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Mechanisms of mast cell activation by bacterial virulence factors

Implications for mucosal infection

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Abstract

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Mast cells are innate immune cells, which can be found in nearly all tissues of the human body. Activated by receptors for IgE or bacterial compounds, mast cells are complex effector cells which respond in many ways to bacterial infection. However, decades of research did not lead to a general model of how mast cells are activated by bacteria. Examples of pathogenic bacteria include the extracellular *Streptococcus equi* subspecies *equi* (*S. equi*) that colonizes the airways in horses by secretion of powerful toxins, and the invasive enterobacterium *Salmonella enterica* subspecies Typhimurium (*S*.Tm). The latter is taken up with contaminated food and invades the epithelium in the distal small and proximal large intestine to reach the deeper layers of the tissue. Mast cells are present as early responders towards both bacteria. In this thesis, I elucidated how pathogenic bacteria activate these versatile immune cells. In **Paper I**, I demonstrated which virulence factors of *S. equi* that activate mast cells, by utilizing bacterial knockout mutants of several virulence factors or a combination of those. While superantigens or the protective capsule did not lead to mast cell activation, removal of the pore-forming toxin streptolysin S led to complete ablation of the mast cell response to *S. equi*, establishing that sublytic pore formation leads to membrane stress, which results in inflammation. To compare our findings from extracellular bacteria with the invasive *S*.Tm, we explored in **Paper II**, if its type-three-secretion system induces a similar sublytic pore-mediated immune activation. We found however, that *S*.Tm triggers mast cell activation by a two-step activation process, involving 1) priming by toll-like receptor (TLR) 4 and 2) effector-induced immunity. While we mainly focused on classical mouse mast cells, we extended our findings further in **Paper III**. In this study, the use of connective tissue-type and mucosal-type mouse mast cells, as well as a human mast cell line broadened the validity of our two-step activation model. We discovered, moreover, that mucosal-like mast cells lack TLR2 and TLR4, making them only responsive to invasive bacteria. Overall, this thesis contributes to our understanding of the general mechanisms for how mast cells are activated by bacteria.

Keywords: Mast cells, bacteria, Salmonella, infection, enterobacteria

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to all people who love science and are driven by curiosity

Thesis Defense

The public defense will be held on **21st of May, 09:00** at **A1:111a, BMC**.

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **von Beek, C**., Waern, I., Eriksson, J., Melo, F.R., Robinson, C., Waller, A.S., Sellin, M.E., Guss, B., Pejler, G. (2019) Streptococcal sagA activates a proinflammatory response in mast cells by a sublytic mechanism. *Cellular Microbiology*, 21, e13064, doi:10.1111/cmi.13064

II. **von Beek, C**., Fahlgren, A., Geiser, P., Di Martino, ML., Lindahl, O., Prensa, G. I., Mendez-Enriquez, E., Eriksson, J., Hallgren, J., Fällman, M., Pejler, G., Sellin, M. E. (2024). A two-step activation mechanism enables mast cells to differentiate their response between extracellular and invasive enterobacterial infection. *Nature Communications*, 15(1), p. 904, doi:10.1038/s41467-024-45057-w.

III. **von Beek, C**., Prensa, G. I., Andersson, J. H. M., Pejler, G., Sellin ME. (2025). Mast Cell Subtype Features Impact the Interplay with Pathogenic *Salmonella* Typhimurium Bacteria. Under review.

List of additional papers not included in the thesis

I. **von Beek, C**., Alriksson, L., Palle, J., Gustafson, A.-M., Grujic, M., Melo, F.R., Sellin, M.E., Pejler, G., (2021) Dynamin inhibition causes context-dependent cell death of leukemia and lymphoma cells. PLOS ONE 16, e0256708.
doi:10.1371/journal.pone.0256708

Contents

Abbreviations

BMMC	Bone marrow-derived (cultured) mast cell
CBMC	Human cord blood-derived mast cell
CLP	Cecal ligation and puncture (mouse model of sepsis)
DAMP	Damage associated molecular pattern
De novo	New, "freshly" synthesized
FimH	Type 1 fimbriae D-mannose specific adhesin
FBS	Fetal bovine/calf serum
IBS	Irritable Bowel Syndrome
IL	Interleukin
In vitro	Outside the natural physiological environment, e.g., in tissue culture
In vivo	Inside the natural physiological environment, e.g., inside an animal
iNTS	Invasive non-typhoidal *Salmonella*
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAPK	Mitogen-associated protein kinase
MC	Mast cell
Mcpt1	(Mouse) Mast cell protease 1
MOI	Multiplicity of infection
Mrgprb2	MAS-related G-protein coupled receptor member b2 (Mouse)
MRGPRX2	MAS-related G-protein coupled receptor member X2 (Human)
NLR	Nod-like receptor
PCM	Peritoneal mast cell
PCMC	Peritoneal cultured mast cell
PGN	Peptidoglycan
PRR	Pattern recognition receptor
rPMC/rPCMC	Rat PMC/PCMC (other mentions are mouse)
SipA	*Salmonella* invasion protein A
SopB	*Salmonella* outer protein B
S. equi	*Streptococcus equi* subspecies *equi*
*S.*Tm	*Salmonella enterica* subspecies *enterica* serovar Typhimurium
SCF	Stem cell factor
SCV	*Salmonella*-containing vacuole
SEA/B	Staphylococcal exotoxin A/B
TLR	Toll-like receptor
TTSS	Type-three-secretion system

Modern naming conventions

Table 1. Historical names of cytokines and their current name.

Previous name	Current name
Tumor necrosis factor α (TNF-α)	Tumor necrosis factor (TNF)
Tumor necrosis factor β (TNF-β)	Lymphotoxin α
Monocyte chemoattractant protein 1 (MCP-1)	Chemokine (C-C motif) ligand 2 (CCL2)
Macrophage Inflammatory Proteins (MIP-1α, MIP-1β)	CCL3, CCL4
Interleukin 8 (IL-8)	Chemokine (C-X-C motif) ligand 8 (CXCL8)

Introduction

Let me take you on a journey through the wonders of immunology. Be warned though, like the interactions of pathogens with the immune system, this journey is not without its hurdles. But so, dear reader, as you imagine yourself traversing this thesis, you can empathize with *Salmonella*, traversing the gastrointestinal tract and invading the gut mucosa such as you dive into new insights within immunology.

In this chapter, I will provide background for the studies I performed and give a general overview about the immune environments, cell types involved, enteropathogenic bacteria as well as experimental models historically used to study the role of mast cells (MCs) in bacterial infection.

Immune environments

The immune system consists of various humoral and cellular components all over the body, which protect the host organism against pathogens such as viruses, protozoan parasites or bacteria. The blood contains mature immune cells such as neutrophils, eosinophils, basophils, B- & T lymphocytes and natural killer cells. In addition, MC progenitors and monocytes can be found, which mature after migration to their target tissues to MCs or dendritic cells and macrophages, respectively. All organs of the body are affected by or affect the immune system. However, different locations and functions shape different tissue environments. Two examples for those organ-specific environments are the skin and the gastrointestinal mucosa. Skin contains connective tissue and covers the whole body. Also, it serves as one of the first barriers which separates the organism from the outside world and microorganisms from the host. Skin consists of several layers, from the epidermis over the dermis to the subcutaneous layers. While the skin mainly provides separation to and protection from the environment, the gastrointestinal mucosa faces a different challenge. While covering an even larger surface than the skin, a delicate balance between protection of the underlying tissues but also absorption of nutrients into the blood in the small intestine needs to be maintained. These two different tasks summarize the main differences between connective tissue, such as found in the deeper layers of the skin, versus mucosal surfaces, the latter being found e.g. in the lungs or the intestines[1].

The intestinal mucosa and its multi-layered protection

The gastrointestinal mucosa maintains a balance between tolerance to microbiota and food antigens and responses to threats. When an enteric pathogen such as *Salmonella enterica* serovar Typhimurium (*S.*Tm) arrives in the small intestinal lumen, it has to cross several layers of defense before it can reach and invade the epithelial monolayer (**Figure 1**). The lumen and outer mucus layers harbor a plethora of microbiota consisting of different bacteria, phages and other microorganisms[1].

