New insights into the antimicrobial effect of mast cells against *Enterococcus faecalis*

Running title: *Interactions between E. faecalis and mast cells*

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ABSTRACT

Enterococcus faecalis has emerged as an important cause of life-threatening multidrug-resistant bacterial infections in the hospital setting. The pathogenesis of enterococcal infections has remained a relatively neglected field despite their obvious clinical relevance. The objective of this study was to characterize the interactions between mast cells (MCs), an innate immune cell population abundant in the intestinal lamina propria, and *E. faecalis.* This study was conducted with primary bone marrow-derived murine MCs. The results demonstrated that MCs exerted an antimicrobial effect against *E. faecalis* that was mediated by both degranulation with the concomitant discharge of the antimicrobial effectors contained in the granules as well as by the release of extracellular traps, where *E. faecalis* was snared and killed. In particular, the cathelicidin LL-37 released by the MCs had potent anti-microbial effect against *E. faecalis.* We also investigated the specific receptors involved in the recognition of *E. faecalis* by MCs. We found that TLRs are critically involved in the MC recognition of *E. faecalis* since MCs deficient in the expression of MyD88, an adaptor molecule required for signalling by most TLRs, were significantly impaired in their capacity to degranulate, to reduce *E. faecalis* growth as well as to release TNF-α and IL-6 after encounter this pathogen. Furthermore, TLR2 was identified as the most prominent TLR involved in the recognition of *E. faecalis* by MCs. The results of this study indicate that MCs may be important contributors to the host innate immune defences against *E. faecalis.*

INTRODUCTION

Enterococcus species are part of the normal intestinal microbiota. From here, they can disseminate and cause a variety of infections in the immune compromised host, including urinary tract infections, bacteremia, endocarditis, and sepsis (1). In recent years, *E. faecalis* has emerged as an important cause of nosocomial infection with mortality rates exceeding 50% in critically ill patients, cancer patients and some transplant patients (2, 3). The intrinsic resistance of *E. faecalis* to several of the most commonly used antibiotics including penicillinase resistant penicillins, cephalosporins, lincosamides, nalidixic acid, low level of aminoglycoside and low level of clindamycin as well as its extreme capacity to acquire resistance to the remaining antibiotic classes such as penicillin, chloramphenicol, tetracyclines, rifampin, fluoroquinolones, aminoglycosides (high levels), and vancomycin (4) by the acquisition of mobile genetic elements (5) are of major concern for most hospitals and healthcare facilities. In particular, the increasing resistance of enterococci to vancomycin and teicoplanin is a challenging and serious public health concern (6).

Asymptomatic *E. faecalis* colonization frequently precedes clinical infection and colonized individuals represent a potential source for the spread of the microorganism. Unfortunately, there is no known effective therapy to decolonize patients with antibiotic-resistant enterococci. The increasing health care problem posed by *E. faecalis* highlights the urgent need for new avenues of therapeutic treatment and/or decolonization. In this regard, greater insight into the host-pathogen interactions during enterococcal infection and a better understanding of the mechanisms of host defence against this pathogen will be the foundation for advances in treatment and prevention modalities.

An important front of host defense in the intestinal mucosa consists of various innate immune cells including mast cell and macrophage, which sense entry of foreign elements into the mucosa and orchestrate an appropriate inflammatory response (7, 8). Mast cells (MCs), in particular, are multifunctional and highly effective tissue dwelling cells, which are considered

important components of the immune system. MCs have an important immunoregulatory function, particularly at the mucosal border between the body and the environment (9). They are located in close proximity to blood vessels, where they can efficiently regulate vascular permeability (10). In addition, MCs are able to modulate the activities of neighbouring effector cells through the release of a broad array of pre-stored or newly synthesized mediators (11). MCs produce four main classes of mediators including a wide variety of cytokines and chemokines, preformed granule-associated mediators, newly generated lipid-mediators and endogenous antimicrobial agents such as antimicrobial peptides or reactive oxygen species (ROS), which can be released from the granules upon activation (12). These mediators have been shown to exhibit various roles in tissue remodelling, angiogenesis, cellular recruitment and/or change of vascular permeability and host defence such as the recruitment of neutrophils to the site of infection (11). Despite their known role in the initiation of allergic reactions, chronic inflammatory processes and activation during certain types of parasitic infections (13, 14), there is now clear evidence that MCs also play a prominent role in the early immune response to invading pathogens (10, 15). MCs can also contribute to host defense by exerting a direct antimicrobial effect against pathogens either through phagocytosis (16) or by releasing extracellular traps (MCET), which are structures composed of DNA, histones, and granule proteins where the pathogens are snared and killed (17, 18). After translocation through the intestinal barrier, *E. faecalis* comes most probably into contact with the MCs present beneath the intestinal epithelium. However, relatively little is known about the MCs interactions with *E. faecalis*. Because MCs play such a prominent role in regulating the immune response, we can predict that these interactions may be of high relevance for fine-tuning the immune response to this invading pathogen. In the current study, we have investigated the dynamic interplay between *E. faecalis* and MCs. Our results show that MCs exert an anti-microbial activity against *E. faecalis* that was mediated by the release of MCET and by the discharge of granular anti-microbial compounds. We also show that

recognition of *E. faecalis* by MCs was mediated by TLRs since the production of cytokines and degranulation of MCs in response to *E. faecalis* was impaired in MCs lacking the common adapter molecule MyD88, which is required for almost all TLRs activation cascades. Furthermore, we could narrow down the recognition of *E. faecalis* by MCs to the TLR2. The results obtained in this study provide new insight into the interplay between *E. faecalis* and components of the innate immune system.

