HHS Public Access

Author manuscript

J Allergy Clin Immunol. Author manuscript; available in PMC 2019 July 01.

Published in final edited form as:

J Allergy Clin Immunol. 2018 July ; 142(1): 120–129.e6. doi:10.1016/j.jaci.2017.07.042.

MAST CELL CHYMASE DECREASES THE SEVERITY OF GROUP B STREPTOCOCCUS INFECTIONS

Claire Gendrin, PhD[1,2,9], **Nicholas J. Shubin, PhD**[2,9], **Erica Boldenow, PhD**[2], **Sean Merillat, BSc**[2], **Morgan Clauson, BSc**[2], **Danny Power, BSc**[2,8], **Kelly S Doran, PhD**[3,4], **Magnus Abrink, PhD**[5], **Gunnar Pejler, PhD**[6,7], **Lakshmi Rajagopal, PhD**[1,2,8,*], and **Adrian M. Piliponsky, PhD**[1,2,*]

[1]Department of Pediatric Infectious Diseases, University of Washington, Seattle, Washington, United States of America

[2]Seattle Children's Hospital Research Institute, Seattle, Washington, United States of America

[3]Department of Biology and Center for Microbial Sciences, San Diego State University, San Diego, CA, United States of America

[4]Department of Pediatrics, University of California San Diego School of Medicine, La Jolla, CA, United States of America

[5]Department of Biomedical Sciences and Veterinary Public Health, Swedish University for Agricultural Sciences, Uppsala, Sweden

[6]Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

[7]The Swedish University of Agricultural Sciences, Department of Anatomy, Physiology and Biochemistry, Uppsala, Sweden

[8]Department of Global Health, University of Washington, Seattle, Washington, United States of America

Abstract

Background—Group B Streptococcus (GBS) or *Streptococcus agalactiae* are β-hemolytic, Gram-positive bacteria that colonize the lower genital tract of women and are frequently associated with infections during pregnancy. Innate immune defenses are critical for controlling

[*]Co-corresponding authors: Adrian M. Piliponsky, Center for Immunity and Immunotherapies, Seattle Children's Research Institute, 1900 9th Ave, Room 721, adrian.piliponsky@seattlechildrens.org, Phone number: 206-884-7226, Fax number: 206-987-7310. Lakshmi Rajagopal, Center for Global Infectious Disease Research, Seattle Children's Research Institute, 1900 9th Ave, Room XXXX, lakshmi rajagopal@seattlechildrens.org, Phone number: 206-884-7336, Fax number: 206-884-7311.

[9]These authors contributed equally to this work

Author contributions:

C.G., E.B., S.M., N.J.S., L.R. and A.M.P designed the experiments. C.G., E.B., S.M., N.J.S., M.C. and A.M.P performed the experiments. M.A., G.P., and K.S.D provided reagents. C.G., E.B., S.M., N.J.S., L.R., and A.M.P analyzed the results and C. G., L.R and A. M. P wrote the manuscript. All authors reviewed the final version of the manuscript.

Disclosure of Conflict of interest: The authors declare that they have no conflict of interests.

GBS dissemination and systemic infection. Mast cells are resident sentinel cells that come into contact with pathogens early during colonization and infection.

Objective—We aimed to investigate the contribution of chymase to systemic GBS infection and rates of preterm birth.

Methods—Pharmacological and genetic approaches using mice deficient in mast cell protease (MCPT)4, the mouse functional homolog of human chymase, were employed.

Results—Our studies show that, in response to GBS, mast cells release a protease with chymotrypsin-like cleavage specificity. Additionally, increased GBS systemic infection and preterm births were observed in MCPT4-deficient mice vs. MCPT4 sufficient mice. We further observed that proteolytic cleavage of the host extracellular matrix protein fibronectin by peritoneal cell-derived mast cell (PCMC) lysates diminished GBS adherence. Consistent with this observation, the increase in GBS dissemination and preterm births observed in MCPT4-deficient mice was abolished when GBS were deficient in expression of the fibronectin binding protein, SfbA.

Conclusions—Taken together, our results suggest that the protective effect of MCPT4 against GBS dissemination and preterm labor can in part be attributed to MCPT4-mediated proteolysis of fibronectin. Our studies reveal a novel role of mast cells in defense against bacterial infections.

Keywords

Group B Streptococcus; mast cells; proteases; mouse mast cell protease 4; chymase; fibronectin

INTRODUCTION

Group B Streptococcus (GBS) or *Streptococcus agalactiae* are β-hemolytic, Gram-positive cocci that commonly reside as commensal organisms in the lower genital tract of adult women. However, GBS infections during pregnancy lead to preterm births, stillbirths or infections in human neonates. In human newborns, GBS infection is associated with pneumonia, bacteremia, sepsis, and meningitis, and these infections have a 10% mortality rate.[1] Morbidities due to GBS infection include delayed development, vision and hearing loss, chronic lung disease, mental retardation, and cerebral palsy.[2] Despite the success of intrapartum antibiotic prophylaxis (IAP) to prevent GBS transmission from colonized mothers to the neonate during labor and delivery, the burden of fulminant, early onset GBS sepsis due to *in utero* infections remains substantial.[3, 4] Additionally, there is no effective therapy to prevent GBS-related stillbirths or preterm births.[3, 4] A better understanding of the nature of GBS virulence factors and their interactions with the host immune system is essential for therapeutic developments.

Mast cells are innate immune cells that are widely distributed in tissues especially at the interface with the external environment. Mast cells are derived from hematopoietic progenitor cells and are best known for their role in IgE-associated allergic disorders.[5] In addition to such detrimental roles, mast cells have a variety of beneficial functions since they induce immune responses that promote host resistance to certain bacterial infections.[5, 6] We recently reported that mast cell degranulation can contribute to the host defense against

systemic GBS infection.[7] However, the mechanisms by which mast cells enhance host resistance to GBS infections remain unclear.

The defining morphological feature of mast cells is their electron-dense secretory granules, which contain large amounts of pre-formed mediators such as biogenic amines, proteoglycans, and cytokines. [8] These granules also contain several mast cell-specific proteases—chymase, tryptase, and mast cell carboxypeptidase A3 (CPA3)—whose release is induced by either IgE-dependent mast cell activation [8] or IgE-independent mechanisms.[9, 10] The overall substrate specificities of the mast cell proteases, in particular that of chymase have been conserved over 200 million years of mammalian evolution.[11] This suggests the presence of a strong selective pressure for maintaining chymase specificity and an important role for mast cell proteases in innate immunity. Based on these notions, we here examined whether mast cell chymase provides protection against GBS infections.

Using mice deficient in MCPT4, the mouse functional homolog of human chymase,[12] we demonstrate that mast cell chymase decreases the severity of systemic GBS infection and rates of preterm birth. These can in part, be attributed to the ability of chymase to downregulate GBS-extracellular matrix (ECM) interactions via proteolytic degradation of fibronectin. These results provide novel insights into mechanisms that can decrease the incidence of GBS infections in humans.

METHODS

Ethics statement

All animal experiments were approved by the Seattle Children's Research Institutional Animal Care and Use Committee (protocols #13311 and #13907) and performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (8th Edition).

Mice

Mast cell protease (MCPT)4-deficient mice (*Mcpt4*$^{-/-}$)[12] were backcrossed with C57BL/6J mice to produce *Mcpt4*$^{-/-}$ mice in the C57BL/6J background. Mice were bred and maintained at the Seattle Children's Research Institute Vivarium. Wild type C57BL/6J mice (*Mcpt4*$^{+/+}$) were purchased from Jackson Laboratories and mated in house for pregnancy as needed.

Bacterial isolates

The wild-type (WT) GBS strain COH1 used in this study is a clinical isolate obtained from infected human newborns.[13] The Δ*cylE*, Δ*covR*, and Δ*covR*Δ*cylE* mutants were previously derived from COH1.[14–16] The GBSΔ*sfbA* mutant was derived from WT GBS COH1 and was previously described.[17] Strains were grown in tryptic soy broth (TSB; Difco Laboratories) at 30° or 37°C in 5% CO_2. Cell growth was monitored at 600 nm.

GBS inoculation

For GBS-induced preterm birth, we utilized a previously established intrauterine model of inoculation[18] rather than a vaginal model of inoculation to minimize discrepancies that can be attributed to differences in mouse vaginal persistence.[19] On day E14.5 of pregnancy, dams were inoculated with 10^7 CFU of GBS (in 100 μl) into the right horn of the uterus between the first (P1) and second (P2) fetal sacs most proximal to the cervix as described.[18, 20, 21] Mice were monitored for 72 h for signs of preterm birth (i.e., vaginal bleeding with pup in birth canal or in cage). At either 72 h or at the onset of delivery (whichever occurred first), fetal survival rates were determined and tissue was collected to assess bacterial load.