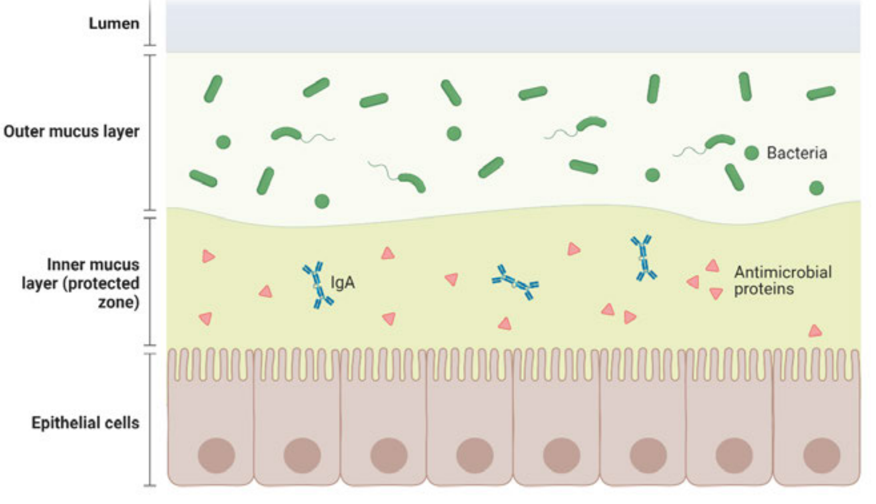

Figure 1. Layers of protection between the intestinal lumen and the epithelial layer. The small intestine consists of several layers of protection against pathogens which stand between the lumen and the epithelial monolayer. The lumen contains nutrients derived from food, but also a diverse set of microorganisms. The outer mucus layer captures most members of the microbiota, while the inner mucus is thicker and serves more as a defense layer against pathogens with IgA and antimicrobial proteins. Adapted from BioRender.

A healthy microbiota provides nutrition to its host and a strong defense against *S.*Tm but can be disturbed by antibiotic intake or inflammation. While swimming through the mucus layer towards the epithelium, the unwanted visitor reaches another, deeper layer of mucus. Here, most microbiota cannot enter and additional defenses such as IgA and antimicrobial proteins like defensins are in place[1]. Largely unimpressed, *S.*Tm will mostly evade those host defenses and travel through the mucus by near-surface swimming[2], investigating possible vulnerabilities in the epithelial layer where it can invade.

The epithelial monolayer in the small intestine consists of a heterogenous cell population of epithelial cells sitting on a basement membrane (**Figure 2**)[1]. Enterocytes are tasked with the absorption of nutrients from the lumen. Microfold (M) cells and dendritic cells sample the epithelial surface to detect microorganisms that traversed the layers of defense above. If an intruder is identified, the dendritic cells will migrate to the Peyer's patches where T and B cells reside together with additional dendritic cells to relay the information to the lymph nodes. Paneth cells guard intestinal stem cells in the crypts by secretion of defensins while goblet cells are the producers of mucus[1].

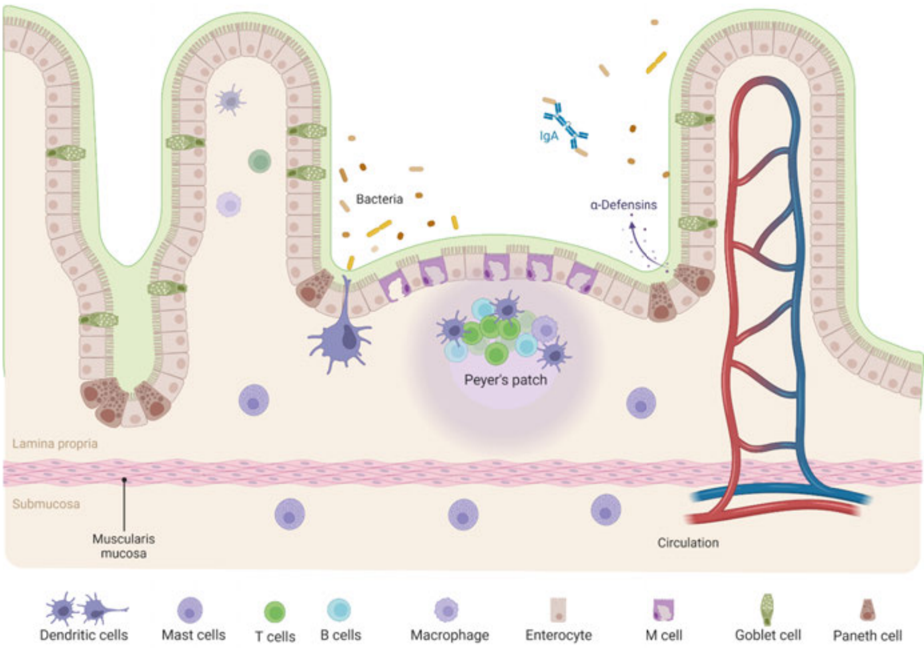

Figure 2. Immune environment in the small intestine. The small intestine contains crypts, harboring stem cells, which are protected by dense layers of mucus (left) and microvilli, protrusions to expand the intestinal surface to maximize nutrient uptake. Peyer's patches (or follicle-associated epithelium generally) do not contain crypts or microvilli but patches of M cells and sampling immune cells, forwarding surveillance to the underlying immune cells. Below the epithelium are the mucosal lamina propria, the submucosa and layers of smooth muscle. Adapted from BioRender.com.

Below the epithelial layer, the lamina propria is found, a loose connective tissue where, besides blood vessels and the Peyer's patches, macrophages and MCs reside. Below the lamina propria, the submucosa, a dense connective tissue, connects to layers of smooth muscle. When a threat needs to be acted on, the resident immune cells are able to induce a potent immune response and recruitment of effector cells from the blood over the circulation to the epithelium[1]. During an infection with *S.*Tm, the immune system responds in various ways, ranging from epithelial cells being expelled into the lumen[3], over macrophages releasing TNF to activate neighboring epithelial cells[4], to the induction of adaptive immunity. In addition, another population of immune cells enters the field: The MC.

Mast cells

MCs are innate immune cells which are strategically located at the interface of the body and the outside environment, populating tissues including the skin and intestines (reviewed in [5]). While MCs can produce a wide array of *de novo* synthesized mediators such as cytokines or prostaglandins, their main characteristic are secretory granules, containing a plethora of preformed mediators such as serine proteases or histamine (**Figure 3**). In a process called "degranulation" those contents can be released into the environment upon stimulation (reviewed in [6]). Due to their location, MCs encounter invading pathogens but also commensal organisms early and are responsible for mounting direct responses to the invader or modulating the immune response towards an ally[5,7]. During embryonal development, the first MCs are derived from the fetal yolk sac and travel as progenitors through the blood to their tissue destination within several hematopoietic waves. Here, they populate the target tissues, mature and maintain themselves by proliferation. This leads to a variety of tissue-dependent phenotypes[8–10]. In addition to tissue resident cells, a second population can be recruited from the adult bone marrow upon inflammation, commonly called "recruited" or "mucosal" MCs. Historically, MCs were described in mice based on their tissue localization (connective tissue or mucosal MCs) and in humans categorized into subtypes based on their protease expression profiles. MCs expressing both tryptase and chymase were associated with the mouse tissue-resident phenotype, while MCs expressing only tryptase were seen as mucosal-like MCs (reviewed in [8]). With the emergence of more sophisticated experimental methods however, this view became challenged. Instead of clearly defined phenotypes, single cell transcriptomics and proteomics indicated a spectrum of different phenotypic states[11–13], particularly in humans. A clearer categorization of MCs takes their origin as well as their local tissue environment into account. Tissue-resident MCs are present in homeostasis, are long lived and proliferate slowly. Recruited MCs, on the other hand, mostly appear when called by T Helper cells to counter an infection by, e.g., a protozoan parasite. Those MCs are recruited to the lung or intestinal mucosa and proliferate rapidly[14].

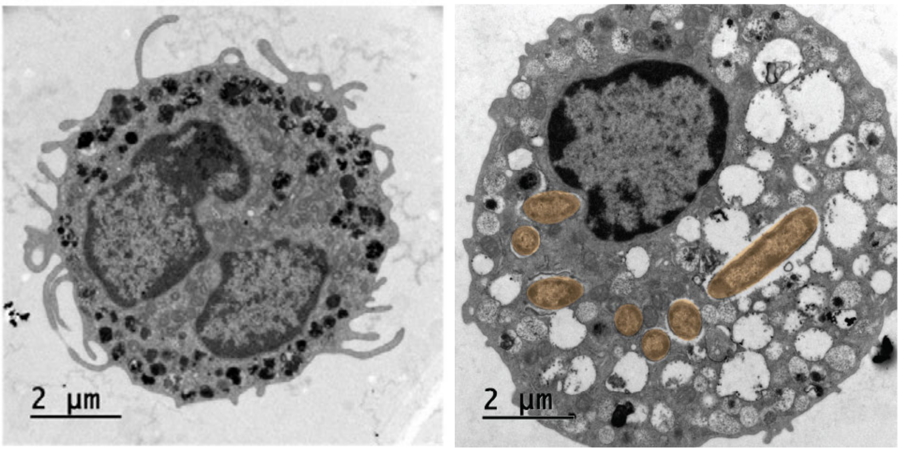

Figure 3. Transmission electron microscopy images of bone marrow-derived mast cells uninfected (left) and infected with *S.*Tm (right). Picture by Grisna I. Prensa and Christopher von Beek.

