1 strain) was isolated from the blood of a patient suffering from streptococcal toxic shock syndrome in Japan in 2006. GAS472 was grown in Todd–Hewitt broth containing 0.2% (w/v) yeast extract (Difco and BBL) in 5% CO₂ at 37 °C without shaking (Matsui et al., 2009).

Infection. The expression of many pathogenic traits of GAS has been shown to depend on the growth phase (Miyoishi-Akiyama et al., 2003). Thus, the hind footpads of 12-week-old female mice were subcutaneously infected with 1 × 10⁷ cfu. GAS472 in stationary growth phase. After s.c. infection, the survival rates were observed every 24 h until 336 h post-infection, and the numbers of viable bacteria in the liver, spleen and popliteal lymph nodes were determined by plating onto sheep blood agar (Eguchi et al., 2007; Kodama et al., 2005).

Macroscopic and microscopic observations. Macroscopic images were obtained with a digital camera (D80; Nikon). For histological examination, a portion of each footpad was fixed with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) and then embedded in paraffin. Tissue sections approximately 5 μm thick were prepared and mounted on glass slides. The slides were stained with haematoxylin and eosin or Giemsa. Alternatively, another portion of each footpad was also fixed with Zamboni’s fixative (2%, v/v, paraformaldehyde and 15%, v/v, saturated picric acid solution in 0.1 M phosphate buffer, pH 7.3) for at least 8 h at 4 °C (Stefanini et al., 1967). The fixed tissue samples were embedded in optimum cutting temperature compound and rapidly frozen by isopentane chilled with liquid nitrogen. The 4 μm cryosections were stained with toluidine blue.

Selective MC reconstitution of W/W' mice. Bone marrow cells were harvested from both 7-week-old female CD46 Tg and C57BL/6 mice and cultured in complete RPMI 1640 supplemented with 10% fetal bovine serum, 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹, 50 μM 2-mercaptoethanol and 4 ng interleukin-3 ml⁻¹ (PeProtec EC). Bone marrow-derived cultured MCs were used at >99% purity, as determined by flow cytometric analysis using FcR1+/ (eBioscience) and c-kit+/b (Beckman Coulter) (Puruta et al., 2006). Bone marrow-derived cultured MCs from CD46 Tg mice were also used to determine the expression of human CD46 by flow cytometric analysis using FITC-conjugated anti-human CD46 mAb (BD Biosciences). At the time of reconstitution, bone marrow cells from W/W' mice were treated with a combination of anti-Thy 1 mAb and guinea pig serum to remove any contamination of T cells. The T cell-depleted bone marrow cells (2 × 10⁶ per mouse) and bone marrow-derived cultured MCs (5 × 10⁶ per mouse) from either CD46 Tg or C57BL/6 mice were injected intravenously and intraperitoneally, respectively, into X-ray-irradiated (4.5 G y x 2; MBR-1520R-3, Hitachi Medical) 6-week-old female W/W' mice. The bone marrow-derived cultured MC-reconstituted W/W' mice were housed for 6 weeks before infection with GAS472.

Statistics. The survival was analysed using a Kaplan–Meier log rank test. Significant differences between the mean ± SD values of different groups were examined using a two-tailed unpaired Student’s t-test. A P-value of <0.05 was regarded as statistically significant.

RESULTS AND DISCUSSION

CD46 Tg mice exhibit degranulation of MCs in the footpad skin sections after s.c. infection with GAS472

Human CD46 was first recognized as an epidermal keratinocyte receptor for the M protein of GAS (Okada et al., 1995). These findings were followed by the discovery that engagement of human CD46 and z5β1 integrin by GAS was required for the efficient invasion of epithelial cells (Rezcallah et al., 2005). Meanwhile, mammalian skin is composed of three layers: the epidermis, dermis and hypodermis (s.c. layer or superficial fascia). The dermis is tightly connected to the epidermis by a basement membrane, and the hypodermis lies beneath the dermis. In addition, muscles are separated from the underlying hypodermis. In our recent study, histological observation of the footpad skin sections of CD46 Tg mice subcutaneously infected with GAS472 revealed that acute inflammation developed in the hypodermis at 6 h post-infection, exfoliation of the epidermis with intracellular oedema and haemorrhaging developed in the dermis at 24 h post-infection, and necrosis of the striated muscle layers developed at 48 h post-infection (Matsui et al., 2009). In the present study, toluidine blue staining of the footpad...
skin sections revealed the presence of dermal MCs in uninfected CD46 Tg mice (Fig. 1a). Then, following the s.c. infection with GAS472, MC degranulation appeared to begin and to progress gradually (Fig. 1b, c, d). These findings provide histological evidence that the extracellular traps by MCs are formed after s.c. infection with GAS472. Therefore, it is suggested that the dermal MCs in CD46 Tg mice might be specialized in many ways to contribute to the innate immune defence system against s.c. infection with GAS472.