For GBS systemic infection, 9- to 12-week-old mice were intravenously injected with 5×10^7 CFU of either WT GBS (COH1) or isogenic GBSΔ*sfbA*. In a separate set of experiments, C57BL/6J mice were administered i.p. with a dose of 20 mg/kg of chymostatin/mouse or vehicle control (DMSO) starting one day prior to WT GBS i.v. inoculation and then every day until euthanasia for a total of three injections. Blood, lungs and spleens were collected aseptically at 48 h post infection from the inoculated mice, tissues were homogenized in sterile PBS and GBS burden was estimated as described.[7]

Other methods

See the Methods section in this article Online Repository www.jacionline.org. for the methods for flow cytometry, generation of PCMCs, measurement of chymotrypsin-like activity from mast cells, assessment of mast cell bactericidal activity, production of reactive oxygen species (ROS) in neutrophils, fibronectin degradation by mast cells and human recombinant chymase and fibronectin binding assays.

Statistical analyses

Statistical tests were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA). SciStatCalc was used for the Barnard's test in which outcome 1 was defined as the occurrence of preterm birth, outcome 2 was the absence of it; categories 1 and 2 were *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice, respectively. Data were analyzed for statistical significance using the Mann Whitney *U*-test, ANOVA or Barnard's test. $P < 0.05$ was considered statistically significant.

RESULTS

Mast cells release MCPT4, a protease with chymotrypsin-like activity, upon GBS exposure

It is unknown whether GBS can induce mast cells to release chymase and/or other proteases with chymotrypsin-like activity. To test this hypothesis, peritoneal cell-derived mast cells (PCMCs) were exposed to ~10^6 CFU of wild type (WT) GBS COH1 (belonging to capsular serotype III, MLST-ST17 clone associated with severe neonatal infections[13, 22,23]). Chymotrypsin-like activity in supernatants and cell lysates from GBS-stimulated mast cells was measured using a previously described chymotryptic activity assay.[24] Our results shown in Fig. 1A indicate that the mildly hemolytic WT GBS strain COH1 induced mast cells to release chymase. The Ca^{2+} ionophore A23187-stimulation was used as a positive control in these assays. Release of chymotrypsin-like activity was also detected when mast cells were

exposed to a hyperpigmented GBS strain (Δ*covR*), which was previously shown to induce mast cell degranulation and release of β-hexosaminidase.[7] However, even non-hemolytic GBS strains (Δ*cylE* and Δ*covR*Δ*cylE*) induced the release of chymotrypsin-like activity from mast cells (Suppl. Fig. E1). Taken together, these data suggest that GBS induces mast cells to release a protease with chymotrypsin-like activity by a mechanism that is independent of hemolytic pigment expression.

We targeted MCPT4, as it has been shown that this protease is the major source for chymotrypsin-like activity in peritoneal cells and ear skin tissue and is the functional mouse homolog to human chymase.[12] Accordingly, chymotrypsin-like activity was not detected in supernatants and cells from PCMC-deficient MCPT4 (*Mcpt4*$^{-/-}$) that were exposed to GBS or the positive control (Ca^{2+} ionophore A23187) (Suppl. Fig. E2). Collectively, these data confirm that GBS triggers PCMCs to release MCPT4, a protease with chymotrypsin-like activity.

MCPT4 decreases adverse birth outcomes associated with GBS infections

Next, we examined if the presence or absence of MCPT4 affects the severity of GBS infections. To this end, we used *Mcpt4*$^{-/-}$ mice, which do not exhibit any marked defect in the expression of proteases with trypsin-like or CPA activity, *e.g.* MCPT6 or CPA3.[12] We first examined if MCTP4 deficiency affected GBS infection associated preterm births using an *in utero* infection model that we previously characterized.[18] In brief, mice on day 14–15 of pregnancy (E14.5) were inoculated *in utero* with approximately 10^7 CFU of WT GBS as described[18] (see Fig. 1B for scheme of *in utero* inoculation). The mice were then monitored for signs of preterm birth (defined as vaginal bleeding or pups in cage) for up to 72 h post-inoculation. Upon signs of preterm birth or at 72 h post inoculation (whichever occurred first), the mice were euthanized, a midline laparotomy was performed, and *in utero* fetal death (IUFD) was recorded prior to subsequent analysis. We observed that the number of dams exhibiting preterm birth was significantly greater in *Mcpt4*$^{-/-}$ mice compared with *Mcpt4*$^{+/+}$ mice that were infected with GBS (Fig. 1C). However, there was no significant difference in bacterial load between fetuses of GBS infected *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice (Fig. 1D). We therefore predicted that MCPT4 confers protective effects to the pregnant dam rather than the fetuses. Consistent with this hypothesis, we found that the GBS burden was significantly increased in the spleens of pregnant *Mcpt4*$^{-/-}$ vs. *Mcpt4*$^{+/+}$ mice (Fig. 1G). Together, these data demonstrate that MCPT4 plays an important role in decreasing the incidence of GBS infection associated preterm births.

MCPT4 decreases bacterial dissemination during systemic GBS infection

To confirm that MCPT4 contributes to decreased systemic GBS infection in adult mice we used a previously described murine model.[7] Briefly, MCPT4 sufficient and deficient mice were intravenously injected with GBS and bacterial burden was estimated in various tissues at 48h post infection (see Materials and Methods). As shown in Figs. 2A–C increased GBS CFUs were observed in the blood, spleens and lungs of *Mcpt4*$^{-/-}$ mice when compared with *Mcpt4*$^{+/+}$ mice. Increased bacterial loads were also observed when using a pharmacological approach in which MCPT4 sufficient mice were treated before and during GBS systemic infection with chymostatin, a selective inhibitor of serine proteases with chymotryptic

activity (Suppl. Fig. E3). Although previous studies have suggested that chymase can mobilize inflammatory cells,[25] neutrophil numbers in the spleens of *Mpct4*$^{-/-}$ mice were not significantly different from those of *Mcpt4*$^{+/+}$ mice at 48h after GBS administration (Fig. 2D). Together, these results indicate that MCPT4 deficiency increases GBS dissemination during systemic infection independent of neutrophil recruitment.

Chymase and MCPT4 are not antimicrobial to GBS

Mast cell proteases with chymotryptic activity have been suggested to be antimicrobial to certain bacterial species.[26] Therefore, we hypothesized that MCPT4 may directly kill GBS and thereby contribute to bacterial clearance. To test this hypothesis, GBS were exposed to PCMCs generated from MCPT4 sufficient and deficient mice and bacterial survival was enumerated after 4h of exposure. The results shown in Fig. 3A indicate that survival of GBS was similar in the presence of PCMCs from *Mcpt4*$^{-/-}$ and *Mcpt4*$^{+/+}$ mice. Further, addition of recombinant human chymase at increasing concentrations did not lead to reduced bacterial survival (Fig. 3A). These data indicate that chymase and MCPT4 do not have a direct bactericidal effect on GBS.

Chymase does not impact generation of reactive oxygen species (ROS) in neutrophils

As chymase can also influence the function of inflammatory cells,[25, 27, 28] we examined if chymase influences neutrophil ROS generation. To test this, human neutrophils were pre-treated with dihydrorhodamine (DHR) and oxidation to fluorescent monohydrorhodamine (MHR) was monitored by flow cytometry immediately and at 1h post incubation with GBS, either in the presence or absence of human chymase. As shown in Fig. 3B, production of ROS by human neutrophils upon exposure to GBS was not significantly impacted by the presence or absence of human chymase. These data together with the *in vivo* observations (Fig. 2D) indicate that chymase does not influence neutrophil function and that MCPT4 does not influence neutrophil recruitment to GBS-infected organs, such as the spleen.

MCPT4 prevents systemic GBS dissemination and preterm birth by disrupting GBS interactions with the extracellular matrix

Previous studies have shown that murine[29] and rat[30] proteases with chymotryptic activity can be secreted by mast cells by an unknown mechanism that may involve piecemeal degranulation. Potentially, such released mast cell proteases can influence both the amount and function of mediators under homeostatic conditions. In line with this notion, fibronectin was proposed to be one of the main physiological targets for chymase[31] and MCPT4 during baseline conditions.[12] Adherence and colonization of bacteria to host cells is a critical first step in GBS pathogenesis. A number of factors expressed on the GBS surface enable the pathogen to bind to ECM proteins, [32] including adhesion factors such as the laminin-binding factor LmbB,[33] the fibrinogen binding proteins FbsA and FbsB,[34, 35] and the fibronectin binding factors BsaB[36] and SfbA.[17] GBS strains deficient in these adhesins exhibit attenuated virulence. Therefore, we hypothesized that MCPT4-mediated proteolysis of fibronectin could impair the ability of GBS to bind to the ECM for subsequent dissemination. To test this hypothesis, we first assessed whether MCPT4 impairs GBS-fibronectin interactions. As shown in Figs. 4A, full length fibronectin was completely degraded by cell lysates generated from *Mcpt4*$^{+/+}$ or *Mcpt4*$^{-/-}$ PCMCs. However, our data

show that MCPT4 is required for the complete degradation of the fragments generated from full length fibronectin. Figs. 4A shows that a fibronectin fragments of approximately 160 kDa and 125 kDa are present in higher amounts in the digests generated by fibronectin incubation with PCMC lysates from *Mcpt4*$^{-/-}$ mice than in the digests generated by fibronectin incubation with PCMC lysates from *Mcpt4*$^{+/+}$ mice. Similar to MCPT4, human recombinant chymase also degraded full length fibronectin and its fragments (Fig. 4C).