Phenotypically, lung and intestinal MCs can both be found in the mucosa but may or may not belong to the recruited phenotype. In addition, their proteoglycan composition as well as protease and cytokine expression levels may vary to a high degree. For example, recruited (mucosal) MCs contain mainly chondroitin sulfate and express the chymases Mcpt1 and Mcpt2, while resident (connective tissue) MCs contain heparin and express mostly the chymases Mcpt4, Mcpt5, the tryptase Mcpt6 and Cpa3[6]. Additionally, MCs possess a wide array of secreted effectors which interact with the local environment. Lee et al. (2013) investigated the small innate intestinal lymphoid cells in mice and demonstrated that a population of cells, expressing Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^-$ $NKp46^-$ $IL\text{-}7R\alpha^-$ $CD34^+$ $\beta7^{int/+}$ (excluding mature immune cell lineages and selecting hematopoietic progenitors expressing beta integrin 7) develop into MCs[15]. This MC progenitor population, residing in the small intestine, expressed high levels of the MC proteases Mcpt1 and Mcpt2 and could differentiate into mature MCs in the presence of IL-3 and SCF. However, when cultured *in vitro* with a cytokine cocktail, expression shifted to Mcpt5 and Cpa3, which indicates a connective tissue phenotype rather than a mucosal one[15]. This demonstrated that under non-inflammatory circumstances, MC progenitors, expressing mucosal proteases are recruited to the small intestine but can change at least partially, into a resident phenotype. Notably however, TGF-β[16] is required for shifting MCs towards a mucosal-like phenotype but was absent in the cytokine cocktail. In addition, MCs interact with nerve endings in the gut and other locations, making them important players in the gut-brain axis[17]. MCs also possess a wide array of surface receptors, ranging from pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) over cytokine receptors and the pseudoallergen receptor MRGPRX2 to the prominent activating receptor FcεRIα, the latter for IgE-mediated activation as seen for example in allergy or defense against parasites[18] (**Figure 4**).

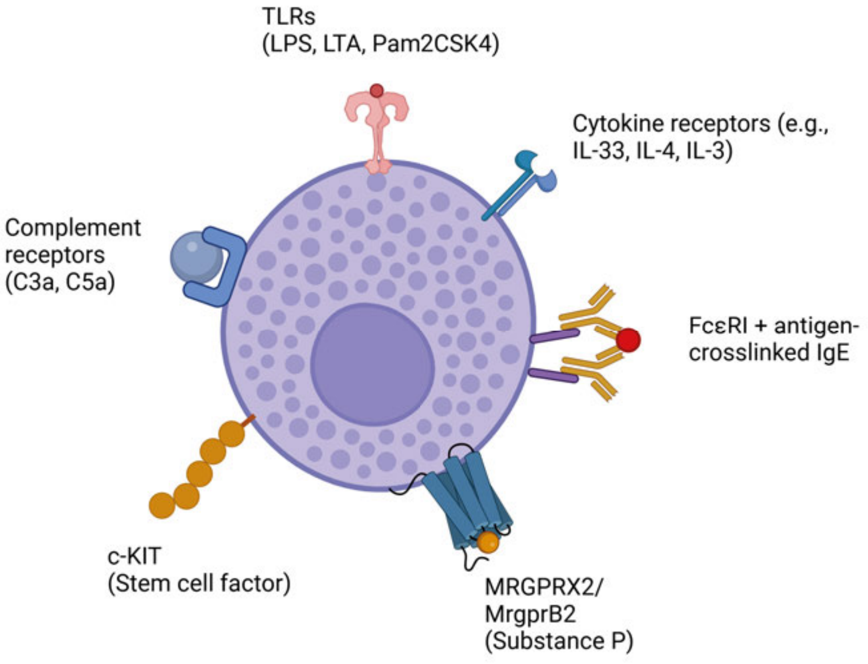

Figure 4. The MC and a (small) selection of its surface receptors. Examples for ligands can be found in brackets. Not all MC phenotypes express all receptors. Created with BioRender.

DAMP associated signaling

Cellular signaling is a process that consists generally of a receptor, binding a ligand, and activating a kinase enzyme, which phosphorylates a target, finally leading to activation or inhibition of a transcription factor and gene expression. In the example of damage associated molecular pattern (DAMP)-mediated immune activation, cytosolic contents which are usually not found in the extracellular space, such as IL-33, HMGB1 or ATP, bind as ligands to DAMP-receptors such as TLRs or the receptor for IL-33 (ST2). The mitogen-activated protein kinases (MAPK), NF-κB or the PI3K/AKT pathways are classically activated by DAMPS, leading to an inflammatory response[19], involving IL-6 and TNF secretion in immune cells.

Cytokines and chemokines

Cytokines are soluble messenger molecules that enable cellular communication by binding to corresponding cell surface receptors expressed on other cells (paracrine) or the own surface (autocrine). Receptors for cytokines exist in different affinities and binding characteristics, enabling signal transduction upon cytokine binding and regulation of gene expression[20]. MCs produce a variety of cytokines, chemokines and growth factors *de novo* in addition to storing some in their granules[21]. Here I will briefly focus on cytokine families important for this work. For a more detailed overview, see [22].

Interleukins (ILs) were originally found in, but are not limited to, leukocytes, and have a plethora of functions, including effects on recruitment, proliferation, and activation of other immune cells. Inflammatory ILs such as IL-6 trigger inflammation by activation and induction of proliferation of B cells, T cells and neutrophils while also suppressing T regulatory cells. Other ILs like IL-10 can have immunosuppressive functions[23,24]. IL-3 is an important MC growth factor, especially for murine MCs. Tumor necrosis factor (TNF) induces tumor cytotoxicity, activation of neutrophils, mediation of adherence and chemotaxis and is clinically most relevant due to its mediation of sepsis. Here, large amounts of TNF are secreted by many different cell types after binding of LPS to TLR4. Chemokines recruit lymphocytes, monocytes, neutrophils and other cells by chemotaxis to target sites. Multiple ligand binding of chemokine receptors allows redundant and overlapping responses. Chemokine ligand 2 (CCL2) promotes inflammation by recruiting T cells and monocytes. CXCL motif chemokines are distinct from CCLs due to their ability to recruit neutrophils. Interferons like IFN-γ provide immune defense against intracellular pathogens. Another core cytokine in MC biology is stem cell factor (SCF), the main MC growth factor[23]. When culturing MCs *in vitro*, different cytokines are combined to cytokine cocktails which induce maturation to the desired phenotype, as for example done in **Paper III**.

Vaccines and Virulence

Vaccines are a tool to achieve active immunization by introducing an antigen, commonly combined with an adjuvant, which enhances and directs the immune response to the antigen. Hence, an ideal vaccine trains the immune system without the risk of a primary infection. Ideally, both T cell immunity and antibody-generating memory B cells are created during the immunization process. While various types of vaccines exist, three main categories can be distinguished for anti-bacterial vaccines: 1) Live vaccines, consisting of a whole (attenuated) organism, brought into the host, 2) dead whole bacteria and 3) components of bacteria. The latter includes purified antigens, modified toxins (toxoids) and recombinant bacterial proteins. The second and third category vaccines are usually better tolerated than live vaccines but require adjuvants for effective immunization. Live vaccines on the other hand, include whole pathogens with reduced fitness so that immunization is achieved with less potent immune activation and side effects compared to infection with the actual pathogen. Examples are non-replicating viruses, bacteria lacking virulence factors or virulence associated genes that are needed to overcome the host's immune defense[25]. Identifying the virulence factors and their mechanism of action on the host organism is crucial to create effective vaccination.

Streptococcus equi

Streptococcus equi subspecies *equi* (*S. equi*) is mainly an extracellular pathogen, belonging to the Gram-positive Lancefield Group C Streptococci[26]. Due to horizontal gene transfer, *S. equi* shares many common virulence factors with other Streptococci such as *Streptococcus pyogenes*[27]. Although there is no evidence for *S. equi* to infect human hosts, the bacteria can be used for the study of streptococcal virulence factors[28].

In horses, *S.* equi causes a respiratory disease called "strangles", first described in 1251 and discussed in the veterinary literature for centuries[26]. Strangles is characterized by a short acute ("strangles") and a longer persistent phase that can become chronic. The infection induces lymph node abscesses which can rupture and suffocate (strangle) the horse, releasing highly infectious pus. After the acute phase, bacterial reservoirs are slowly released by balls of dried pus (chondroids) from the guttural pouches[29]. This chronic infection leads to persistent, symptom-free carriers that remain infectious to other horses.

S. equi is equipped with several virulence factors typical for group C Streptococci such as SeM, a fibrinogen- and immunoglobulin-binding factor that has homologues in other Streptococci such as *S. zooepidemicus* and *S. pyogenes*. In addition, equibactin is secreted by *S. equi* for iron acquisition[30], the phospholipase A_2 toxins SlaA and SlaB probably contribute to membrane rupture in host cells[31] and IdE1 and IdE2 are IgG endopeptidases which inhibit recognition and targeting of bacteria[32,33]. Fim1 aids attachment to horse tissue[34]. Further, *S. equi* express Streptolysin S, a hemolytic toxin and the super antigens SeHILM, inducing strong immune activation by crosslinking T cell receptors[35]. To prevent opsonization and phagocytosis, *S. equi* possesses a hyaluronic acid capsule[36].