MATERIALS AND METHODS

Bacteria. *E. faecalis* strain DSMZ 20478 and OG1RF as well as different clinical isolates kindly provided by M. Probst-Kepper (Städtisches Klinikum Braunschweig, Germany) were used in this study. Stock cultures were maintained at –80°C, and cultured at 37 °C in BHI-medium for 6 h. Bacteria were harvested at mid-log phase (absorption OD_{600nm}~0.5), centrifuged for 10 minutes at 4000 rpm, washed with sterile PBS and adjusted to 10% transmission at 600 nm. Bacteria suspensions were further diluted in cell culture medium to achieve the required concentrations. For some experiments *E. faecalis* was co-cultivated with LL-37 (AnaSpec, San Jose, USA) add to the indicated concentrations for 90 min.

Mice. Inbred, pathogen-free, 8 to 12 week-old C57BL/6 mice were purchased from Harlan-Winkelmann (Borchen, Germany). C57BL/6 $TLR2^{-/-}$ mice were a gift from S. Weiss (Helmholtz Center for Infection Research, Braunschweig, Germany), C57BL/6 $MyD88^{-/-}$ mice were kindly provided by T. Sparwasser (TWINCORE, Hannover, Germany). Mice were maintained under standard conditions and according to institutional guidelines. All experiments were approved by the ethical board Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Oldenburg in Germany.

Generation of bone marrow-derived MCs. MCs were isolated and differentiated as previously described (18). Briefly, mice were sacrificed by CO_2 asphyxiation and the femurs and tibias were removed. The bone marrow was harvested by repeated flushing with Iscove's modified Dulbecco's medium (IMDM). Bone marrow cells were incubated in IMDM supplemented with 10% FCS, 2 mM L -glutamine, 1 mM pyruvate, 100 U/ml of penicillin, 100 µg/ml of streptomycin and 20 U/ml of recombinant murine IL-3 (BioLegend, San Diego, USA) for 21 days. Non-adherent cells were transferred to fresh culture plates every 2–3 days to remove adherent macrophages and fibroblasts. The purity of the resulting cell population

consisted of 98% of MCs as determined by flow cytometry analysis using anti mouse CD117 antibody (Caltag Laboratories, Hamburg, Germany) and a FACSCalibur TM flow cytometer (Becton Dickinson, San Jose, Calif., USA).

MCs infection assay. BMMCs were harvested, washed twice with IMDM without antibiotics and seeded in 48-well tissue culture plates at a density of $2x10^6$ cells/ml. *E. faecalis* was added to wells containing BMMCs at a multiplicity of infection (MOI) of 1 bacterium per cell (1:1) or to wells containing medium alone. The MOI of 1:1 was chosen based on a previous report (18) to ensure optimal conditions for the investigation of extracellular killing mechanisms elicited by MCs. Kinetics of bacterial growth in the presence or absence of MCs was monitored at the indicated time points of infection by determination of colony forming units (CFU) in the cell cultures. The total amount of bacteria in the wells was determined by plating serial dilutions of 20 µl volume of each well. The percentage of bacterial growth inhibition in the presence of MCs was calculated using the bacterial growth based on CFU after 90 min with the following formula: (CFU in medium alone – CFU in medium+MCs / CFU in medium alone) *x* (100).

In some experiments, BMMCs were treated for 30 min prior to infection with 1 µM Ca^{2+} ionophore ionomycin (Sigma, Deisenhofen, Germany), 100 µM cromolyn (Sigma, Deisenhofen, Germany) and/or 50 mU of micrococcal nuclease (New England Biolabs, Frankfurt am Main, Germany).

Toluidine stain. A total of $5x10^5$ MCs per well were infected with *E. faecalis* at a MOI of 10:1 and incubated for 2 h at 37°C, 5% CO_2. MCs were then fixed with 3% paraformaldehyde (PFA) for 10 min at RT and 100 µL of the MCs suspension was centrifuged on slides at 150 *x* g for 5 min and dried on air at RT. 5 µL of 0.5% toluidine-blue in PBS was placed on the slides and incubated further for 5 min at RT. After the incubation

step, slides were rinsed with water until the blue color disappeared and analyzed by light microscopy (Zeiss Axiophot microscope with an attached Zeiss Axiocam HRc digital camera and Axiovision software 4.8,. Carl Zeiss, Oberkochen, Germany).