Then, we investigated whether MCPT4-mediated fibronectin cleavage generates fragments that are impaired in their ability to bind GBS. Increased GBS CFU were recovered from microtiter wells coated with fibronectin that has been pre-incubated with lysates from *Mcpt4*$^{-/-}$ PCMC when compared with fibronectin that had been pre-incubated with lysates from *Mcpt4*$^{+/+}$ PCMC (Figs. 5A and 5B) indicating that MCPT4 can reduce GBS adherence to fibronectin via proteolytic cleavage of this extracellular matrix component.

We think that the reduced fragmentation of fibronectin by *Mcpt4*$^{-/-}$ PCMCs is not a consequence of an off-target effect of the mutation in MCPT4 because similar results were obtained by adding chymostatin to *Mcpt4*$^{+/+}$ PCMC lysates to inhibit chymotryptic activity (Suppl. Figs. E4A and E4B). Furthermore, recombinant human chymase addition to PCMC lysates generated from MCPT4-deficient mice confers these lysates with the ability to degrade fibronectin (Suppl. Fig. E4C).

Overall, these data indicate that chymase degrades fibronectin into smaller fragments that have impaired ability to support GBS adherence.

Mcpt4$^{-/-}$ mice have been reported to exhibit higher fibronectin amounts.[12] Our data suggest that GBS interactions with the ECM might be favored in these mice leading to increased systemic infection and preterm birth. To test this hypothesis, we inoculated MCPT4 sufficient and MCPT4-deficient mice with a GBS strain deficient in expression of the fibronectin binding protein SfbA (GBSΔ*sfbA*[17]). Notably, GBSΔ*sfbA* was as effective as WT GBS in its ability to induce the release of a protease with chymotrypsin-like activity from PCMCs (Suppl. Fig. E5). As shown in Figs. 6A–C, bacterial CFUs in blood, spleens and lungs were significantly lower in *Mcpt4*$^{-/-}$ mice infected with GBSΔ*sfbA* compared to *Mcpt4*$^{-/-}$ infected with WT GBS. No significant differences in bacterial CFUs were observed in *Mcpt4*$^{-/-}$ mice vs. *Mcpt4*$^{+/+}$ mice that were inoculated with GBSΔ*sfbA* or when these groups were compared to *Mcpt4*$^{+/+}$ mice infected with WT GBS. These data indicate that increased amounts of intact fibronectin in *Mcpt4*$^{-/-}$ mice may promote interactions with bacterial fibronectin binding proteins such as SfbA leading to increased GBS dissemination and virulence in contrast to wild type mice where fibronectin is continuously degraded by the action of MCPT4.

We next examined GBS infection associated preterm births in pregnant *Mcpt4*$^{-/-}$ mice infected with GBSΔ*sfbA*. MCPT4-deficient mice infected with GBSΔ*sfbA* did not exhibit signs of preterm births (Fig. 6D). Moreover, CFU counts of GBSΔ*sfbA* in the maternal spleen of *Mcpt4*$^{-/-}$ mice were similar to those in *Mcpt4*$^{+/+}$ mice (Fig. 6E). Taken together, these data indicate that MCPT4-mediated proteolysis of fibronectin contributes to diminished GBS dissemination and preterm births.

DISCUSSION

Mast cells are important effector cells that participate in innate and adaptive immunity.[37] No human has been found who lacks mast cells. Located at strategic sites within epithelial and mucosal surfaces, mast cells perform sentinel roles in combating numerous pathogens, in part, via their exocytosed heparin•protease complexes. [38] By using mice with c-kit independent mast cell deficiency, we recently showed that mast cells are required for an effective immune response during systemic GBS infections.[7] GBS are Gram-positive bacteria that frequently colonize the lower genital tract of healthy women but cause severe infections during pregnancy, leading to preterm birth, stillbirth, or early-onset newborn infections. A critical role for mast cells in the protection against a pathogen that can potentially endanger species propagation, such as GBS, is consistent with the ancient origin of mast cells that precedes the development of adaptive immunity. Despite this observation, we could not identify mast cell mediators and/or mechanisms by which mast cells confer protection against GBS. To address this question, we focused our attention on mast cell-restricted proteases. In mast cells, these proteases can account for up to 35% of the total cellular protein, and the absolute majority of these belong to the chymotrypsin-related serine protease family.[39] The overall substrate specificity of chymase has been conserved for over 200 million years of mammalian evolution.[11] This suggests the presence of a strong selective pressure for maintaining chymase specificity and an important role for this protease in innate immunity. In agreement with this hypothesis, it has been shown that MCPT4, the mouse functional homologue of chymase, [12] can protect against toxicity of certain venoms via proteolytic cleavage. However, much less is known about chymase's role in the protection against bacterial pathogens. In fact, it was shown only recently that MCPT4 can reduce bacterial burden by regulated shedding of bladder epithelial cells in a murine model of urinary tract infection.[40]

Considering that the female genital tract is populated by mast cells that express chymase in their granules,[41] we decided to examine the role of this protease during infection with GBS. For this purpose, we used mice deficient in MCPT4. Using an *in utero* infection model, we found that the number of dams exhibiting preterm birth was significantly greater in $Mcpt4^{-/-}$ mice compared with $Mcpt4^{+/+}$ mice that were infected with GBS (Figs. 1C), with significantly higher bacterial dissemination to the maternal spleen. However, there was no significant difference in bacterial load between the fetuses of GBS infected $Mcpt4^{+/+}$ and $Mcpt4^{-/-}$ mice (Fig. 1D). This observation is consistent with the fact that very low chymase activity levels are observed during embryonic development (E15-E18[42]). These findings indicate that maternal GBS systemic dissemination facilitated by MCPT4 deficiency can contribute to adverse birth outcomes. Notably, maternal sepsis due to GBS has been associated with adverse birth outcomes in humans.[43] We also confirmed MCPT4's ability to decrease systemic GBS dissemination in adult mice with a systemic infection murine model. The next question in our study was to determine how MCPT4 exerts its protective role against GBS. It has been shown that intracellular IL-15 expression in mast cells can transcriptionally limit the amount of MCPT2, resulting in decreased mast cell-associated chymotrypsin-like activity *in vitro*, decreased mast cell antibacterial properties, and reduced survival of mice subjected to the cecal ligation and puncture model of experimental sepsis.[26]

These findings prompted us to examine the possibility that chymase has a direct killing effect against GBS. However, our *in vitro* data with human recombinant chymase or PCMCs that express high levels of chymotrypsin-like activity[44] do not support this hypothesis. Although these observations indicate that chymase does not participate in direct GBS killing, we cannot rule out the possibility that chymase indirectly contributes to GBS clearance by modulating the bactericidal activity of other mediators, such as eotaxins or defensins via proteolytic cleavage.[45]

Neutrophils and macrophages play critical roles in the host immune defense against GBS infection.[27, 28, 46] Moreover, it has been shown that chymase can induce leukocyte migration and activation.[25] Despite these observations, we found no evidence that chymase can enhance myeloid cell function to clear GBS. In fact, chymase did not enhance the ability for neutrophils to produce ROS (Fig. 3B), one of the main neutrophil mediators with GBS-killing activity.[47] Moreover, neutrophil accumulation in the spleen of GBS-infected mice was not affected by MCPT4 deficiency (Fig. 2D).

Constitutively released chymase can have an impact on tissue homeostasis. For example, it has been shown that MCPT4 can have an impact on normal connective tissue homeostasis of the skin,[12] on baseline intestinal barrier function[48] and on bone mass.[49] Mechanistically, these effects can be attributed to chymase's proteolytic activity when a substrate associated with the disruption of the homeostatic function can be identified. *In vitro* data with MCPT4-deficient cells have shown that fibronectin may be one of the main physiological targets for chymase. [12] This observation is relevant to our understanding of how chymase protects against GBS dissemination since the GBS surface enables the pathogen to bind to ECM proteins, partially via fibronectin binding factors such as BsaB[36] and SfbA. Accordingly, we demonstrated that MCPT4 degrades fibronectin to fragments with a reduced ability to bind GBS.

We were able to rescue MCPT4-deficient mice from systemic infection and high preterm birth rates when the mice were infected with a mutant strain of GBS that does not express SfbA. As far as we know, this is the first evidence revealing that MCPT4-fibronectin interactions can have an impact on host immunity against bacterial infections. However, our findings do not preclude additional mechanisms by which chymase or SfbA can influence GBS disease severity (*e.g.* chymase mediated proteolytic degradation of other targets and/or SfbA binding to other ECM components).

In summary, we demonstrate that MCTP4 is critical to prevent GBS infection-associated disease severity. Given that maternal sepsis can predispose infants to adverse outcomes, our studies suggest a beneficial role of chymase in decreasing maternal GBS sepsis and infant morbidity. It has been suggested that mast cell proteases can promote inflammation during bacterial infections via activation of pro-inflammatory mediators by limited proteolysis. However, several studies also indicate that mast cell proteases can inactivate the same pro-inflammatory mediators via degradation. Our findings in this study point at a different mechanism whereby mast cells release chymase to reduce host susceptibility to a systemic bacterial infection. As a consequence, a more limited inflammatory response will be required to clear the infection, thereby reducing the risks associated with hyper-

inflammation and sepsis in the pregnant dam, which can lead to increased risk of preterm birth. Hence, our findings provide novel insight into a mechanism by which mast cells contribute to innate defense against bacterial infection. As an extension of our findings, we envision future therapeutic strategies by which the antibacterial action of chymase is exploited.