In 2010, Rönnberg et al. studied the response of bone marrow-derived MCs (BMMCs) and peritoneal cell-derived MCs (PCMCs) to *S. equi* and observed a slow degranulation and histamine release[37]. While there was no evidence for phagocytosis, MCs responded to live bacteria with secretion of cytokines including IL-6, TNF and IL-13, which was partially dependent on cell-cell contact. This response was mainly attributed to TLR2 and TLR4, using respective knockout BMMCs. Further, a microarray analysis revealed the overexpression of several transcription factors (e.g., nuclear receptor subfamily 4 “Nr4a3”), cell signaling processes (e.g., Map3k8, Rasgef1b) and proteolytic enzymes.

To serve the need for a better tolerated strangles vaccine and to investigate the virulence- and contributing factors during infection, the Waller lab created single- and multi-deletion mutants of *S. equi* strain 4047[38]. Besides knockouts of certain virulence factors mentioned above, the metabolic genes *aroB* and *pyrC* were deleted, leading to requirement of aromatic amino acid and pyrimidine intermediates from the host and subsequently reducing fitness *in vivo*. In addition, lack of *recA* makes mutant strains highly susceptible to sunlight and interferes with transmission to other horses and spreading infection. The goal was an efficient and sufficiently tolerated live vaccine, a safer alternative to the market for strangles vaccines. However, despite deleting six virulence factors or associated genes, a clinical study with Welsh mountain ponies indicated protection against strangles but did not show the desired clearance rate in the majority of the ponies[38].

In **Paper I**, we followed up on the previous research to understand how MCs are activated by specific virulence factors of *S. equi*.

Salmonella and other enterobacteria

Enteropathogenic bacteria are commonly infecting humans and other animals through the fecal-oral route by contaminated food or water. Symptoms range from diarrhea over stomach cramps to more severe disease outcomes[39,40]. Invasive enterobacteria commonly interfere with the host cytoskeleton by manipulating cellular Rho GTPases with secreted effector proteins. In case of *Shigella*, *Salmonella* and enterohemorrhagic *Escherichia coli*, this leads to forced uptake. *Yersinia pseudotuberculosis,* on the other hand, employs its effectors YopE and YopH to inhibit uptake into macrophages[41,42] and enteropathogenic *E. coli* trigger pedestals to bind tightly to the surface of epithelial cells[39].

The genus *Salmonella* consists of Gram-negative, rod-shaped and flagellated bacteria. As a facultative intracellular pathogen, the species *Salmonella enterica* includes the subspecies *enterica* (*Salmonella enterica* subsp. *enterica*), which is able to infect warm-blooded animals and causes a diverse range of diseases with a variety of different serovars. Classically, *Salmonella* was shown to invade non-phagocytic cells such as epithelial cells by its effectors *Salmonella* invasion protein A (SipA), *Salmonella* outer protein B (SopB), SopE, and SopE2, to induce actin rearrangements, so called *ruffles*, which take up the bacteria. However, this process depends on cell type, transformative status of the cell as well as the physiological environment[43]. The interaction of *S*.Tm and epithelial cells of various origins has been studied extensively in the past[44].

The delivery of effector proteins is either done by bacterial secretion and host cell endocytosis or by active injection. Several Gram-negative bacteria evolved for use of the latter - the type-three-secretion system (TTSS)[45].

The Type-Three-Secretion System

The TTSS is a needle-like structure which penetrates the host membrane and delivers proteins from the cytosol of the bacteria to the cytosol of the host cell (**Figure 5)**. Both the amount of strategies to manipulate the actin skeleton as well as the bacteria utilizing them, are diverse[45]. Rho GTPase functions include regulated endocytosis, filopodial arrangement and apoptosis. This makes them a valuable target for interference of cellular processes. Once inside the host cell, invasive bacteria employ two strategies: vacuolar or cytosolic life styles[46].

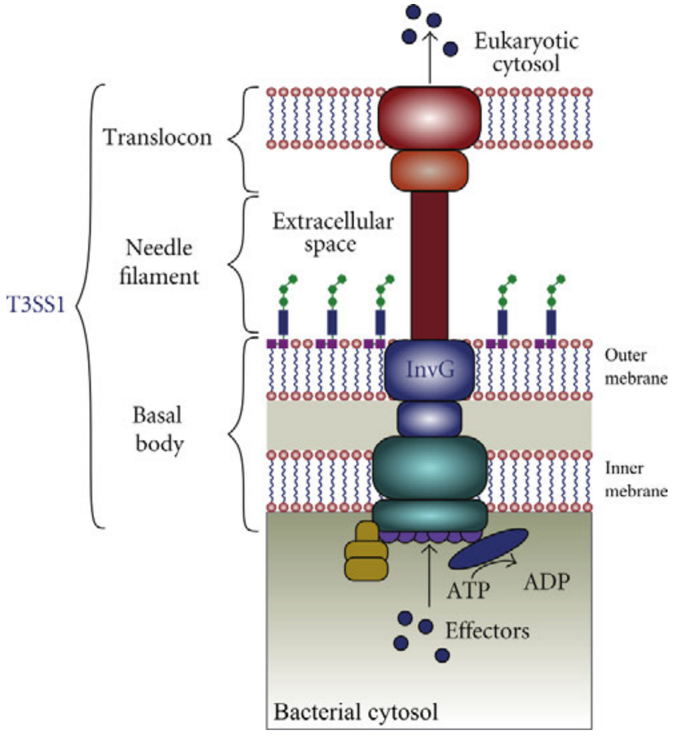

Figure 5. **The *S.*Tm type-three-secretion system 1.** The TTSS-1 acts like a needle, bridging the extracellular space from the bacterial cytosol into the host (eukaryotic) membrane to translocate effector proteins. Notice the structural core protein InvG, required for TTSS assembly. Adapted from[47] and printed with permission.

Intracellular lifestyle: Vacuolar and cytosolic

Once taken up into the endocytic vacuole, bacteria such as *Salmonella* can hijack and modify it into a *Salmonella*-containing vacuole (SCV). This process enables the bacteria to be largely unrecognized by cytosolic PRRs, such as nucleotide-binding oligomerization domain-like receptors (NLRs), or caspases such caspase 4/11 that sense cytosolic LPS directly[46]. However, nutrients are limited due to the need of transportation across the vacuolar membrane, and bacterial replication inside the vacuole can be slow or stalled[46]. To gain nutritional access, *S*.Tm exploits the host's endosomal system by forming interconnected tubular vesicles to enable intravacuolar growth[48]. Vacuolar PRRs pose an additional risk of discovery. Bacterial ssRNA and CpG DNA fragments can be detected by TLR3/7/8 and TLR9, respectively[46].

As most *S*.Tm prefer vacuolar housing, other bacteria such as *Listeria monocytogenes* or *Shigella flexneri* favor cytosolic residency. This lifestyle includes a rapid rupture of the vacuole after bacterial uptake and direct access to cytosolic nutrients[46]. However, cytosolic defense mechanisms such as the PRRs mentioned above need to be evaded, for instance by secreted effector proteins, such as the caspase 4/11-inhibiting OspC3 by *S. flexneri*[49,50].

Historically, it was believed that an intracellular pathogen is limited to one niche. In recent years however, a body of literature indicated cell type-dependent life styles and for *S*.Tm and *L. monocytogenes*, a heterogenous life style was unraveled where subpopulations of the same bacterium can reside in both vacuolar and cytosolic niches within the same cell. This strategy enhances intracellular survival and spread to other cells as well as increased resistance against host responses. Notably however, being exposed to both vacuolar and cytosolic immune responses inside the same cells increases the risk for detection[46]. So far, cytosolic survival and replication of *S*.Tm was only shown for epithelial cells but not for fibroblasts or macrophages[51]. In the latter cases, cytosolic bacteria were growth-restricted or lead to rapid cell death. Additionally, one needs to keep in mind, that for *S*.Tm to successfully adapt to the cytosol, host cell death avoidance needs to be accomplished, which is easier to achieve in continuous cell lines which intrinsically have less sensitive thresholds for cell death. One example is the cervical carcinoma-derived HeLa cell line, commonly used as a cellular infection model. Nevertheless, some primary MC cell lines also tolerate intracellular *S*.Tm for more than 24 h as shown in **Paper II**.

Clinical manifestations of *Salmonella* infection in humans

Generally, there are two types of *Salmonella* causing disease in humans: The invasive *Salmonella enterica* serovars Typhi and Paratyphi, causing typhoid-fever-like systemic disease and the non-invasive *Salmonella enterica* serovar Typhimurium, causing gastroenteritis. However, recent epidemics in Africa revealed another category: invasive non-typhoidal *Salmonella* (iNTS). Overall, non-typhoidal *Salmonella* are self-limiting in immunocompetent individuals, but can progress to invasive disease when comorbidities such as HIV or Malaria are present[52].

Gastroenteritis by *S.*Tm is typically caused by ingestion of contaminated animal products such as eggs, milk or meat with a variable infectious dose ranging from tens to hundreds of thousands of bacteria, depending on the strain or the immunocompetence of the infected host[52]. As described above, *S.*Tm travels through the gastrointestinal system until reaching the small intestine in humans or the caecum in mice where it colonizes the intestinal lumen and invades the epithelium. To overcome the resident microbiota, *S.*Tm utilizes the proinflammatory environment[53], created by the acute immune responses towards *S.*Tm, to outcompete the resident bacteria. This results in symptoms commonly including nausea, stomach cramps, diarrhea[52] and a strong behavioral impulse to improve food hygiene in future meals.