β-Hexosaminidase-Assay. MCs were centrifuged at progressing times after stimulation, and degranulation was determined by assessing the percentage of β-hexosaminidase released into the culture supernatant according to the method of Kuehn et al. (19). Briefly, the cell pellets were lysed with distilled H_2O and the extracts were analyzed for the total β-hexosaminidase activities. A 50 µl volume of cell culture lysates and 100 µl of 2 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidase in 0.04 M sodium citrate buffer (pH 4.5) were added to each well of a 96-well plate, and color was allowed to develop for 30 min at 37 °C. The enzyme reaction was terminated by adding 50 µl 0.4 M glycine-NaOH (pH 10.7). The absorbance at 405 nm of each sample was measured with a 96-well TECAN Sunrise reader (Tecan Group Ltd., Männedorf, Switzerland). Total release of β-hexosaminidase was calculated as percentage of the maximum release of C48/80 degranulated cells, untreated MCs served as positive controls.

Cytokines determination. The determination of IL-6 and TNF-α levels was performed by specific ELISA, using matched antibody pairs and recombinant cytokines as standards. Briefly, ninety six–well microtiter plates were coated overnight at 4°C, with the corresponding purified anti-human capture monoclonal anti-IL-6, or TNF-α antibody (Pharmingen, San Jose, California, USA) at a concentration of 2 µg/ml in sodium bicarbonate buffer over night at 4°C. The wells were washed and then blocked with 1% bovine serum albumin-PBS before the serum samples and the appropriate standard were added to each well. Biotinylated rat monoclonal anti-IL-6 or-TNF-α antibody (BD Pharmingen) at 2 µg/ml was

added as the second antibody. Detection was performed with streptavidin-peroxidase, and the plates were developed by use of TMB.

qRT-PCR. Total RNA was prepared using the GeneJET RNA Purification Kit (Fisher Scientific – Schwerte, Germany). RNA was reverse transcribed with RT (Hoffman La Roche, Basel Switzerland) and cDNA synthesis was performed with a Gibco RT-PCR kit according to the manufacturer's instructions. The single-stranded cDNA was then subjected to PCR amplification under standard reaction conditions. The PCR primer sequences for the TLR-2 gene were as followed: forward: 5`-AAG AGG AAG CCC AAG AAA GC-3; reverse: 5`-ACC CAA AAC ACT TCC TGC TG-3`and forward: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'; reverse: 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'. The sequences for the housekeeping gen *β-actin* are as followed, forward: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3'; reverse: 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'. The resultant PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and photographed (Gel Doc™ XR System, BioRad, Hercules/Kalifornien, USA).

Generation of skin air pouches. Mice were anesthetized with Isofluran (Isoba; Essex Tierarznei, München, Germany) and infected subcutaneously with $5x10^7$ CFU of GFP-expressing *E. faecalis*. Mice were killed by CO_2 inhalation at 2 h after bacterial inoculation and the infiltrating inflammatory cells were isolated from the side of infection by extensively rinsing with warm IMDM medium. Inflammatory cells were incubated for 5 min at 4 ° C with anti-CD16/CD32 antibodies to block the FcR, followed by PE-conjugated anti-CD117 antibodies. After incubation for 30 min at 4 °C, cells were washed and flow cytometry analysis was performed using a FACSCalibur TM (Becton Dickinson). MCs were gated

according to their expression of CD117 antigen (FL2). MCs associated with green-labeled enterococci were identified by the expression of green fluorescence (FL1).

Live/Dead staining. MCs were seeded on glass coverslips, incubated for 5 minutes and then infected with *E. faecalis* at a MOI of 10 bacteria per mast cell. After 2 h incubation at 37°C in a 5% CO_2 atmosphere, gentamycin (100 µg/ml) was added to kill non-phagocytosed bacteria and MCs were further incubated for 2 h at 37°C, 5% CO_2. Discrimination between intact and damaged MCs was performed using the LIVE/DEAD viability/cytotoxicity kit for animal cells (Molecular Probes, Leiden, The Netherlands) according to manufacturer's instructions. Briefly, infected MCs were washed with PBS and incubated with 250 µl of PBS containing 2 µM Calcein-AM and 8 µM ethidium homodimer-1. Cells were incubated at room temperature for 15 minutes and photographed under fluorescein isothiocyanate illumination in an epifluorescence microscope (Zeiss Axiophot, Zeiss, Germany) coupled to a camera (model AxioCam HRc, Zeiss) and analysed by AxioVision Software 4.8 (Zeiss).

Visualization of MCs extracellular traps. To examine the release of extracellular traps by fluorescence microscopy, BMMCs were seeded on poly-L-lysine-covered glass cover slips, infected with *E. faecalis* at a MOI of 1:1 and fixed with 4% paraformaldehyde at 4 h of infection. BMMCs were then stained using the LIVE/DEAD cell viability kit for mammalian cells (Invitrogen, Karlsruhe, Germany) following the manufacturer's recommendations and examined using a Zeiss Axiophot microscope with an attached Zeiss Axiocam HRc digital camera and Axiovision software 4.8 (Carl Zeiss, Oberkochen, Germany). LIVE/DEAD BacLight TM Bacterial viability kit (Invitrogen) was used in some experiments to determine the viability of *E. faecalis* following the recommendations of the manufacturer.