METHODS

Flow cytometry

Single cell suspensions were stained with the viability marker FVS510 (BD Biosciences) for 15 min on ice. Then, the cells were blocked with unconjugated anti-CD16/CD32 for 10 min and were stained with allophycocyanin-labeled anti-CD11b (2 μg/ml; clone M1/70, BD Biosciences) and phycoerythrin-labeled Ly6G antibodies (1 μg/ml; clone 1A8, BD Biosciences) for 15 min. Cells were fixed overnight with 1% paraformaldehyde. Cells were collected using LSR II instrument (BD Bioscience) with FACSDiva software and analyzed using FlowJo (software version 8.8.7, Tree Star).

Generation of peritoneal cell-derived mast cells (PCMCs)

Peritoneal cells from *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice were maintained *in vitro* for 3–8 weeks in medium containing 10ng/ml IL-3 (Miltenyi Biotec, Auburn, CA) and 50 ng/ml SCF (Miltenyi Biotec) until the mast cells represented >95% of the total non-adherent cells according to May-Grünwald-Giemsa staining.

Measurement of chymotrypsin-like activity from mast cells

About 10^5 PCMCs were exposed to GBS WT COH1 or isogenic Δ*sfbA,* Δ*cylE,* Δ*covR,* and Δ*covR*Δ*cylE,* and supernatants and cells were assessed for chymotrypsin-like activity levels. Briefly, the GBS strains indicated above were grown to an optical density at 600 nm (OD_{600}) of 0.3 and washed in PBS. 10^6 CFU were incubated with the PCMCs at a multiplicity of infection (MOI) of 10 for 4 h at 37ºC in DMEM+10%FBS. The calcium ionophore A23187 was used as a positive control and used at a concentration of 5 μM (2.5 ng/μl). Chymotrypsin-like activity was assessed using methods described.[1] Briefly, the cells were centrifuged and the NaCl concentration of the first supernatant (S1) was adjusted to 1M. The cell pellets were resuspended in fresh DMEM+10%FBS containing 1M NaCl, and the samples were centrifuged again. After removing the second supernatant (S2), the cells were resuspended again in DMEM+10%FBS containing 1M NaCl and 0.5% Triton X-100 to lyse the cell pellets (P). The S1, S2 and P samples were sonicated for 4 sec using a W-385 ultrasonic processor (Heat systems, Ultrasonics inc) that is equipped with a microprobe. The chymotrypsin-like activity was then assessed by addition of the substrate CS-PSA (DiaPharma) at a final concentration of 360 μM, and by monitoring the change in absorbance at 405 nm. The % activity released was calculated using the following formula: (S1+S2)/(S1+S2+P)*100.

Assessment of mast cell bactericidal activity

GBS WT (COH1) were grown to OD 0.3 and washed 2 times with PBS. Approximately 1.5×10^4 GBS CFU were added to 1.5×10^5 PCMCs (MOI=0.1) and incubated for 4 h at 37ºC

in DMEM+10%FBS. The same amount of bacteria was also treated with 0.1, 0.5 or 2.5 μg of the human recombinant chymase (catalog number C8118, Sigma). Surviving bacteria were enumerated at the end of the experiment by plating 10-fold serial dilutions on TSA. Survival indexes (SI) were calculated as the ratio of the CFU recovered after incubation with either PCMCs or human chymase to the CFU recovered in media only.

Production of reactive oxygen species (ROS) in neutrophils

Neutrophils were isolated from fresh human adult blood as described previously.[2] Briefly, approximately 10 ml of blood was collected from independent healthy human donors in EDTA tubes (BD Bioscience). The MACSxpress neutrophil isolation kit was used to isolate the neutrophils, as per manufacturer's instructions (Miltenyi Biotec). The cells were then pelleted and the red blood cells were lysed in 0.15 M NH_4Cl, 1mM $NaHCO_3$. For ROS generation studies, neutrophils were washed and resuspended in with Hanks Buffered Salt Solution (HBSS) at a density of 1×10^6 cells/ml. They were incubated with dihydrorhodamine 123 (catalog number D1054, Sigma) at a concentration of 84 μM at 37°C for 20 min. Oxidation to fluorescent hydrorhodamine 123 was monitored by flow cytometry immediately and at 1h post incubation with bacteria (MOI = 100) at 37°C. PMA (phorbol-myristate-acetate) was used at a concentration of 20 nM, and 0.5 μg human chymase were used for the corresponding treatments.

Fibronectin degradation by mast cells and human recombinant chymase

Given its high degree of homology between mouse and human fibronectin, the latter was used for the *in-vitro* studies. BLAST results indicated that both human and mouse fibronectin have protein sequences that contain 2477 amino acids (blast.ncbi.nlm.nih.gov) with 91% identical homology (2251/2461 identical amino acids) and 96% positive homology (2363/2461 amino acids with similar physical-chemical properties).

Two micrograms of human plasma fibronectin (catalog number F2006, Sigma) were incubated with 63.5 ng of human recombinant chymase or PCMC lysates for 6 h at 37°C. For the preparation of PCMC lysates, approximately 2.5×10^5 PCMCs obtained from either *Mcpt4*$^{+/+}$ or *Mcpt4*$^{-/-}$ mice were lysed in 2.5 μl of Tyrode's buffer containing 0.1% Tx-100.

In a separate set of experiments, chymostatin (1mM) was added to *Mcpt4*$^{+/+}$ PCMCs and human recombinant chymase (500ng) was added to *Mcpt4*$^{-/-}$ PCMCs to rule out potential off-target effects of the *Mcpt4* mutation. Fibronectin and its degradation fragments generated by MCPT4 proteolytic activity (160kDa, 140kDa, 125kDa and 110kDa) were visualized by western blot analysis using a fibronectin rabbit polyclonal antibody (Ab2413, Abcam). Band intensity was determined by western blot densitometry analysis using NIH Image J. Fibronectin fragment abundance relative to abundance of full length fibronectin was calculated using the following formula: Intensity of a band that corresponds to fibronectin fragment/Intensity of full length fibronectin (220 kDa) *100.

Fibronectin binding assays

The effect of mouse MCPT4 on GBS-fibronectin interactions was assessed using a modified version of a protocol previously described.[3] Briefly, a 96 well plate was coated overnight at

4°C with either intact fibronectin or fibronectin fragments (obtained by incubation of 5 μg fibronectin with the PCMC lysate preparations described above). WT GBS COH1 was grown to O.D 0.3 and washed 2 times with PBS. The fibronectin-coated plates were washed 3 times with PBS and approximately 10^5 CFU of GBS WT COH1 were added for 30 min at 37°C. The plates were then washed 3 times and the adherent bacteria were recovered by trypsinization for 10 min at 37°C. Bacteria were enumerated using 10-fold serial dilutions and plating.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work was supported by funding from the National Institutes of Health; Grants R01AI100989, R21AI109222 and R01AI112619 to L.R., Grant R01HL113351 to A.P and Grant R01NS051247 to K.S.D. The NIH training grant T32 HD007233 (PI: Lisa Frenkel) supported E. B. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

We thank Michelle Coleman for technical assistance. We also thank Jay Vornhagen and Kristina Adams Waldorf for insightful discussions.

Abbreviations used

GBS	Group B Streptococcus
IAP	intrapartum antibiotic prophylaxis
CPA3	mast cell carboxypeptidase A
ECM	extracellular matrix
MCPT	mast cell protease
WT	wild type
CFU	colony forming units
PCMCs	peritoneal cell-derived mast cells
SCF	stem cell factor
MOI	multiplicity of infection
SI	survival indexes
ROS	reactive oxygen species

References

1. Edmond KM, Kortsalioudaki C, Scott S, Schrag SJ, Zaidi AK, Cousens S, et al. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet. 2012; 379:547–56. [PubMed: 22226047]

2. Ledger WJ. Perinatal infections and fetal/neonatal brain injury. Current Opinion in Obstetrics and Gynecology. 2008; 20:120–4. [PubMed: 18388810]

3. Verani JR, McGee L, Schrag SJ. Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. MMWR Recomm Rep. 2010; 59:1–36.

4. Weston EJ, Pondo T, Lewis MM, Martell-Cleary P, Morin C, Jewell B, et al. The Burden of Invasive Early-onset Neonatal Sepsis in the United States, 2005–2008. Pediatric Infectious Disease Journal. 2011; 30:937–41. [PubMed: 21654548]

5. Galli SJ, Tsai M. Mast cells in allergy and infection: versatile effector and regulatory cells in innate and adaptive immunity. Eur J Immunol. 2010; 40:1843–51. [PubMed: 20583030]

6. Johnzon CF, Ronnberg E, Pejler G. The Role of Mast Cells in Bacterial Infection. Am J Pathol. 2016; 186:4–14. [PubMed: 26477818]

7. Gendrin C, Vornhagen J, Ngo L, Whidbey C, Boldenow E, Santana-Ufret V, et al. Mast cell degranulation by a hemolytic lipid toxin decreases GBS colonization and infection. Sci Adv. 2015; 1:e1400225. [PubMed: 26425734]