The streptomycin pretreatment model of oral *Salmonella* infection in mice

To gain physiological insight into the underlying mechanism of *S.*Tm assault on the epithelium and the interplay with the host, (genetically modified) mouse models are a valuable tool. However, mice are naturally resistant to oral *S.*Tm enterocolitis infection, while other modes of entry do not recapitulate the human disease[54,55]. On the other hand, many inbred mouse strains lack a fully functional *Nramp* gene ("*Nramp*$^{-}$") and develop systemic typhoid fever-like disease with colonization of the Peyer's patches and lymph nodes through invasion of dendritic cells in response to *S.*Tm infection. Finally, this results into infection of liver and spleen, leading to high mortality within days. Still, those mice do not permit efficient growth of *S.*Tm in the intestine and infection does not resemble the phenotype of human enterocolitis[55–57]. Therefore, many early contributions to our understanding of *S.*Tm enterocolitis were derived from bovine infection models. Such models are however not suited for creation of knockout animals and are due to their nature difficult to handle.

In order to create a murine model of *S.*Tm enterocolitis similar to human disease, Barthel et al. developed in 2003 the streptomycin pretreatment model of oral *Salmonella* infection in mice[55]. 24 h prior to oral introduction of *S.*Tm, mice are pretreated with 20 mg streptomycin to disrupt the resident microbiota. This breaks the microbiota-mediated colonization resistance against *S.*Tm[53]. Since the *S.*Tm lab strains SL1344 and 14028 are naturally streptomycin resistant, their colonization ability is unimpaired. Giving ~10^3-10^8 *S.*Tm to mice, within 24-48 h a prominent inflammation of the intestines with colonization of the caecum is established. *Nramp*$^{+}$ strains such as CBA can be monitored far longer, such as 42 days in our own experiments as demonstrated in **Paper II**. This is in contrast to C57BL/6, which succumb to systemic infection within days[55].

Bacterial culture conditions and virulence

As introduced above, bacterial pathogens are evolutionary adapted to their host environment. Hence, it is important to sense where in the host a bacterium is at a certain time to modulate protein expression appropriately. However, the induction of virulence by specific culture conditions is sometimes overlooked in studies. This can lead to radically different phenotypes during infection experiments and likely contributes to the reason why many bacteria are believed to be non-invasive towards MCs[58]. In addition, part of the impression that MCs react highly specific to certain bacterial strains while acting different to closely related strain[59] might be due to the lack of virulence-inducing bacterial culture conditions.

To optimally induce virulence in *S.*Tm, three strategies are employed simultaneously: *S.*Tm overnight cultures are grown for exactly 12 h on a shaking wheel incubator (i) and subcultured for 4 h to achieve an inoculum in late-exponential phase (ii). In addition to this, a stressor, such as 300 mM NaCl is present in the LB growth medium (common LB receipts include 8.56-171 mM NaCl, corresponding to 0.5-10 g/l) (iii). In contrast to 16 h overnight cultures in LB without stressor, ca. 50% of bacteria will express the TTSS and invasion into 30-60% of MCs can be observed[60]. Mild aeration and growth to late exponential phase is generally a good method to enhance virulence in the *S.*Tm population[61].

To study *S.*Tm in the lab, we have commonly used the strains SL1344 and 14028.

Mast cells during gastrointestinal infection

Under healthy conditions, intestinal MCs reside in the submucosa or lamina propria[1]. An increased number of MCs and their degranulation in the intestine is a common observation in various infectious diseases, such as *Campylobacter*-induced hemorrhagic enteropathy in pigs[62] or *Shigella* infection in humans[63]. Raqib et al. (2003) demonstrated that MCs are more active in children suffering from *Shigella* infection, compared to adults and contribute to a longer infection[64]. In addition, infection with Shiga toxin-expressing *Shigella dysenteriae* was reported to result in leukotriene C4 secretion in feces of infected patients, which was hypothesized to be derived mainly from degranulated mucosal MCs, activated by the toxin[65]. In recent years, research of the impact of MCs in infection shifted from the observation of number and secreted mediators to sophisticated models of MC interaction. Important examples include MCs bridging innate effector function with adaptive humoral immunity.

Following the route of the pathogen as discussed above, incoming pathogens entering the intestines encounter mucus and IgA as early defense barriers. It was recently discovered that MCs act in concert with Peyer's patches to modulate B cell numbers, IgA subtype repertoire and IgA levels by the secreted metabolite 5-HIAA, acting on dendritic cells by the GPR35 receptor[66]. Bacterial infection in the gut or presence of their toxins was also shown to create high levels of pathogen-specific IgE[67]. MCs are therefore able to stimulate adaptive immune responses to bacteria such as *Citrobacter rodentium*, but also to harmless food antigens, increasing the risk for food allergies and irritable bowel syndrome (IBS). Extending to the acute infection, MCs were shown to remain alert, leading to potential proinflammatory environments favoring immune pathologies. At the end of an infection, MCs can modulate the gut to either stay in a hyperalert state which can be one of the underlying factors for IBS, or calm down the inflammation.

Mast cell activation by bacteria

In this chapter, I am aiming to give a comprehensive overview of when and how MCs are activated by various bacteria. Since I believe that large disparities of MC phenotypes observed in the literature are dependent on the MC model used, I sorted the mouse infections by standardized models and the tissue culture infections by cell type.

In mice

Historically, MCs are largely associated with their detrimental effects in allergy or defense against eukaryotic parasites. The study of the role of MCs in bacterial infection emerged relatively recently in the 1990s[68]. Although gastrointestinal infections in mice were studied in large depths as seen for example by the common application of the streptomycin pretreatment mouse model of *S.*Tm infection in mice[55], not many studies were published about the role of MCs. Instead, a couple of MC-deficient mouse model strains as well as standardized procedures were investigated. These mainly include the models of polymicrobial sepsis and peritonitis.

In the following section, I summarize the interaction of MCs and bacteria in these different animal models and go briefly through the general mechanisms of how bacterial virulence factors trigger MCs activation. Regarding the mouse model systems, it is important to acknowledge limitations of mouse strains and experimental designs. MC knockouts in mice follow two main strategies: Targeting the MC receptor c-kit (CD117), or using protease promoters such as Mcpt5 targeting CTMCs. Initially, MCs were knocked out by utilizing a mouse model, harboring a spontaneous mutation which affects global kit expression using the $Kit^{W/W-v}$ (W/W^v) mice with a strategy for reconstitution of MCs by transplantation of BMMCs[69]. However, this model possesses two important caveats since kit expression is not limited to MCs and BMMC reconstitution does not phenocopy WT mice, leading to hematologic abnormalities independent of MCs[59,70]. In response, Kit^{W-sh} "sash" mice were bred as a better kit-dependent model lacking sterility and anemia[71]. However, inherited side effects of kit deficiency were still present. Sash mice were for instance shown to have distinct populations of myeloid cells which is MC independent and can hinder the investigation of MC-specific phenotypes[72]. For a more detailed overview, I refer to [73].

Defined peritonitis models

The peritoneum is the serous membrane which lines the abdominal cavity. A bacterial peritonitis (inflammation of the peritoneum) can be induced by injecting bacteria intraperitoneally. This model was utilized for many MC infection mouse models, assessing short term (1-48 h) effects on CTMCs. For instance, Malaviya et al. (1994) demonstrated MC degranulation in the peritoneum by adhesive type 1 fimbriae expressing ($FimH^+$) *E. coli*[68,74] and Edelson et al. (2004) established that *L. monocytogenes* is activating peritoneal MCs which trigger an immune response depending on the integrin α2β1 and leading to IL-6 production and neutrophil recruitment[75]. In a *Staphylococcus aureus* model of peritonitis, however, no changes in the immune response between mice lacking MCs and WT mice were observed[76]. Further, no effects of MC deficiency were observed in mice infected with mastitis isolate strains of *E. coli* and *S. aureus*[77].

Cecal ligation and puncture (CLP) mouse model of acute septic peritonitis

Historically, many of the first studies in the study of MCs in bacterial infection were performed in the CLP model. CLP induces an artificial breach of the intestinal barrier, resulting in severe sepsis caused by translocation of the resident intestinal microbiota into the blood. In these studies, the composition of the microbiota is not defined, and the model system differs in severity between some research groups[78,79].