Double immunofluorescence microscopy. 5 x 10^5 MCs were seeded on poly-L-lysine treated coverslides and infected with *E. faecalis* at a MOI of 10 to 1. After 90 min incubation, the coverslides were rinsed and cells were fixed with 3.7% formaldehyde. For double immunofluorescence staining, extracellular bacteria were stained with polyclonal rabbit anti-*E. faecalis* antibodies, followed by Alexa green-conjugated goat anti-rabbit antibodies (Sigma, Deisenhofen, Germany). After several washes, the cells were permeabilized by 0.01% Triton X-100 in PBS and washed again and intracellular bacteria were stained by anti-*E. faecalis* antibodies, followed by Alexa red-conjugated goat anti-rabbit antibodies (Sigma). The fluorescence images were obtained using a Zeiss Axiophot microscope with an attached Zeiss Axiocam HRc digital camera and Axiovision software 4.8 (Carl Zeiss, Oberkochen, Germany)

Electron microscopy. For scanning electron microscopy, samples were fixed with 5% formaldehyde and 2% glutaraldehyde in cacodylate buffer for 1 h on ice and washed in TE buffer. Dehydration was performed with a graded series of acetone, critical-point dried with CO_2, and sputter coated with gold-palladium before examination in a Zeiss field emission scanning electron microscope Merlin at 5 kV using the Everhart-Thornley SE detector and the in-lens secondary electron (SE) detector in a 25:75 ratio.

Statistics. Data were analyzed by using Excel 2007 (Microsoft Office) or GraphPad Prism 5.0 (GraphPad sofware). All data are presented as mean ± SD. Comparison between groups was made by use of *t*-test or one-way ANOVA or non-parametric Mann-Whitney test. *P* values ≤ 0.05 were considered as significant.

RESULTS

***E. faecalis* interacts with MCs *in vitro* and *in vivo*.** Scanning electron microscopy was used to visualize the interactions of *E. faecalis* with MCs, in *in vitro* cultures. As shown in Fig. 1A and 1B, *E. faecalis* attached to the surface of MCs. To demonstrate that this interaction also occurs in an *in vivo* environment, GFP-expressing *E. faecalis* was injected subcutaneously in the back of mice and the inflammatory cells recruited to the site of infection were collected at 2 h after bacterial inoculation. Infiltrated cells were stained with PE-conjugated anti-CD117 antibodies to identify the MC population by flow cytometry. Anti-CD117 antibody was specific for MCs and did not bind *E. faecalis* (supplementary Fig 1). The results showed that approximately 28% ± 2% of MCs isolated from the infected skin contained *E. faecalis*-GFP (Fig. 1C). As MCs have been previously shown to induce growth inhibition of several pathogens (17, 18), we determined if MCs were also capable to influence the growth of *E. faecalis* in *in vitro* cultures. For this purpose, the growth of *E. faecalis* was determined over time in the presence and absence of MCs. Results in Fig. 1D show that MCs were also very efficient at inhibiting the growth of *E. faecalis.* MCs were capable to inhibit the growth over a wide range of clinical isolates (Fig.1E).

TLR2 signaling is crucially involved in MCs recognition of *E. faecalis*. As TLR2 is among the most important pattern recognition receptors for gram-positive microorganisms, we examined the potential contribution of TLR2 to recognition of *E. faecalis* by MCs. First we investigated the potential contribution of TLRs to the recognition of *E. faecalis* by MCs. For this purpose, we characterized the response of MCs derived from MyD88$^{-/-}$ mice, an adaptor molecule required for signaling by most TLRs, to *in vitro* challenge with *E. faecalis*. MyD88$^{-/-}$ MCs were significantly impaired in their capacity to degranulate in response to *E. faecalis* as measured by the release of β-hexasominidase (Fig. 2A). MyD88$^{-/-}$ MCs were also less effective than wild-type MCs to inhibit *E. faecalis* growth (Fig. 2B).

MCs deficient in the expression of TLR2 also exhibited significantly lower level of degranulation (Fig. 2A) and growth inhibitory effects against *E. faecalis* (Fig. 2B) than wild-type MCs. Furthermore, *E. faecalis* co-cultured with wild-type MCs showed vesicle-like structures on the bacterial surface (Fig. 2C) which were less evident on bacteria cultivated with $TLR2^{-/-}$ MCs (Fig. 2D) or untreated control bacteria (Fig 2E). The number of enterococci displaying vesicle-like structures after co-cultivation with either wild type MCs, $TLR2^{-/-}$ MCs or cell culture medium was quantified by scanning electron microscopy after counting more than 300 individual organisms in 10 selected chains. A significantly higher percentage of *E. faecalis* bacteria producing vesicles was found after co-cultivation with wild type MCs (89.9%) in comparison to $TLR2^{-/-}$ MCs (12.2%) or control bacteria (6.4%) ($p < 0.005$. *E. faecalis* co-cultured with wild type MCs vs $TLR2^{-/-}$ MCs and $p < 0.005$ for *E. faecalis* co-cultured with MCs vs medium alone).

The release of inflammatory cytokines such as IL-6 (Fig. 3A) and TNF-α (Fig. 3B) by MCs in response to *E. faecalis* was also mediated by the TLR2 signaling pathway since MCs deficient in the expression of MyD88 or TLR2 released significantly lower levels of these cytokines after exposure to *E. faecalis*. Furthermore, the pathogen induced the up-regulation of TLR2 in MCs as demonstrated by the increased level of *tlr2* mRNA in MCs after exposure to *E. faecalis* (Fig. 3C).