**MCs suppress the GAS472-induced destruction of the muscle layer**

Quite a few reports have previously shown that MC deficiency results in a markedly elevated susceptibility to a host of different bacterial and parasitic infections in addition to GAS infection. For example, MC-deficient mice are much more susceptible to infection caused by *Salmonella enterica* serovar Typhimurium (Chatterjea *et al.*, 2005), *Mycoplasma pneumoniae* (Xu *et al.*, 2006), *Citrobacter rodentium* (Wei *et al.*, 2005), *Helicobacter felis* (Velin *et al.*, 2005), *Haemophilus influenzae* (Ebmeyer *et al.*, 2005), *Listeria monocytogenes* (Gekara & Weiss, 2008), *Pseudomonas aeruginosa* (Siebenhaar *et al.*, 2007), *Leishmania major* (Maurer *et al.*, 2006) and *Plasmodium berghei* (Furuta *et al.*, 2006). In order to clarify the role of MCs in mice during GAS infection, MC-deficient W/W and their control +/+ mice were subcutaneously infected with GAS472. Although the necrotizing lesions were observed in the feet of both W/W (Fig. 2b) and +/+ (Fig. 2f) mice at 72 h post-infection, the destruction of muscle layers in the skin sections was recognized in W/W mice (Fig. 2c) but not in +/+ mice (Fig. 2g). In contrast, large numbers of inflammatory cells were visible around the muscle layers of the footpad skin sections of +/+ mice (Fig. 2g), whereas only small numbers of inflammatory cells were recognized around the destructed muscle layers in the footpad skin sections of W/W mice (Fig. 2c). Much of this variation would be associated with differences in bacterial amounts within tissues. Indeed, although clusters of streptococci were found at the destructed muscle layers in W/W mice (Fig. 2d), bacteria were detected only at the dermis in +/+ mice (Fig. 2h) at this stage. In addition, W/W mice had a significantly higher number of viable bacteria in the samples of the liver, spleen and popliteal lymph nodes compared with +/+ mice 72 h after s.c. infection with GAS472 (Fig. 3). These findings suggest that the severity of necrosis in the s.c. tissues and muscle layers of the feet of mice during GAS infection correlated with the number of disseminated bacteria in the involved tissues.

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**Fig. 1.** Footpad skin sections of CD46 Tg mice. Toluidine blue-stained footpad skin sections from uninfected CD46 Tg (a) and GAS472-infected CD46 Tg (b, c, d) mice at 24 h (b), 48 h (c) or 72 h (d) after s.c. infection with 1×10⁷ c.f.u. GAS472. Arrowheads indicate the MCs. Original magnifications, ×200.
MCs do not contribute to the survival rates of mice after s.c. infection with GAS472

Based on the fact that W/Wv mice had a significantly higher number of viable bacteria in the deep tissues compared with +/+ mice (Fig. 3), a clear difference in mortality rates would be expected between W/Wv and +/+ mice after s.c. infection with GAS472. However, we found that there was no significant difference in the mortality rates between W/Wv and +/+ mice (Fig. 4a). In addition, there was no significant difference in the mortality rates between the CD46 Tg mouse bone marrow-derived cultured MC-reconstituted W/Wv and non-Tg mouse bone marrow-derived cultured MC-reconstituted W/Wv mice after s.c. infection with GAS472 (Fig. 4b). In fact, there were no significant differences in mortality between any two of the four groups. Meanwhile, as compared with the necrotizing lesions of surviving mice after s.c. infection with GAS472, the necrosis of the skin, s.c. tissue, muscle and even bones of the feet of W/Wv mice progressed more rapidly after infection. In contrast, NF developed only partially in the feet of non-Tg mouse bone marrow-derived cultured MC-reconstituted W/Wv mice after infection (Fig. 5). Apparently, the adoptively transferred MCs reduced progressive tissue necrosis in the feet of W/Wv mice after infection.