8. Pejler G, Ronnberg E, Waern I, Wernersson S. Mast cell proteases: multifaceted regulators of inflammatory disease. Blood. 2010; 115:4981–90. [PubMed: 20233968]

9. Maurer M, Wedemeyer J, Metz M, Piliponsky AM, Weller K, Chatterjea D, et al. Mast cells promote homeostasis by limiting endothelin-1-induced toxicity. Nature. 2004; 432:512–6. [PubMed: 15543132]

10. Metz M, Piliponsky AM, Chen CC, Lammel V, Abrink M, Pejler G, et al. Mast cells can enhance resistance to snake and honeybee venoms. Science. 2006; 313:526–30. [PubMed: 16873664]

11. Reimer JM, Enoksson M, Samollow PB, Hellman L. Extended substrate specificity of opossum chymase--implications for the origin of mast cell chymases. Mol Immunol. 2008; 45:2116–25. [PubMed: 18022236]

12. Tchougounova E, Pejler G, Abrink M. The chymase, mouse mast cell protease 4, constitutes the major chymotrypsin-like activity in peritoneum and ear tissue. A role for mouse mast cell protease 4 in thrombin regulation and fibronectin turnover. J Exp Med. 2003; 198:423–31. [PubMed: 12900518]

13. Martin TR, Rubens CE, Wilson CB. Lung antibacterial defense mechanisms in infant and adult rats: implications for the pathogenesis of group B streptococcal infections in the neonatal lung. J Infect Dis. 1988; 157:91–100. [PubMed: 3275727]

14. Whidbey C, Harrell MI, Burnside K, Ngo L, Becraft AK, Iyer LM, et al. A hemolytic pigment of Group B Streptococcus allows bacterial penetration of human placenta. J Exp Med. 2013; 210:1265–81. [PubMed: 23712433]

15. Rajagopal L, Vo A, Silvestroni A, Rubens CE. Regulation of cytotoxin expression by converging eukaryotic-type and two-component signalling mechanisms in Streptococcus agalactiae. Mol Microbiol. 2006; 62:941–57. [PubMed: 17005013]

16. Lembo A, Gurney MA, Burnside K, Banerjee A, de los Reyes M, Connelly JE, et al. Regulation of CovR expression in Group B Streptococcus impacts blood-brain barrier penetration. Mol Microbiol. 2010; 77:431–43. [PubMed: 20497331]

17. Mu R, Kim BJ, Paco C, Del Rosario Y, Courtney HS, Doran KS. Identification of a group B streptococcal fibronectin binding protein, SfbA, that contributes to invasion of brain endothelium and development of meningitis. Infect Immun. 2014; 82:2276–86. [PubMed: 24643538]

18. Whidbey C, Vornhagen J, Gendrin C, Boldenow E, Samson JM, Doering K, et al. A streptococcal lipid toxin induces membrane permeabilization and pyroptosis leading to fetal injury. EMBO Mol Med. 2015; 7:488–505. [PubMed: 25750210]

19. Patras KA, Wang NY, Fletcher EM, Cavaco CK, Jimenez A, Garg M, et al. Group B Streptococcus CovR regulation modulates host immune signalling pathways to promote vaginal colonization. Cell Microbiol. 2013; 15:1154–67. [PubMed: 23298320]

20. Hirsch E, Saotome I, Hirsh D. A model of intrauterine infection and preterm delivery in mice. Am J Obstet Gynecol. 1995; 172:1598–603. [PubMed: 7538729]

21. Elovitz MA, Wang Z, Chien EK, Rychlik DF, Phillippe M. A new model for inflammation-induced preterm birth: the role of platelet-activating factor and Toll-like receptor-4. Am J Pathol. 2003; 163:2103–11. [PubMed: 14578208]

22. Musser JM, Mattingly SJ, Quentin R, Goudeau A, Selander RK. Identification of a high-virulence clone of type III Streptococcus agalactiae (group B Streptococcus) causing invasive neonatal disease. Proc Natl Acad Sci U S A. 1989; 86:4731–5. [PubMed: 2660146]

23. Pritzlaff CA, Chang JC, Kuo SP, Tamura GS, Rubens CE, Nizet V. Genetic basis for the beta-haemolytic/cytolytic activity of group B Streptococcus. Mol Microbiol. 2001; 39:236–47. [PubMed: 11136446]

24. Schwartz LB, Riedel C, Caulfield JP, Wasserman SI, Austen KF. Cell association of complexes of chymase, heparin proteoglycan, and protein after degranulation by rat mast cells. J Immunol. 1981; 126:2071–8. [PubMed: 6164718]

25. Tani K, Ogushi F, Kido H, Kawano T, Kunori Y, Kamimura T, et al. Chymase is a potent chemoattractant for human monocytes and neutrophils. J Leukoc Biol. 2000; 67:585–9. [PubMed: 10770293]

26. Orinska Z, Maurer M, Mirghomizadeh F, Bulanova E, Metz M, Nashkevich N, et al. IL-15 constrains mast cell-dependent antibacterial defenses by suppressing chymase activities. Nat Med. 2007; 13:927–34. [PubMed: 17643110]

27. Christensen RD, Rothstein G. Efficiency of neutrophil migration in the neonate. Pediatr Res. 1980; 14:1147–9. [PubMed: 7465286]

28. Currie AJ, Curtis S, Strunk T, Riley K, Liyanage K, Prescott S, et al. Preterm infants have deficient monocyte and lymphocyte cytokine responses to group B streptococcus. Infect Immun. 2011; 79:1588–96. [PubMed: 21300777]

29. Scudamore CL, Pennington AM, Thornton E, McMillan L, Newlands GF, Miller HR. Basal secretion and anaphylactic release of rat mast cell protease-II (RMCP-II) from ex vivo perfused rat jejunum: translocation of RMCP-II into the gut lumen and its relation to mucosal histology. Gut. 1995; 37:235–41. [PubMed: 7557574]

30. Wastling JM, Scudamore CL, Thornton EM, Newlands GF, Miller HR. Constitutive expression of mouse mast cell protease-1 in normal BALB/c mice and its up-regulation during intestinal nematode infection. Immunology. 1997; 90:308–13. [PubMed: 9135562]

31. Okumura K, Takai S, Muramatsu M, Katayama S, Sakaguchi M, Kishi K, et al. Human chymase degrades human fibronectin. Clin Chim Acta. 2004; 347:223–5. [PubMed: 15313162]

32. Vornhagen J, Adams Waldorf KM, Rajagopal L. Perinatal Group B Streptococcal Infections: Virulence Factors, Immunity, and Prevention Strategies. Trends Microbiol. 2017

33. Spellerberg B, Rozdzinski E, Martin S, Weber-Heynemann J, Schnitzler N, Lutticken R, et al. Lmb, a protein with similarities to the LraI adhesin family, mediates attachment of Streptococcus agalactiae to human laminin. Infect Immun. 1999; 67:871–8. [PubMed: 9916102]

34. Jacobsson K. A novel family of fibrinogen-binding proteins in Streptococcus agalactiae. Vet Microbiol. 2003; 96:103–13. [PubMed: 14516712]

35. Schubert A, Zakikhany K, Schreiner M, Frank R, Spellerberg B, Eikmanns BJ, et al. A fibrinogen receptor from group B Streptococcus interacts with fibrinogen by repetitive units with novel ligand binding sites. Mol Microbiol. 2002; 46:557–69. [PubMed: 12406229]

36. Jiang S, Wessels MR. BsaB, a novel adherence factor of group B Streptococcus. Infect Immun. 2014; 82:1007–16. [PubMed: 24343649]

37. Galli SJ, Nakae S, Tsai M. Mast cells in the development of adaptive immune responses. Nat Immunol. 2005; 6:135–42. [PubMed: 15662442]

38. Wong GW, Zhuo L, Kimata K, Lam BK, Satoh N, Stevens RL. Ancient origin of mast cells. Biochem Biophys Res Commun. 2014; 451:314–8. [PubMed: 25094046]

39. Hellman L, Thorpe M. Granule proteases of hematopoietic cells, a family of versatile inflammatory mediators - an update on their cleavage specificity, in vivo substrates, and evolution. Biol Chem. 2014; 395:15–49. [PubMed: 23969467]

40. Choi HW, Bowen SE, Miao Y, Chan CY, Miao EA, Abrink M, et al. Loss of Bladder Epithelium Induced by Cytolytic Mast Cell Granules. Immunity. 2016; 45:1258–69. [PubMed: 27939674]

41. Theoharides TC, Stewart JM. Genitourinary mast cells and survival. Transl Androl Urol. 2015; 4:579–86. [PubMed: 26813805]

42. Combs JW, Lagunoff D, Benditt EP. Differentiation and proliferation of embryonic mast cells of the rat. J Cell Biol. 1965; 25:577–92. [PubMed: 4284572]

43. Kalin A, Acosta C, Kurinczuk JJ, Brocklehurst P, Knight M. Severe sepsis in women with group B Streptococcus in pregnancy: an exploratory UK national case-control study. BMJ Open. 2015; 5:e007976.