MCs release antimicrobial extracellular traps (MCETs) in response to *E. faecalis*.

We next investigated the potential mechanism used by MCs to inhibit growth of *E. faecalis.* To determine if killing of *E. faecalis* by MCs was dependent of phagocytic uptake, MCs were infected with *E. faecalis* for 90 min and the amount of internalized bacteria determined by double immunofluorescence microscopy. The fluorescence microscopy photographs depicted in Fig. 4A show that *E. faecalis* is scarcely internalized by MCs. These results suggest that the

antimicrobial effect of MCs against *E. faecalis* was essentially mediated by extracellular mechanisms.

As it has been previously reported that MCs release MCETs with antimicrobial effect (17), we evaluated whether MCs released ETs after encounter *E. faecalis.* MCs were infected for 3 h with *E. faecalis* or medium alone, fixed, and processed for immunofluorescence microscopy. Histone staining reveals formation of MCETs by MCs in co-culture with *E. faecalis* (Fig. 4C) but not by MCs cultured in medium alone (Fig. 4B). Quantification of MCETs released after exposure to *E. faecalis* is shown in Fig. 4D. MCs stimulated for 3 h with PMA served as positive control.

We next determined the potential contribution of MCETs to the *E. faecalis* growth inhibition exerted by the MCs. For this purpose, we analysed the viability of *E. faecalis* cultured in the presence of MCs for 3 h using Live/Dead® staining. The confocal microscopy picture depicted in Fig. 5A shows that *E. faecalis* microorganisms entrapped in the MCETs were dead (arrows). However, we observed that a proportion of free *E. faecalis* were also dead, suggesting that MCs also killed *E. faecalis* in a MCETs-independent manner (Fig, 5B). To determine the proportional contribution of MCETs to the growth inhibition of *E. faecalis,* MCs were co-cultivated with *E. faecalis* at a MOI of 1:1 for 90 min in the presence or absence of 50 mU of endonuclease, which has been shown to dismantel MCETs by digesting the nuclear back bone (17). Destruction of MCETs resulted in only partial growth inhibition of *E. faecalis* by MCs suggesting that an additional mechanism was also involved (Fig. 5C).

Release of antimicrobial compounds by MCs contributes to the growth inhibition of *E. faecalis*. We next determined whether the additional mechanism used by MCs to inhibit the growth of *E. faecalis* was mediated by the release of antimicrobial compounds contained in the MCs granules. Indeed, toluidine-blue staining indicated a massive degranulation of MCs after encounter *E. faecalis* (Fig 6B). Toluidin-.stained uninfected MCs are shown in Fig

6A for comparison. The degranulation of MCs was also evidenced by scanning electron microscopy (Fig. 6C-E). We then determined the contribution of antimicrobial compounds released by MCs after degranulation to the growh inhibition of *E. faecalis* using MCs, which have been treated with cromolyn, a stabilizing agent that inhibits MC degranulation. Treatment with cromolyn strongly reduces the capacity of MCs to inhibit the growth of *E. faecalis.* Thus *E. faecalis* inhibition by MCs was reduced to 27.4 fold when MCs were treated with cromolyn to block degranulation (Fig. 6F).

Together these results indicate that the antimicrobial effect exerted by MCs against *E. faecalis* was mediated in part by the release of MCETs and in part by the release of antimicrobial compounds after degranulation. To confirm this postulation, we determined the levels of growth inhibition of *E. faecalis* by degranulated MCs and in the presence of nuclease to dismantle the MCETs. MCs treated with cromolyn and nuclease was completely impaired in their capacity to inhibit the growth of *E. faecalis* (Fig. 6G). These results confirmed the components of both MCETs and release of granule compounds to the anti-microbial effect of MCs against *E. faecalis.*

The cathlecidin LL-37 exhibits strong anti-microbial effects against *E. faecalis*. One important antimicrobial factor released by MCs is the cathlicidins LL-37 (20, 21). Therefore, we examined the effect of LL-37 on *E. faecalis.* As shown in Fig. 7A, LL-37 exerted a strong anti-microbial effect against *E. faecalis* at a concentration of 10 µg/ml. This effect was also observed when different clinical isolates of *E. faecalis* were tested (data not shown). *E. faecalis* incubated in the presence of LL-37 (10 µg/ml) displayed the same membrane vesicle formation as it was already observed on bacteria co-cultivated with MCs, non-treated *E. faecalis* bacteria served as controls (Fig. 7B, C). The number of enterococci displaying vesicle-like structures after cultivation in the absence or in the presence of LL-37 (10 µg/ml) was quantified by scanning electron microscopy after counting 100 individual

bacteria (supplementary figure 3). A significantly higher percentage of *E. faecalis* bacteria produced vesicles after treatment with LL-37 (94.4%) compared to bacteria grown in medium alone (5.7%) ($p < 0.0005$).