**Fig. 2.** Representative appearance of the hind feet with histological observations of W/Wv and +/+ mice. Macroscopic images of the feet from uninfected control W/Wv (a) and +/+ (e) mice, followed by the feet from infected W/Wv (b) and +/+ (f) mice at 72 h after s.c. infection with 1 × 10⁷ c.f.u. GAS472. Haematoxylin and eosin-stained (c, g) or Giemsa-stained (d, h) footpad skin sections of s.c. infected W/Wv (b, c, d) and +/+ (f, g, h) mice are also shown. The black arrowheads indicate the necrotizing lesions (b, f). The red arrowheads and black arrows indicate the inflammatory cells and the striated muscle layers, respectively (c, g). The red arrows indicate the clusters of streptococci (d, h). Original magnifications, ×100 (c, g), ×200 (h) and ×400 (d).

**Fig. 3.** Bacterial counts in tissues of W/Wv and +/+ mice. W/Wv (open columns) and +/+ (closed columns) mice were subcutaneously infected with 1 × 10⁷ c.f.u. GAS472. At 72 h postinfection, the numbers of viable bacteria in the liver (1), spleen (2) and popliteal lymph node (3) samples were determined by plating. Data represent the mean value of the number of bacteria per tissue sample ± SD. *1, P = 0.009; *2, P = 0.017 (W/Wv mice vs +/+ mice). ND, Not detected. Each group has six mice.
s.c. infection with GAS472. Therefore, it was concluded that even though MCs can reduce progressive tissue necrosis, they do not participate in the improvement of host mortality due to GAS infection. Moreover, human CD46 does not play a crucial role in the MC-mediated innate immune defence during GAS infection.

**Fig. 4.** Comparison of survival rates between $W/W^r$ and $+/+$ mice or between the CD46 Tg mouse bone marrow-derived cultured MC-reconstituted $W/W^r$ and non-Tg mouse bone marrow-derived cultured MC-reconstituted $W/W^r$ mice. The mice infected subcutaneously with $1 \times 10^7$ c.f.u. GAS472 were monitored every 24 h for survival during the 336 h study. ○, $W/W^r$ mice ($n=12$); ●, $+/+$ mice ($n=12$); □, CD46 Tg mouse bone marrow-derived cultured MC-reconstituted $W/W^r$ mice ($b; n=12$); ■, C57BL/6 bone marrow-derived cultured MC-reconstituted $W/W^r$ mice ($b; n=12$). (a) $P=0.47$ ($+/+$ mice vs $W/W^r$ mice); (b) $P=0.91$ (CD46 Tg mouse bone marrow-derived cultured MC-reconstituted $W/W^r$ vs C57BL/6 bone marrow-derived cultured MC-reconstituted $W/W^r$ mice).

**Fig. 5.** Representative appearance of hind foot lesions of $W/W^r$ and non-Tg mouse bone marrow-derived cultured MC-reconstituted $W/W^r$ mice. Macroscopic observations of the feet of $W/W^r$ (a, b, c) and non-Tg mouse bone marrow-derived cultured MC-reconstituted $W/W^r$ (d, e, f) mice at 72 h (d), 120 h (a), 168 h (b, e) or 336 h (c, f) post-infection with $1 \times 10^7$ c.f.u. GAS472.
Local MCs play a limited role in innate immunity

In human patients, NF due to GAS infection is defined pathologically by a deep-seated infection of the s.c. tissue that results in the progressive destruction of fascia and fat, with relative sparing of the skeletal muscle (Bisno & Stevens, 1996; Filbin et al., 2009; Fustes-Morales et al., 2002; Leitch et al., 2000). In the present study, GAS472 infection of W/W mice resulted in typical rhabdomyolysis in the muscle layers at 72 h post-infection (Fig. 2c), followed by the progressive destruction of skin, s.c. tissue, muscle and even bone (Fig. 5a, b and c), which indicates that the infected W/W mice developed progressive and widespread tissue necrosis in their feet that was involved in myonecrosis and osteonecrosis, as well as NF. In the present murine experimental model of GAS infection, we showed that MCs play a key role in the reduction of progressive tissue necrosis caused by s.c. infection with GAS472 (Figs 2 and 5). Even so, a vital question still remains: namely, why was there no significant difference in the mortality rates between the W/W and +/+ mice (Fig. 4a)? MCs are preferentially located at sites with higher risks of bacterial infection. MC numbers within the whole skin tissue are highest in the most superficial skin layers and lowest in the subcutis, and they increase with the distance of the anatomical site from the body centre (Maurer & Metz, 2005). The MC-mediated protection against GAS infection should be linked to MC degranulation at an early stage after infection (Fig. 1). In our view, MCs are able to trap and kill bacteria, but their relatively low numbers (e.g. less than 5% of skin cells) and their finite mobility prevent them from playing a major role in the direct bactericidal action in deep tissues.

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