44. Malbec O, Roget K, Schiffer C, Iannascoli B, Dumas AR, Arock M, et al. Peritoneal cell-derived mast cells: an in vitro model of mature serosal-type mouse mast cells. J Immunol. 2007; 178:6465–75. [PubMed: 17475876]

45. Gela A, Kasetty G, Jovic S, Ekoff M, Nilsson G, Morgelin M, et al. Eotaxin-3 (CCL26) exerts innate host defense activities that are modulated by mast cell proteases. Allergy. 2015; 70:161–70. [PubMed: 25377782]

46. Boldenow E, Gendrin C, Ngo L, Bierle C, Vornhagen J, Coleman M, et al. Group B Streptococcus Circumvents Neutrophils and Neutrophil Extracellular Traps during Amniotic Cavity Invasion and Preterm Labor. Sci Immunol. 2016:1.

47. LeVine AM, Reed JA, Kurak KE, Cianciolo E, Whitsett JA. GM-CSF-deficient mice are susceptible to pulmonary group B streptococcal infection. J Clin Invest. 1999; 103:563–9. [PubMed: 10021465]

48. Groschwitz KR, Ahrens R, Osterfeld H, Gurish MF, Han X, Abrink M, et al. Mast cells regulate homeostatic intestinal epithelial migration and barrier function by a chymase/Mcpt4-dependent mechanism. Proc Natl Acad Sci U S A. 2009; 106:22381–6. [PubMed: 20018751]

49. Lind T, Gustafson AM, Calounova G, Hu L, Rasmusson A, Jonsson KB, et al. Increased Bone Mass in Female Mice Lacking Mast Cell Chymase. PLoS One. 2016; 11:e0167964. [PubMed: 27936149]

REFERENCES

1. Schwartz LB, Riedel C, Caulfield JP, Wasserman SI, Austen KF. Cell association of complexes of chymase, heparin proteoglycan, and protein after degranulation by rat mast cells. J Immunol. 1981; 126:2071–8. [PubMed: 6164718]

2. Boldenow E, Gendrin C, Ngo L, Bierle C, Vornhagen J, Coleman M, et al. Group B Streptococcus Circumvents Neutrophils and Neutrophil Extracellular Traps during Amniotic Cavity Invasion and Preterm Labor. Sci Immunol. 2016:1.

3. Mu R, Kim BJ, Paco C, Del Rosario Y, Courtney HS, Doran KS. Identification of a group B streptococcal fibronectin binding protein, SfbA, that contributes to invasion of brain endothelium and development of meningitis. Infect Immun. 2014; 82:2276–86. [PubMed: 24643538]

Clinical implications

Future therapeutic strategies based on the antibacterial action of chymase may be useful to decrease GBS systemic infections and preterm birth.

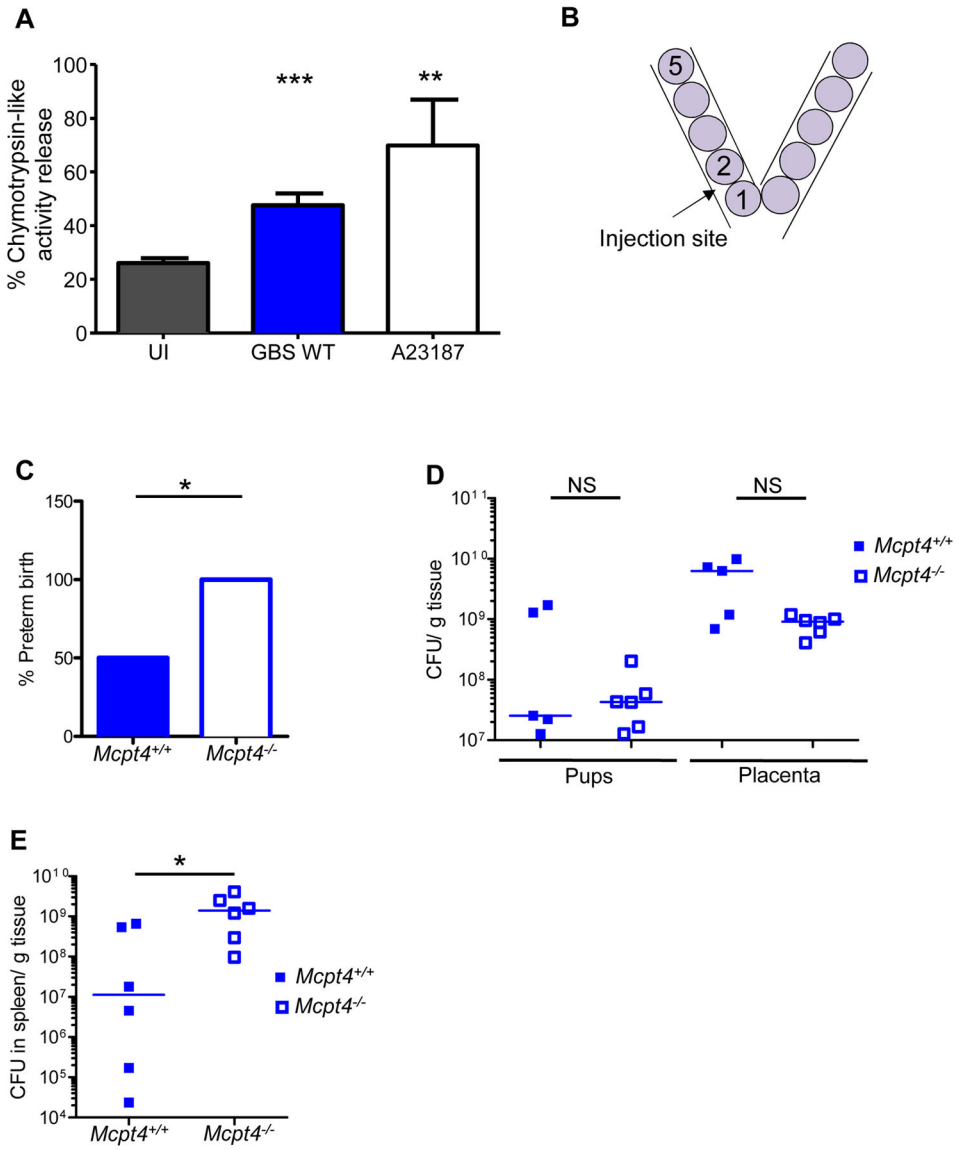

Figure 1. GBS induces the release of a mast cell protease with chymotrypsin-like activity and MCPT4-deficient mice exhibit increased rates of GBS infection associated preterm birth

(A) Approximately 10^5 PCMCs were treated with 10^6 CFU of WT GBS (COH1) at a MOI of 10 for 4h. The calcium ionophore A23187 (5 μM) was included as a positive control. Percentage of chymotrypsin-like activity release was assessed by measuring chymotrypsin-like activity in the mast cell supernatants and the cell lysates. Data shown were obtained from four independent experiments ($n = 3$; $^{**}P < 0.01$, $^{***}P < 0.001$, Students t test; error bars, ±SEM).

(B–F) Pregnant, chymase proficient, wild type C57BL6/J mice (*Mcpt4*$^{+/+}$) or MCPT4-deficient mice in C57BL6/J background (*Mcpt4*$^{-/-}$) were injected *in utero* with 10^7 CFU of GBS WT COH1 (n=6/group). Mice were monitored for signs of preterm birth up to 72 h post inoculation. Surgery and GBS inoculation for each pregnant mouse were performed

independently. (B) Scheme of pup numbering *in utero* and injection site between fetuses P1 and P2 is shown.

(C) Percent preterm birth in MCPT4-sufficient (*Mcpt4*$^{+/+}$) or MCPT4-deficient (*Mcpt4*$^{-/-}$) mice. Preterm birth was defined as vaginal bleeding with pup in birth canal or in cage before three days post-infection. * $P < 0.05$, Barnard's test.

(D) Bacterial burden in representative fetal pups and their corresponding placenta from *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice infected with GBS WT COH1 (n = 5–6/group; of note, pups that were delivered preterm were excluded from CFU enumeration. CFUs are not significantly different (NS= not significant) between any of the groups (median is shown).

(E) Bacterial burden in maternal spleen from pregnant *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice infected with WT GBS (n = 6/group, median is shown, * P <0.05, Mann Whitney test).