DISCUSSION

The nosocomial human pathogens *E. faecalis* and *E. faecium* are classically considered as the main cause of enterococcal bacteremia. Enterococci are the third most common pathogens isolated from human bloodstream infections (22-25) and it has been previously reported that up to 90% of enterococcal infections in human are caused by *E. faecalis* (26). Despite bloodstream infections they are also important causes of urinary tract infections, endocarditis, intra-abdominal and pelvic infections not only in the hospital environment but even more concerning in the community environment (27). The high propensity of these pathogens to acquire and express new antibiotic resistance determinants further increases their ability to sustain antibiotic selection, impeding bacterial clearance and thus promoting gastrointestinal colonization (28). Furthermore, transfer of transposable mobile genetic elements carrying antibiotic resistance determinants from *E. faecalis* to other bacterial genera by broad host range conjugative elements, aggravates the problems associated with the spreading of multidrug resistant pathogens (5).

Despite the clinical importance of *E. faecalis* infections little is known about the interactions of *E. faecalis* with the host immune system. This knowledge could lead to new strategies to improve the natural host resistance to this emerging pathogen. Investigations to unravel the interaction of enterococci with host immune cells have been strongly focused on the role of professional phagocytotic cells. In this regard, several reports have highlighted the crucial role of polymorphonuclear neutrophils in the early control of *E. faecalis* (29). However, little is known about the interactions of *E. faecalis* with other important immune cell types with high immune modulatory functions like MCs. MCs are inflammatory cells which are typically located immediately beneath the epithelial surfaces exposed to the outer environment such as the skin, the mucosa but also the respiratory, genitourinary and gastrointestinal tract (10). Because many of these sites are also common entry ports for pathogens, MCs represent one of the first immune cells encountered by an invading pathogen

438 and therefore initiating the inflammatory immune response against these microbes (30).
439 Recent evidences have suggested that MCs have a beneficial contribution to both innate and
440 adaptive immunity during infection (31). In this study, we performed an in-depth
441 characterization of the interactions of *E. faecalis* with MCs. The results demonstrated that
442 MCs exhibited a remarkable anti-microbial effect against *E. faecalis*.

443 MCs have been shown to be able to kill bacteria by two different mechanisms (16): (1)
444 phagocytosis (mainly Gram-negative bacteria) and/or (2) extracellular either by the release of
445 anti-microbial peptides such as LL-37 by degranulation or by the formation of MCETs (17) or
446 a combination of both (18). We found no evidence that *E. faecalis* could actively invade MCs
447 as it was previously demonstrated for other Gram-positive pathogens like *Staphylococcus*
448 *aureus* (18) or Gram-negative bacteria like *Escherichia coli* (32). These pathogens are able to
449 persist for long time periods intracellularly within MCs without losing viability by gaining
450 access into MCs by a rout distinct from the classical endosome-lysosome pathway.

451 Our study shows that MCs became activated after encountering *E. faecalis* and exerted a
452 direct extracellular antimicrobial activity against this pathogen that was mediated by various
453 extracellular mechanisms. One of those mechanisms involves the formation of MCETs, where
454 *E. faecalis* microorganisms are trapped and killed. However, the level of MCET formations
455 was not as pronounced as it has been reported for other pathogens like *Streptococcus*
456 *pyogenes* (17) and therefore killing of *E. faecalis* by MCETs cannot account for the fully anti-
457 microbial effect of MCs observed in this study. This was further confirmed by the diminished
458 but still significant anti-microbial effect of MCs after dismantling the MCETs by nuclease
459 treatment. The reason why only a percentage of MCs in the cultures released MCETs after
460 encounter *E. faecalis* is not yet clear, however, this is likely to reflect a heterogeneity in the
461 physiological status of the MCs in the culture. A similar phenomenon has been reported in
462 extracellular traps formation by neutrophils (33).

The second extracellular mechanism used by MCs to control *E. faecalis* consisted in the discharge of granule anti-microbial compounds. Several studies have shown that activation of MC degranulation leads to an effective killing of various Gram-positive as well as Gram-negative bacteria (34). In the present study, blocking MC degranulation by cromolyn strongly impaired the anti-microbial effect exerted by MCs against *E. faecalis*, which argues for a predominant contribution of MC degranulation to the antimicrobial response of MCs to *E. faecalis*. MC degranulation is generally accompanied by the release of anti-microbial peptides such as cathepsin G or cathelicidins (20, 35), which are known to inhibit growth of various Gram-positive bacteria including *S. pyogenes* or *S. aureus* (20, 36, 37). Our results show that *E. faecalis* was very sensitive to the anti-microbial effect of cathelicidin LL-37, indicating a potential major role of this anti-microbial peptide in the anti-microbial activity of MCs against *E. faecalis*, which has been suggested in other studies (38-41). Scanning electron microscopy examination revealed that *E. faecalis* exposed to either MCs or to LL-37 microorganism developed vesicle-like structures on the bacterial surface. Such vesicles have been described mainly for Gram-negative microorganism like *E. coli* (42), *Salmonella enterica* (43) or *Pseudomonas aeruginosa* (44) and some Gram-positive microorganisms like *S. aureus* (45, 46), *Streptococcus pneumoniae* (47), Mycobacteria (48) and *Bacillus anthracis* (49). The function of these vesicles is not yet clear but they may be related to bacterial responses towards environmental stress, which in our study might be produced by the anti-microbial compounds of MCs acting in the bacterial cell-wall.