The first CLP study in mouse concerning the role of MCs in bacterial infection was done by Echtenacher et al. (1996) in WBB6F1 W/W^v mice[80]. The authors showed a higher mortality in MC-deficient mice, which could be counteracted by transplantation of BMMCs. Notably, the model induced severe sepsis, killing all W/W^v mice within 5 days, while only 25% of WT mice experienced mortality. However, an anti-TNF antibody highly reduced the effect of transplanted BMMCs on mortality while injection of recombinant TNF delivered protection even in absence of MCs. Prodeus et al. (1997) followed up on the study by investigating the effect of CLP in complement-deficient mice, lacking either C3 or C4[81]. They demonstrated that, in CLP, the complement is needed for activation and degranulation of peritoneal MCs and TNF secretion in peritoneal lavage, followed by recruitment of neutrophils and clearance of bacteria[81]. The mechanism of how MCs are activated during CLP was later shown to be dependent specifically on the complement receptors CD21/CD35 and CD19[82]. Another study, conducted by Maurer et al. (1998), showed that subcutaneous injections of recombinant SCF increased the number of peritoneal and skin MCs in mice and enhanced survival in CLP[83]. In addition, the Mac-1$^{-/-}$ mouse model with MC deficiency in peritoneum but not in jejunum or caecum was evaluated by Rosenkranz et al. (1998). Subjecting Mac-1$^{-/-}$ mice to CLP revealed a higher mortality compared to WT mice due to decreased neutrophil numbers and decreased bacterial clearance[84]. Further downstream, it was indicated that MCs stimulate P-selectin secretion, supporting a CCL3-dependent recruitment of neutrophils in CLP[85]. It is also noteworthy that the beneficial effect of transplanted BMMCs in W/W^{v} mice depends highly on TLR4[86].

In summary, in these models of CLP, early MC-derived TNF is important for recruitment of neutrophils which clear bacterial loads and enhance survival. Thereby, MCs are indirectly activated via the complement system, and directly via TLR4 and recruitment via Mac-1. However, a word of caution shall be given in the interpretation of MC-deficiency in W/W^{v} mice. While the studies above all showed a clear increase of mortality in CLP for the MC-deficient phenotype in WBB6F1 genetic background, Shelley et al. (2003) observed no significant differences in C57BL/6 background[87]. They also showed that WT mice have a five times higher number of peritoneal MC baseline levels in C57BL/6 compared to WBB6F1 mice. Further evaluations by Piliponsky et al. (2010) showed that the protective effect of MCs in CLP seen in WBB6F1 mice can only be reproduced in a model of moderately severe sepsis, while in severe sepsis a reconstitution of WT BMMCs in WBB6F1 W/W^{v} was insufficient to rescue the protective phenotype[78]. In addition, the group showed that C57BL/6 sash mice exhibited reduced survival in *moderately severe* CLP compared to WT mice, which was entirely independent of TNF, and that recruited neutrophil numbers do not depend on MC-derived TNF. In *severe* CLP however, MCs contributed directly or indirectly through other cell types with higher levels of TNF, but neutrophil recruitment did not depend on it. Hence, the (protective) role of MCs in CLP and its mediation by TNF depends on mouse strain, nature of kit mutation and the experimental protocol of CLP. Other MC effectors contributing positively or negatively to CLP, include neurolysin[88], histamine, IL-6[89] and Mcpt4[90] while receptors include CD300a[91,92], CD300f[93] or the IL-33 receptor ST2[94]. Lastly, *E. coli* and *Enterococcus faecium* can protect mice in CLP by suppressing MC activation[95].

What can we learn from MC studies in CLP? Most importantly, CLP is a model of polymicrobial sepsis and should not be used to generalize the role of MCs in bacterial infection. Further, *in vivo* studies in mice have the great potential of delivering physiologically relevant knowledge but vary between different factors among experiments inside the same CLP mouse model. In addition, it is hard to derive mechanistical insights which are undoubtedly derived from MCs, when a strong inflammation is induced which involves many other immune cells. In CLP, the effector cells which mediate bacterial clearance are neutrophils, but to which level their recruitment depends on MCs might differ depending on the experimental setup. Concludingly, reconstitution of BMMCs into mice lacking MCs does not result in a MC phenotype identical to that in WT animals[78]. Mechanistic insights about MC receptors and effectors which are generally valid over different protocols and severities within the CLP model, are difficult to verify, as demonstrated by the diverse literature about the role of MC-derived TNF.

Models of LPS administration in mice

In addition to infection of rodents with bacteria, much focus was set on the administration of single bacterial components to mice, most prominently lipopolysaccharide (LPS), to elucidate the immune response based on one single element. Although the literature is a rich source of reports about LPS administration into animals, the role of MCs was assessed only in a minority of studies. Examples include a sepsis model of *E. coli* LPS injection into rats, where degranulation and a MC-dependent effect on bacterial translocation and gut-permeability was observed, which could be suppressed by the MC stabilizer doxantrazole[96]. BRX-537A, a calcium ionophore, induced a similar response on gut permeability as LPS. After 3 h, a spike of rat MC protease 2 could be observed in the colon. Aschenbach et al., (2003) fed LPS from a clinical isolates of *S.*Tm to pigs, which showed a mild histamine release but no MC degranulation[97] in strong contrast to strong proinflammatory responses seen by others, e.g., in models of sepsis (see above). Enteral infusion of *E. coli* into piglets, however, increased the relative amount of degranulated MCs in a model of necrotizing enterocolitis[98].

Toxins and quorum sensing molecules activating mast cells in mice

In a model of *Clostridium difficile* toxin A-mediated intestinal inflammation in W/W^{v} mice[99], MCs were shown to promote neutrophil infiltration and fluid secretion. Also, Staphylococcal enterotoxin A was demonstrated to degranulate intestinal mucosal MCs in a peritoneal injection model of Asian house shrews[100]. Pundir et al. (2019) established that MCs can recognize quorum sensing molecules from different bacteria such as *S. pyogenes*, *Streptococcus pneumoniae* or *E. faecium* by the Mrgprb2/X2 receptors[101]. Mrgprb2 deficient mice were shown to be more susceptible to peritoneal infection than were WT mice.

Interaction of mast cells and adaptive immunity during bacterial infection

Due to their high affinity IgE receptors (FcεRIα), MCs bridge to adaptive immunity. Bacteria-specific IgE adds another potent possibility of MC activation to the innate repertoire. Starkl et al. (2020) showed that *S. aureus* skin infection leads to pathogen-specific IgE on MC surfaces, enabling them to counteract a second infection by inhibition of growth and *S. aureus* toxin activity[102]. In addition, as discussed above, MCs play a role in food allergy and IBS which can be triggered and/or exacerbated by bacterial infection with *C. rodentium* or ingestion of *Staphylococcus* enterotoxin B when certain food antigens are introduced [67].

Words of caution: Stress-induced mast cell responses in laboratory animals

When studying the role of MCs in animals, some caveats need to be considered. MCs act during stress as effector cells of the gut-brain axis[103,104]. Hence, the MC-dependent stress response alone can be sufficient for intestinal inflammation in rats[105]. This is particularly important in the gastrointestinal mucosa where MCs reside in close proximity to nerve endings[106] and can contribute to psychologically stress-induced physiological changes, such as IBS[107].

In tissue culture

Animal models are valuable tools to gain insight into systemic immune responses, especially those involving immune cell recruitment. Tissue culture, however, enables research on molecular mechanisms, requiring high cell yields and helping to study MC characteristics and responses isolated from other cell types, which would be present in tissue. When studying MCs *in vitro*, one can generally utilize three different types of cellular models: 1) Cell lines, which are inexpensive and easy to maintain, or 2) freshly isolated mature primary MCs, which require availability of tissue, time, sophisticated isolation procedures and ethical approval. 3) A middle ground between those models is the use of cultured primary MCs, which are easier to derive and maintain than their fresh counterparts. Those usually show physiological characteristics closer to MCs *in vivo* compared to cell lines. For an overview of *in vitro* MC cultures, see[108].

Continuous cell lines

Commonly used MC lines are the rat RBL-2H3 line and the human cell lines HMC-1, LUVA and LAD2. The mouse cell line MC/9 is rarely used in research on bacterial infection since the availability of primary mouse MCs. The following sections will briefly introduce the respective cell lines and give an overview about their behavior in bacterial infection or response to bacterial components. For more information about MC cell lines, see[109].

HMC-1

The human MC line 1 (HMC-1), isolated from a patient with MC leukemia, was the first human cell line showing MC markers and is historically the most widely used MC-like cell line[110]. HMC-1 are immature cells, which express c-kit and low levels of β-tryptase, have heparin and chondroitin sulfate in their granules but no α-tryptase, and lack expression of FcεRI. HMC-1 cells are unable to react to IgE and they express CD68, a marker for monocytes/macrophages as well as eosinophil markers. Based on these characteristics, HMC-1 cells are considered as being immature and do not reflect a MC phenotype seen for instance in human skin MCs[111]. The sub clone HMC-1 (5C6) was created to enhance tryptase and FcεRI expression[112] to increase maturity of the cell line. Concludingly, many publications titling "human mast cells" which solely rely on HMC-1 need to be read critically.