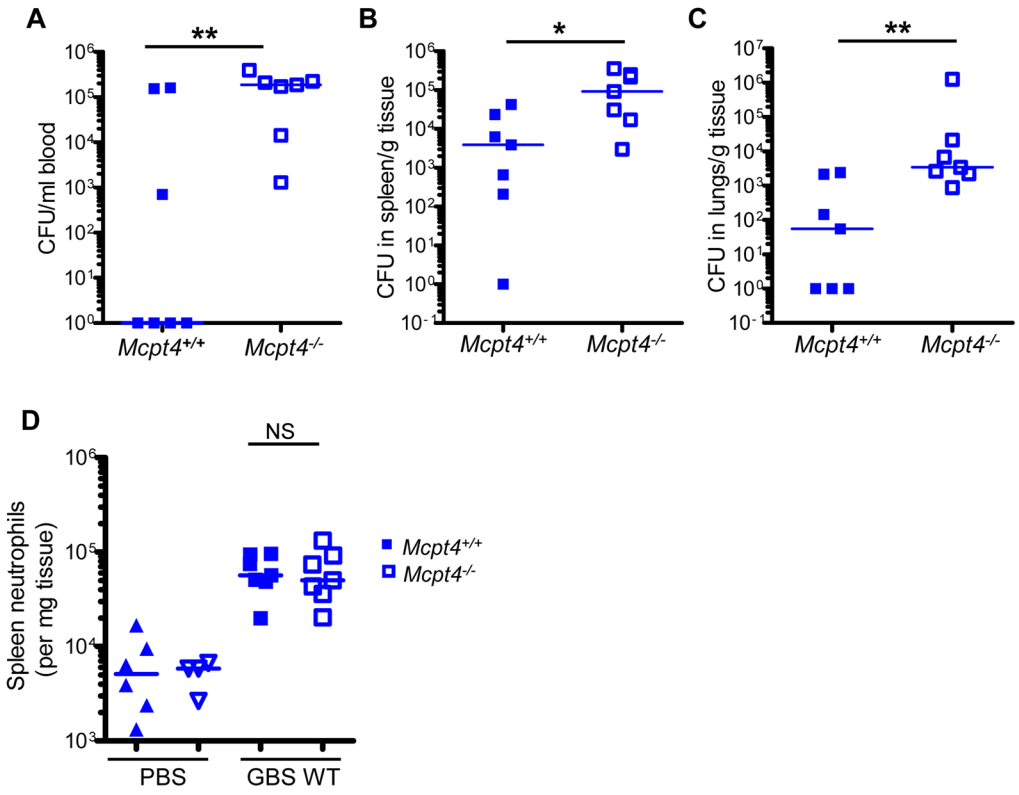

Figure 2. MCPT4-deficient mice exhibit increased GBS dissemination during systemic infection independent of neutrophil recruitment

(A–C) MCPT4-sufficient (*Mcpt4*$^{+/+}$) and MCPT4-deficient (*Mcpt4*$^{-/-}$) mice were intravenously infected with 5×10^7 CFU WT GBS COH1. At 48 h after infection, bacterial burden was evaluated in blood, spleen and lungs. Data shown are from a representative experiment (out of two independent experiments) performed with seven animals per group. The Mann-Whitney test was used for comparisons between the two groups. Medians are indicated (*$P < 0.05$; **$P < 0.01$, ***$P<0.005$).

(D) Percent neutrophils (Ly6G$^+$ CD11b$^+$ cells) in the spleens of uninfected and WT GBS infected *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice (NS= not significant, Mann Whitney test).

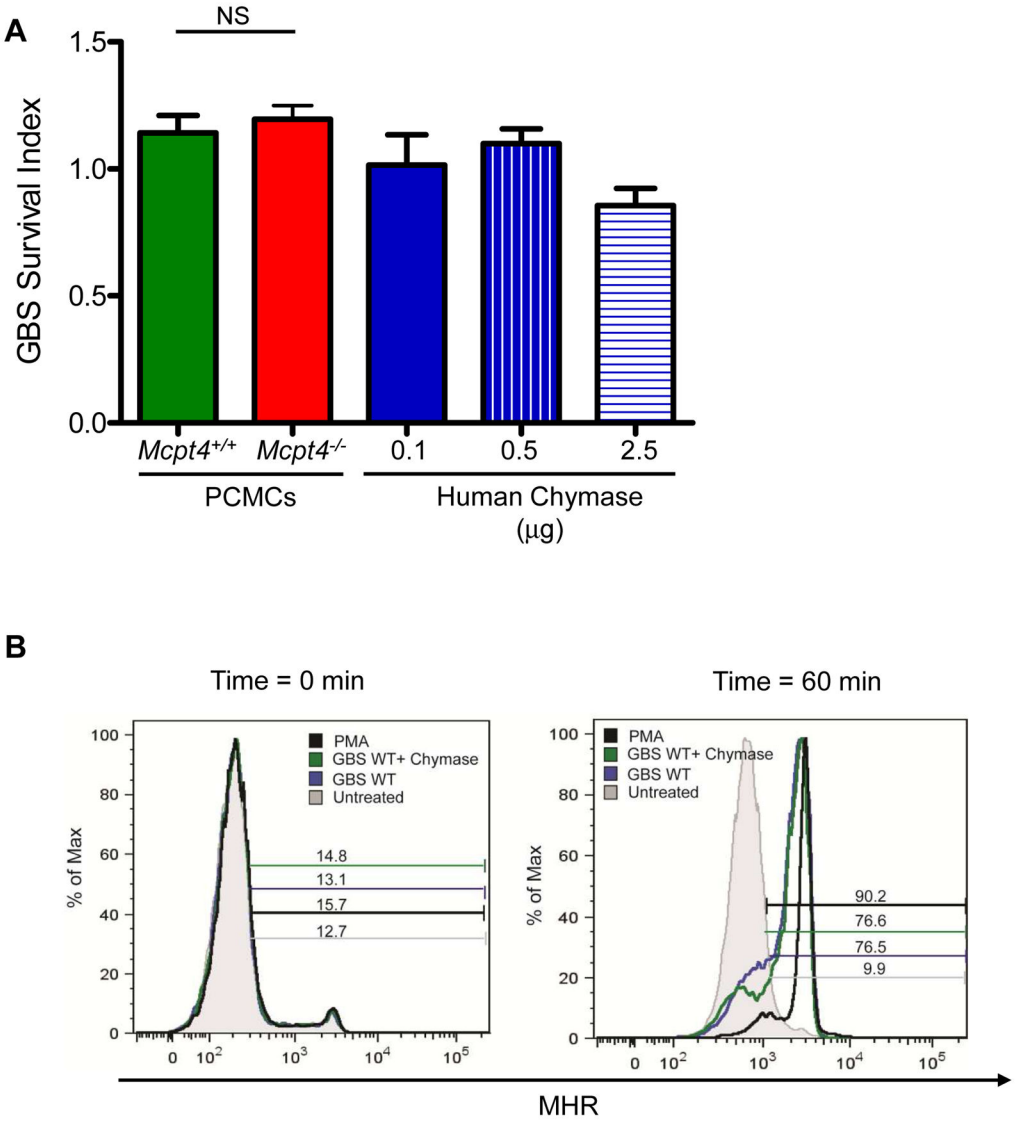

Figure 3. Chymase and MCPT4 do not exhibit direct antibacterial activity and chymase does not diminish neutrophil ROS production in response to GBS

(A) Approximately 1.5×10^4 CFU of WT GBS were incubated with either 1.5×10^5 PCMCs (MOI=0.1) from *Mcpt4*$^{+/+}$ or *Mcpt4*$^{-/-}$ mice or with human recombinant chymase (0.1 µg, 0.5 and 2.5 µg) for 4 h. Surviving bacteria were enumerated at the end of the experiment. Survival index (SI) represents the ratio of the GBS CFU recovered after incubation with either PCMCs or human chymase to the GBS CFU recovered in media alone. Data represent the average of three independent experiments (error bars ± SEM). NS= Not significant, ANOVA.

(B) Purified adult human neutrophils were pretreated with 84 µM dihydrorhodamine 123 and were then either left untreated or treated with WT GBS COH1 at MOI 100 in the presence and absence of human recombinant chymase (0.5 ug). PMA (20 nM) was included as a positive control. Oxidation to fluorescent mono-hydrorhodamine 123 (MHR) was monitored

at 0 and 60 min after treatment, respectively. Gates reflect percent cell numbers. Data shown are representative of two independent experiments.