There are multiple direct and indirect pathways by which MCs can be selectively activated by pathogens. These include TLRs, co-receptors and complement receptors, among which the TLR family occupies a central position. Signalling via these receptors guides the immune system to produce an effective immune response to invading pathogens. We also investigated in this study the receptors involved in the recognition of *E. faecalis* by MCs. Using MCs deficient in MyD88, a critical adaptor molecule for most TLRs, we demonstrated

that recognition of *E. faecalis* by MCs was largely mediated by TLRs. Among the different TLRs, we selectively evaluated the role of TLR2 because of the important role in the recognition of Gram-positive microorganisms. TLR2 signalling has been reported to be critically involved in the control of other pathogens like *Francisella tularensis* by MCs (50). Furthermore, it has been demonstrated that lipoteichonic acid of *E. faecalis* induces the expression of chemokines in macrophages mainly via the TLR2/PAFR signalling pathway (51). Also the involvement of other TLRs in the immune response to enterococci have been demonstrated in recent studies, involving the recognition of enterococcal nucleic acids by the endosomal TLRs 7 and 9 in macrophages (52). Our results reveal an important role of TLR2 signaling for activation of MCs in response to *E. faecalis.* Thus, MCs deficient in the expression of TLR2 were significantly impaired in their capacity to release inflammatory cytokines such as IL-6 and TNF-α. IL-6 release by MCs was recently shown to be important to regulate the selective influx of dendritic cells (DCs) into inflamed lymph nodes, thus enhancing opportunities for effective T cell-DC interaction and therefore for mounting an effective adaptive immune response to bacterial pathogens (53). In particular, the release of TNF-α by local MCs can be seen as an important protective mechanism elicit by MCs during enterococcal infection, because TNF-α serves as an important chemo attractant for phagocytic cells like PMNs and macrophages which are involved in the elimination of this pathogen. Furthermore, the anti-microbial effect of MCs against *E. faecalis* was strongly reduced in $TLR2^{-/-}$ MCs in comparison with wild-type MCs. We also found that $TLR2^{-/-}$ MCs exhibited a similar capacity to release MCETs than wild-type MCs (data not shown), we speculated that MC degranulation and release of anti-microbial compounds may be driven by TLR2 signaling. Indeed, $TLR2^{-/-}$ MCs were strongly impaired in their capacity to degranulate after encounter *E. faecalis.* Interestingly, our results also showed that MCs up-regulated TLR2 expression after encountering *E. faecalis*. Increasing the availability of TLR2 by the MCs can serve to amplify the magnitude of the inflammatory response mediated by this receptor.

In summary, this study provides for the first time experimental evidence that MCs exert antimicrobial activity against *E. faecalis* and thereby supporting a protective effect of this immune cell type against this important pathogen. Further studies are however needed to elucidate the further facets of MCs-*E. faecalis* interactions as well as the bacterial determinants involved in MC activation. Understanding the mechanisms and scope of the contribution of MCs to host defense will be crucial to understand in respect to regulate their activity therapeutically during bacterial infections.

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548 **FIGURE LEGENDS**

549

550 **FIGURE 1.** Association of *E. faecalis* with MCs. (A) Scanning microscopy picture of *E.*
551 *faecalis* attached to the surface of MCs (bacteria are indicated by white arrows). (B) High
552 magnification of a MC harboring *E. faecalis* (white arrow). Bars represent 3 µm in (A) and 2
553 µm in (B). (C) Histogram analysis showing MCs associated with *E. faecalis* during *in vivo*
554 infection. Mice were subcutaneously inoculated with 5 $x10^7$ GFP-expressing *E. faecalis*,
555 infiltrated cells were isolated from the site of bacterial inoculation at 2 h of infection and the
556 amount of MCs (CD117+) containing *E. faecalis* was determined by flow cytometry. The
557 percentage of MCs containing *E. faecalis* was determined by the increase in green-
558 fluorescence (GFP) within the gated CD117+ cell population. One representative experiment
559 out of three is shown. (D) Growth kinetics of *E. faecalis* in the presence (squares) or absence
560 (circles) of MCs (MOI 1:1). A compilation of three independent experiments is shown. *, $p <$
561 0.05. (E) Growth inhibition (%) of *E. faecalis* clinical isolates in the presence of MCs
562 determined at 90 min of co-culture. Percentage of bacterial growth inhibition in the presence
563 of MCs was calculated using net bacterial growth based on CFU after 90 min with the
564 following formula: (CFU in medium alone – CFU in medium+MCs / CFU in medium alone)
565 *x* (100). Each bar represents the mean ± SD of triplicates from three independent experiments.

566

567 **FIGURE 2.** MCs derived from wild type $TLR2^{-/-}$ or $MyD88^{-/-}$ mice were impaired in their
568 capacity to (A) degranulate and (B) to inhibit growth of *E. faecalis* (120 min of infection).
569 Each bar represents the mean ± SD of quadruplicates from two independent experiments. *, p
570 < 0.05; **, $p < 0.01$; ***, $p < 0.001$. Scanning microscopy picture of *E. faecalis* co-cultivated
571 with either wild type MCs (C), $TLR2^{-/-}$ MCs (D) or medium alone (E) (white arrows indicate
572 membrane vesicle formation in the surface of *E. faecalis*). Bars represent 1 µm in (C), (D) and
573 (E).