Nevertheless, as the first human MC line, HMC-1 contributed to many early findings of cellular MC responses to bacteria. It was demonstrated that HMC-1 express MHC class II[113] and were able to present the superantigen staphylococcal enterotoxin A (SEA) to T-cells[114], which was also shown for enterotoxin B (SEB) by another group[115]. However, SEB did not induce MC degranulation[116]. On infection with *S. aureus*, HMC-1 cells were demonstrated to release extracellular traps, capturing and killing bacteria[117]. Intriguingly, *S. aureus* could evade this mechanism by invading HMC-1 where they persisted intracellularly for at least five days, with intracellular survival in the cytoplasm depending on α-hemolysin. The recombinant effector toxins of *S. aureus* PSMα1, PSMα3 and δ-hemolysin were shown to kill HMC-1 while PLV and α-hemolysin did not lead to cell death or tryptase release[118]. HMC-1 degranulated upon *Pseudomonas aeruginosa* infection due to the bacterial quorum sensing N-(3-oxododecanoyl) homoserine lactone[119]. Further, stimulation with *E. coli* expressing the streptococcal pyrogenic exotoxin B (streptococcal cysteine protease) but not LPS induced histamine release by HMC-1 cells after 20 min of exposure, although with unclear effects on viability[120].

Besides studies on the early activation assessed by degranulation, HMC-1 were widely used to study the late response of bacteria on MCs, based on cytokine secretion. For example, Krishnaswamy et al. (2003) demonstrated that dead *Moraxella catarrhalis* and *Neisseria cinerea* induced IL-6 and CCL2 secretion in HMC-1 cells, which was dependent on NF-κB while LPS downregulated CCL2 secretion[121]. Notably, MCs formed tight cell aggregates with each other around the bacteria. Using HMC-1 derived HMC-1 5C6, Lin et al. (2003) showed that *P. aeruginosa* but not *Legionella pneumophilia* induced CCL20 secretion[122]. Further, live *P. aeruginosa* induced apoptosis via its exotoxin A, depending on the caspases 3 and 8 by downregulating FLIP, and the

Bcl family as regulators of mitochondrial membrane integrity which was claimed as the responsible mechanism for the secretion of IL-6 in mouse cells[123–125]. *C. difficile* was shown to degranulate HMC-1 cells via its toxins TcdA and TcdB which reorganized the actin cytoskeleton upon endocytic uptake, leading to Erk1/2 and p38 phosphorylation and consequently CXCL8 secretion[126]. HMC-1 cells could also be activated by sublytic concentrations of pneumolysin or *S. pneumoniae,* leading to release of LL-37 and LTC_4 and consequent killing of bacteria, whereas no release of histamine or cytokines could be found after 1 h of infection[127]. *S. pneumoniae*-based cytotoxicity on HMC-1 cells was mediated by H_2O_2 and high concentrations of pneumolysin.

The role of TLRs in this cell line are controversial. HMC-1 cells were shown to express TLR2, which lead to internalization of peptidoglycan and subsequent detection by NOD2 which induced degranulation[128]. In contrast, Kulka et al. (2004) reported neither TLR2 transcription nor protein expression in HMC-1 cells[129]. In contrast, another research group demonstrated that Pam3CSK4, a TLR1/2 agonist and LPS induced IL-6 secretion in HMC-1 cells[130].

In summary, HMC-1 cells have contributed to the literature about bacteria-mediated MC activation with many different mechanisms. Investigators used cytokine panels or looked at single cytokines, which lead to single or multiple reports of the respective cytokine. HMC-1 cells were shown to phagocytose *E. coli* based on opsonization or on FimH expression while they were also able to present antigen of bacterial toxins to T-cells. However, one needs to keep in mind that some of the observed properties might be associated to the immature and partially monocytic phenotype of HMC-1 cells. Intriguingly, LPS- and TLR-based mechanisms for the recognition of bacteria by HMC-1 cells seem to have rather a minor impact on cytokine secretion.

LAD2

In the beginning of the new millennium, models with a higher physiological resemblance of MCs were available for mice in form of PCMCs or BMMCs as well as for rats including rat PMCs. However, for human MCs, the only convenient model available was still HMC-1. Efforts to create a human MC line which resembles the actual MC phenotype seen *in* and *ex vivo*, led to the development of the Laboratory of Allergic Disease (LAD) cell lines, from which LAD2 became most prominent[131]. LAD2 was created from the bone marrow of a sarcoma/leukemia patient, is dependent on SCF and possesses a doubling time of two weeks. In contrast to HMC-1 cells, LAD2 expresses most MC markers such as functional FcεRI but possesses, similar to HMC-1, levels of tryptase and chymase which are magnitudes lower than seen in human skin MCs[111].

As a relatively novel and easy model for the study of human MCs, LAD2 cells were also employed to study the effect of bacteria on MCs. It was shown that LAD2 cells express functional TLR2 and TLR9, as well as low levels of the complement receptor CR3, the expression of which could be enhanced by the TLR-2 ligand lipoteichoic acid (LTA, TLR2/6) while peptidoglycan (PGN, TLR1/2) or LPS (TLR4) or did not[132]. Accordingly, pretreatment of LAD2 cells with LTA enhanced phagocytosis of opsonized *S. aureus*[132]. This was shown to depend on CD11b, a subunit of the complement receptor 3. Following incubation with opsonized *S. aureus*, LAD2 cells secreted TNF and IL-4[132]. In addition, the same study showed that LAD2 cells can internalize *E. coli,* which could be blocked by addition of cytochalasin D. Depending on the structure of the lipid A in LPS of *Porphyromonas gingivalis*, it could suppress degranulation by the defensins LL-37 (human cathelicidin) and human beta-defensin 3 via MRGPRX2[133]. Live *Acinetobacter baumannii* and *P. aeruginosa* induced TNF and CXCL8 when LAD2 were infected in human serum[134]. Intriguingly, *A. baumannii* adhered to LAD2 cells by binding FcγRII (CD32) which was responsible for the observed response. Karpov et al. (2018) demonstrated that LAD2 secrete TNF upon treatment with PGN, LPS, Pam3CSK4 and LTA[135]. Lastly, polymyxin B and E were shown to trigger a degranulation via MRGPRX2[136].

In conclusion, LAD2 cells can phagocytose *E. coli* or opsonized bacteria, similar to HMC-1 cells. In addition, LAD2 cells can also be activated by pore-forming toxins such as pneumolysin or protein fractions from *H. pylori*. However, in line with HMC-1 cells, dead bacteria or LPS promoted mostly weak activation of LAD2 cells. Intriguingly, bacterial components binding to MRGPRX2 might be a novel mechanism of recognition of bacteria, or their products.

LUVA

LUVA cells are untransformed, spontaneously emerged cells from the culture of CD34$^+$ enriched mononuclear hematopoietic progenitor cells from a blood donor in the presence of IL-3, IL-6 and SCF[137]. They possess several MC markers such as tryptase, CPA3, functional FcεRI and unmutated c-kit. The authors claim that LUVA cells are comparable to human cord blood derived MCs. While I am not aware of bacterial infection studies with LUVA cells in the literature, we demonstrated in our own lab that LUVA cells respond to *S.*Tm with TNF secretion in a similar way as BMMCs, but they did not secrete IL-6 in response to bacterial challenge (see **Paper II**). In addition, LUVA cells did not express TLR4 or respond to LPS but could be activated by the TLR2/6 agonist Pam2CSK4, leading to TNF secretion (**Paper III**).

RBL-2H3

Rat basophilic leukemia cells (RBL) are phenotypically an intermediate between basophils and MCs and were derived from a basophilic tumor in leukemic rats, induced by β-chlorethylamine[138,139]. RBL-2H3 cells can be used to study IgE and FcεRI-dependent mechanisms of MCs and basophils but are rarely deployed in studies of bacterial infections. Their main advantage over other models lies in their strong potential for genetic modification (reviewed in [140]).

There is a controversy about the responsiveness of RBL-2H3 to LPS and how it is mediated. For example, the cells degranulated in response to *S. aureus* enterotoxin B but not to LPS from *S.*Tm[141]. However, LPS from *E. coli* induced IL-13 and TNF secretion by distinct mechanisms and was shown to derive from TLR4/MyD88 signaling[142]. In contrast to these findings, RBL-2H3 cells were shown to lack TLR4 expression and LPS from *E. coli* did neither promote IL-13 nor TNF release, while isolated rat peritoneal MCs transcribed TLR4 and released TNF[143]. It is argued that, in different labs, deviating phenotypes of RBL-2H3 cells were generated due to differences in culture conditions, which might explain contradictory results[144,145]. A follow-up study showed that although TLR4 is transcribed in RBL-2H3, it is not functional due to the lack of CD14 and MyD88 expression[144]. This controversy indicates clearly that RBL-2H3 do not present a convenient model for the study of host-pathogen interactions if the response can be PRR-dependent. However, some responses of the cell line reflect characteristics of primary rat MCs. For example, in RBL-2H3 cells [146] and rat peritoneal MCs, degranulation is inhibited by *C. difficile* TcdB[147]. Furthermore, Fc receptor-mediated phagocytosis requires CDC42 and Rac1 and was demonstrated to be inhibited by TcdB in RBL-2H3 cells [148]. In addition, studies employing live bacteria mainly showed RBL-2H3 activation by special virulence factors. For example, live but not heat-killed *Mycoplasma pneumoniae* could infect RBL-2H3 cells and induce degranulation as well as expression of IL-4, IL-6 and TNF[149], which was connected to the bacterial adhesin P1, allowing surface colonization[150]. A similar pattern was seen for viable *S. penumoniae* strains which induced a degranulation response without cytotoxicity but did not induce IL-6 or TNF expression, in contrast to LPS[151]. Further, the vacuolar cytotoxin A (VacA) from *H. pylori* activated RBL-2H3 cells by induction of calcium oscillations, leading to degranulation and expression of TNF[152]. Another example is *Vibrio vulnificus* which was shown to bind to RBL-2H3 cells and induce actin aggregation via the RtxA1 toxin, resulting in cell death without prior degranulation[153]. Choi et al. (2013) demonstrated degranulation of RBL-2H3 cells upon infection with *E. coli* but not *S.*Tm[154]. Also, Wesolowski and Paumet (2014) demonstrated an inhibition of RBL-2H3 cell degranulation by *E. coli* in the exocytic SNARE mechanism when using a multiplicity of infection (MOI) of 10,000 bacteria per MC, which is less likely to be physiologically relevant[155]. Recently, it was also shown that *Streptococcus oralis* can kill MCs by H_2O_2 induction[156]. Remarkably, the authors did not observe MC mediator release from RBL-2H3 cells during cell death.