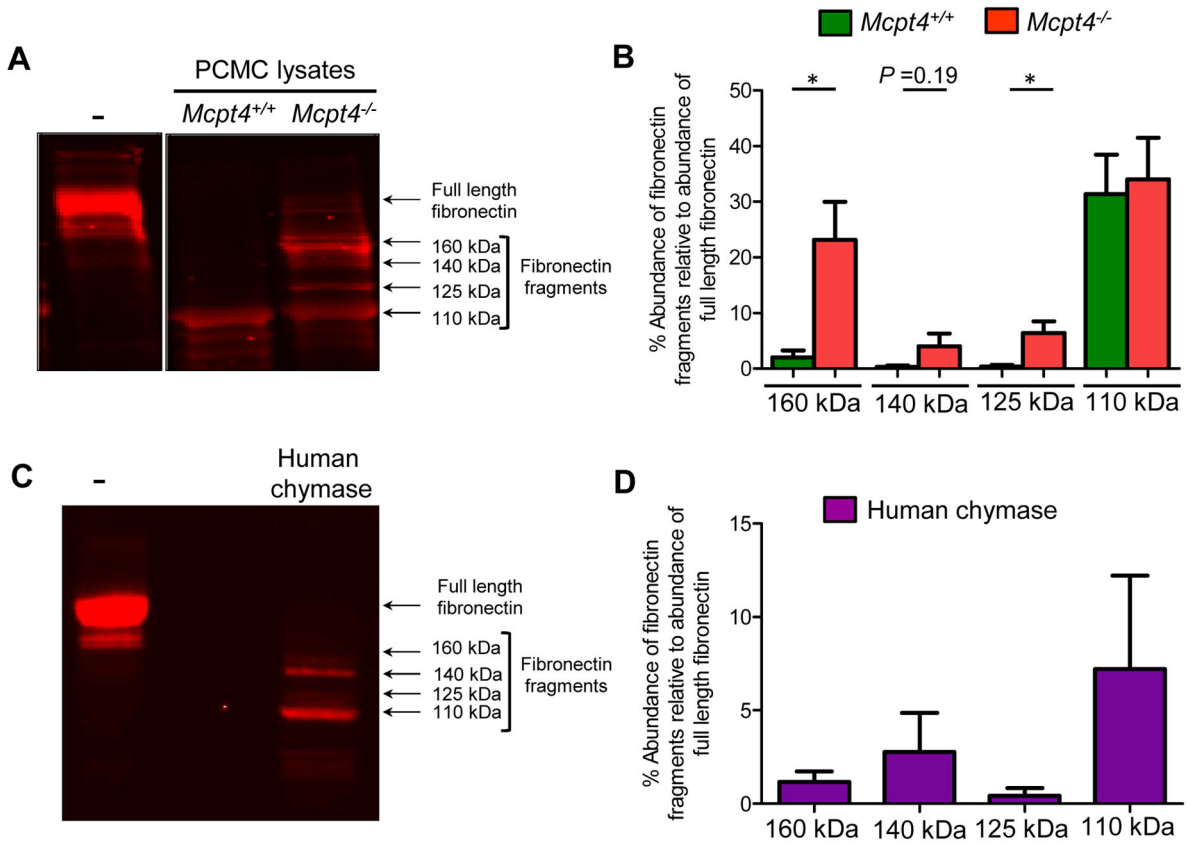

Figure 4. Chymase and MCPT4 degrade full length fibronectin and fibronectin fragments derived from fibronectin proteolytic cleavage

(A) PCMCs lysates from *Mcpt4*$^{+/+}$ or *Mcpt4*$^{-/-}$ mice (lysates from 2.5 × 10^5 cells) were incubated with 5μg of human plasma fibronectin. Fibronectin and its degradation fragments were then visualized by western blot analysis.

(B) % Abundance of fibronectin fragments (approximately 160kDa, 140kDa, 125kDa, and 110kDa) relative to the abundance of full length fibronectin. Data represent the average of five independent experiments (error bars ± SEM). A student's t-test was used for comparison between the two groups.

(C) Human recombinant chymase (human chymase, 63.5ng) was incubated with 2 μg of human plasma fibronectin. Fibronectin and its degradation fragments (approximately 160kDa, 125kDa, and 110kDa) were then visualized by western blot analysis.

(D) % Abundance of fibronectin fragments (approximately 160kDa, 140kDa, 125kDa, and 110kDa) relative to the abundance of full length fibronectin. Data represent the average of four independent experiments (error bars ± SEM).

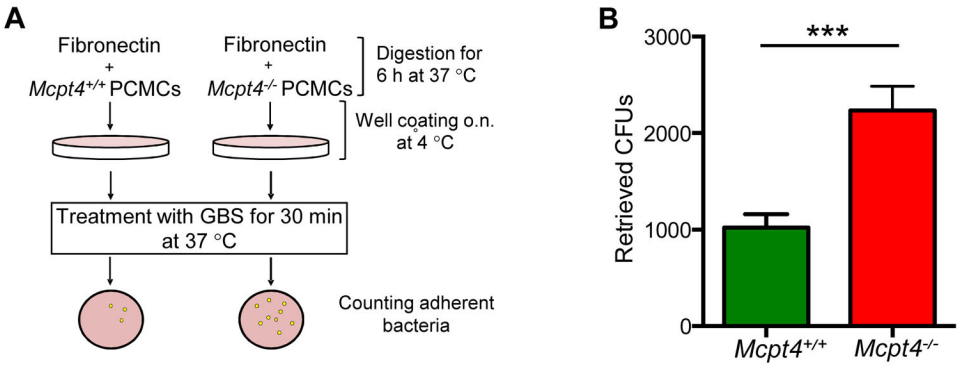

Figure 5. MCPT4 mediated-fibronectin degradation via proteolytic cleavage prevents GBS adherence

(A) Scheme describing the assay used to evaluate the role of chymase proteolysis of fibronectin on GBS adherence. Microtiter plates were coated overnight at 4°C with fibronectin or fibronectin fragments (obtained by incubation of 5 μg fibronectin with lysates generated from 2.5×10^5 chymase deficient or proficient PCMCs). Plates were then treated with WT GBS COH1 for 30 min at 37°C. After washing non-adherent bacteria, adherent bacteria were trypsinized and enumerated by plating. Data are shown in panel B.

(B) GBS adherence to fibronectin that was pretreated with PCMC lysates generated from *Mcpt4*$^{+/+}$ or *Mcpt4*$^{-/-}$ mice. Data represent the average of three independent experiments (error bars ± SEM). A student's t-test was used for comparison between the two groups. (*** $P<0.005$).

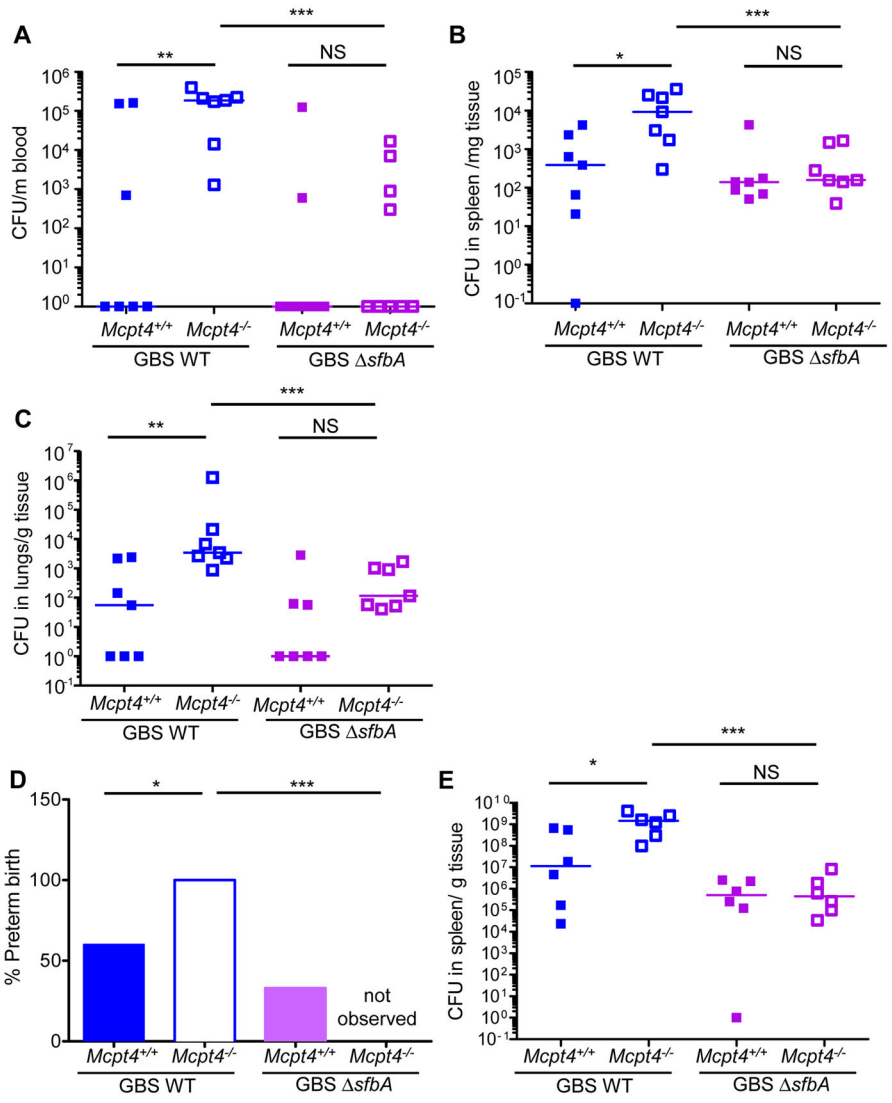

Figure 6. Chymase diminishes GBS infection by inhibiting GBS interactions with the extracellular matrix

(A–C) *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice were intravenously infected with 5 × 10^7 CFU of GBS WT or GBSΔ*sfbA*. At 48h after infection, bacterial burden was evaluated in blood, spleens and lungs. Data shown are from a representative experiment (out of two independent experiments) performed with seven animals per group. The Mann-Whitney test was used for comparisons between the two groups. Medians are indicated (*$P < 0.05$; **$P < 0.01$, ***$P<0.005$, NS= Not significant).

(D–E) Pregnant *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice were injected *in utero* with 10^7 CFU of GBS WT COH1 or GBS GBSΔ*sfbA* (n=6/group). Mice were monitored for signs of preterm birth up to 72 h post inoculation. Surgery and GBS inoculation for each pregnant mouse were performed independently.

(D) Percent preterm birth in *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice infected with WT GBS or GBSΔ*sfbA*. Preterm birth was defined as vaginal bleeding with pup in birth canal or in cage before three days post-infection. * $P < 0.05$, * $P < 0.005$ Barnard's test.

(E) Bacterial burden in maternal spleen from pregnant *Mcpt4*$^{+/+}$ and *Mcpt4*$^{-/-}$ mice infected with WT GBS or GBSΔ*sfbA*. (Median is shown, * P <0.05, *** P <0.005, NS= not significant, Mann Whitney test).