574 **FIGURE 3.** MCs derived from either wild type, TLR2$^{-/-}$ or MyD88$^{-/-}$ mice were altered in
575 their capacity to release (A) IL-6 and (B) TNF-α in response to *E. faecali*s. Each bar
576 represents the mean ± SD of three independent experiments. **, $p < 0.01$. (C) Expression of
577 *tlr2* mRNA on MCs after infection with *E. faecalis*. MCs were exposed to *E. faecalis* for 2 h,
578 washed and further incubated in the presence of gentamicin for 6 h and 24 h. Total RNA was
579 isolated followed by quantitative measurement of *tlr2* mRNA expression in MCs after
580 infection with *E. faecalis* by qRT-PCR. *β-actin* served as internal control and the results of the
581 qRT-PCR were calculated according to the ΔΔCT method (38). One representative result out
582 of three is shown.
583
584 **FIGURE 4.** Double immunofluorescence staining of *E. faecalis*-infected MCs (A) and MCs
585 released extracellular traps after exposure to *E. faecalis.*. MCs were infected with *E. faecalis*
586 at a MOI of 10 to 1 for 90 min incubation and stained for double immunofluorescence.
587 Extracellular bacteria were stained by anti-*E. faecalis* antibodies, followed by Alexa green-
588 conjugated goat anti-rabbit antibodies (ii). MCs were then permeabilized by 0.01% Triton X-
589 100 in PBS and intracellular bacteria were stained by anti-*E. faecalis* antibodies, followed by
590 Alexa red-conjugated goat anti-rabbit antibodies (iii). The DNA is stained in blue (i). The
591 lack of red bacteria in the merged multi fluorescence picture in (iv) shows that *E. faecalis* is
592 located mainly extracellular. MCs were seeded on poly-L-lysine coated glass slides and (B)
593 left untreated or (C) infected with *E. faecalis* for 3 h. MCs were then fixed with 4%
594 paraformaldehyde and examined by immunofluorescence microscopy after Histone
595 (red)/DAPI (blue) staining. Bars represent 10 µm. (D) Microscopic quantification of MCETs
596 released per field of view (40x magnification) in untreated control, *E. faecalis*-infected (MOI
597 10:1) and PMA- (200µM) treated MCs. Each bar represents the mean ± SD of three
598 independent experiments. **, $p < 0.01$; ***, $p < 0.001$.
599

FIGURE 5. Live/Dead® staining of MCs infected with *E. faecalis.* (A and B) MCs were seeded on poly-L-lysine coated glass slides, then infected with *E. faecalis* for 3 h at a MOI 10 : 1, fixed with 4% paraformaldehyde and examined by immunofluorescence microscopy after staining with Live/Dead® reagents. Dead bacteria appear red and viable bacteria appear green. Arrows indicate dead bacteria . Bars represent 10 µm in (A) and 5 µm in (B). (C) Growth kinetics of *E. faecalis* in medium alone (circles), in medium + nuclease (triangles), or in co-culture with either untreated (squares) or nuclease-treated (inverted triangles) MCs. Each point represents the mean ± SD of triplicates from three independent experiments. *, $p < 0.05$.

FIGURE 6. Contribution granule components released by MCs to the antimicrobial effect against *E. faecalis.* Uninfected MCs (A) or MCs were infected for 3 h with *E. faecalis* (B) were fixed and stained with toluidine-blue. (C-E) Scanning electron photographs showing an uninfected MC (C) and a MC degranulating after infection with *E. faecalis* at a MOI of 10:1 (bacteria on the MC surface are indicated by a white arrow) (D). An enlarged view of *E. faecalis* on the MC surface is shown in (E). Bars (A) and (B): 10 µm and Bars (C) and (D): 5µm; e: 1µm). (F) Growth kinetics of *E. faecalis* in medium alone (circles), in medium + cromolyn (triangles), or in co-culture with either untreated (squares) or cromolyn-treated (inverted triangles) MCs. Each point represents the mean ± SD of triplicates from three independent experiments. (G) Growth of *E. faecalis* in medium alone (white bar) or co-cultured with MCs either untreated (dark gray bar) or treated with cromolyn (light grey bar), nuclease (hatched bar) or both (black bar) for 90 min. Each point represents the mean ± SD of triplicates of three independent experiments. *, $p < 0.05$.

FIGURE 7. Anti-microbial effect of LL-37 against E. *faecalis.* (A) Growth of *E. faecalis* in the presence of different concentrations of cathelicidine LL-37 for 120 min. Each bar represents the mean ± SD of quadruplicates from two independent experiments. *, $p < 0.05$.

Scanning electron microscopy photographs of (B) untreated or (C) LL-37-treated (10 µg/ml) *E. faecalis*. Arrows indicate membrane vesicle formation on the surface of *E. faecalis*. Bars represent 1 µm in (B) and (C). Insertion shows enlarged scanning electron micrograph. Arrows indicated vesicle formation on the bacterial surface. Bar represents 1 µm (C, insertion).

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