In conclusion, RBL-2H3 cells were demonstrated to be a reasonable model for the study of single bacterial virulence factors but is unable to respond to bacteria via PRRs, although this might depend on the cultured phenotype.

Importantly, deriving claims solely based on RBL-2H3 cells for bacterial infection in "mast cells" or "rodent mast cells" should be viewed critically.

MC/9

MC clone 9 (MC/9) is a clonal murine cell line derived from fetal liver and feature phenotypic characteristics of immature mouse MCs *in vivo*[157,158]. In addition, maturation can be induced by sodium butyrate, which results in the formation of granules containing low levels of histamine but no heparin or expression of FcεRI. In contrast to primary murine cells, MC/9 is rarely used in studies of bacterial infection.

For instance, Talkington and Nickell (1999) demonstrated that live and dead *Borrelia burgdorferi* induce TNF secretion and low levels of degranulation in MC/9 cells, which was dependent on bacterial surface proteins, but not to LPS or recombinant OpsA, a surface protein known to activate other immune cells by TLR2 and CD14-dependent mechanisms[159]. Later, the authors showed transcription of CCL3, CCL4 and TCA3 by MC/9, and IL-6 expression after infection with live *B. burgdorferi* which was dependent on FcγRIII[160]. This seems plausible due to a study showing that MC/9 cells transcribe TLR2, TLR4 and TLR6 as well as the adaptors MD-2 and MyD88 but lack CD14 on their surface[161]. However, the group showed secretion of IL-6 and TNF-α after incubation with *E. coli* LPS and *S. aureus* PGN. In another study, MC/9 cells reacted with IL-6 secretion when infected with *Pseudomonas aeruginosa,* which was dependent on protein kinase Cα associated with the phosphatase 2A catalytic unit[123]. Finally, *Bacillus anthracis* lethal toxin induced cytotoxicity in MC/9 cells[162]. Intriguingly, pre-stimulation of MC/9 cells with 100 ng/ml LPS reduced TNF secretion upon antigen stimulation dramatically, mediated by SOCS-1 and SOCS-3 induction[163], while TLR antagonists were reported to increase the antigen-induced cytokine secretion[164].

Overall, MC/9 were shown to transcribe most TLR components and adaptors, but it is unclear how LPS acts on this cell line and what the consequences are. At least activation by *P. aeruginosa* depended on a secreted toxin.

Primary cell lines

Cells, freshly isolated from tissue are called primary cells. Their cultured derivatives are called primary cell lines. Fresh cells usually preserve most of the characteristics they showed *in vivo* with decreasing resemblance over time in culture. Importantly, while PCMCs are cultured mature MCs, BMMCs and MCs derived from human cord or peripheral blood mature in culture, leading to a less defined MC phenotype than PCMCs. Historically, the first primary cell type commonly used were rat peritoneal MCs while nowadays mouse and human cells dominate in studies of bacterial infection.

Rat peritoneal mast cells

In rats, the most common primary MCs are rat peritoneal MCs (rPMCs), which are derived from peritoneal lavage and isolated from other peritoneal cells[165]. Culturing rPMCs results in peritoneal cell-derived MCs (rPCMCs). Rat bone marrow-derived MCs were also investigated[166], but rarely applied in studies of bacterial infection. rPCMCs are an early model of MC culture *in vitro*, employed for many investigations on histamine release upon bacterial infection.

A high number of hemolysins from a range of bacteria was studied in this model. For instance, Gross-Weege et al. (1988) examined the effect of 27 clinical *E. coli* isolates and their supernatants on histamine secretion[167]. Intriguingly, only bacteria expressing or supernatants containing hemolysin were able to induce histamine in rPCMCs and hemolysin expression correlated with histamine release. Importantly, the authors did not assess cell death, which could be responsible for the release of pre-stored histamine, nor did they observe any contribution of type I or type P fimbriae[168,169]. In addition, *Serratia marescens* and *Serratia liquefaciens* which express cell bound hemolysin, activated rPCMCs solely via hemolysin, demonstrated by unresponsiveness to bacterial supernatant of those bacteria[168,170]. Further evidence was shown for the histamine releasing capacity of *L. monocytogenes* and *Aeromonas hydrophila* producing listeriolysin and aerolysin, respectively[168]. *V. vulnificus* cytolysin only induced histamine secretion in rPCMCs when applied in cytotoxic concentrations, leading to cell lysis[171]. In addition, hemolysin-mediated histamine release was also shown for *P. aeruginosa*[172] while in contrast, Friedl et al. (1992) observed an inhibitory effect of *P. aeruginosa* on histamine release[173].

Studies on effects of cell wall components demonstrated that rPCMCs secreted histamine in response to live *S. aureus* and to its cell wall fraction or extracted peptidoglycan, which was lectin-mediated[174,175]. Live *H. pylori*, its bacterial extract or LPS did not induce histamine on their own, but inhibited histamine release by other stimuli[176] which was also demonstrated for *C. difficile* toxin B[146]. Also LTA from *Bacillus subtilis* and *S. aureus* did not trigger histamine release in rPCMCs but instead induced release of cysteinyl leukotrienes [132].

Moving from the sole assessment of histamine release to secretion of cytokines, Leal-Berumen et al. (1994) demonstrated in a hallmark study that *E. coli* LPS can induce IL-6 secretion in rPCMCs without prior release of histamine[177]. Release of IL-6 was dependent on fetal bovine serum, presence of Ca^{2+} in the medium and could be inhibited by pertussis toxin. These findings revealed that when observing MC activation, a focus on preformed mediators such as histamine or tryptase might be insufficient. As also mentioned for MC/9 cells, Talkington and Nickell (1999) showed that live and dead *B. burgdorferi* induce TNF secretion and low levels of degranulation in MCs, which was dependent on bacterial surface proteins but not to LPS or recombinant OpsA, a surface protein known to activate other immune cells by TLR2 and CD14-dependent mechanisms[159]. Lastly, a recent intriguing finding demonstrates that opsonized *E. coli* are taken up into phagosomes, which kill the bacteria and partially dissolve, presenting the content to NOD1 and NOD2 which migrate from the granule matrix, granular surface or cytosolic vesicles to the vacuole[178].

In summary, there are three main insights gained about MC activation by bacteria from rPCMCs: (i) their histamine release by degranulation or cell death upon encounter of hemolytic toxins from a wide range of bacteria, (ii) histamine induction to cell wall components of *S. aureus* such as peptidoglycan but inhibition of degranulation as response to *H. pylori* or its LPS, (iii) histamine-unrelated induction of IL-6 or leukotrienes as response to *E. coli* LPS or LTA from *B. subtilis* or *S. aureus,* respectively, and lastly, how rPCMCs process phagocytosed *E. coli* and respond to it by NOD1 and NOD2. Although rPCMCs transcribe the TLR1-6 and TLR8[145], they were not shown to have a major contribution of recognizing bacteria in rPCMCs.

Mouse bone marrow-derived mast cells

Bone marrow (cell)-derived MCs (BMMCs) are one of the most utilized models for *in vitro* MC research. BMMCs are easy to obtain and generate high cell numbers in culture after four weeks of maturation of bone marrow aspirate in the presence of IL-3, SCF or both cytokines. Their phenotype can be shifted further towards mucosal- or connective tissue-like phenotypes with specific cytokine cocktails, as done in **Paper III**. While BMMCs are sometimes claimed to possess a mucosal phenotype, BMMCs cultured under these standard conditions lack the expression of Mcpt1 and have high levels of CPA3. Therefore, this MC model shows an immature phenotype and does not fully resemble neither mucosal nor connective-tissue MCs.

One of the first studies of the MC response to bacteria was done by Malaviya et al. (1994). They showed that *E. coli* expressing recombinant FimH as well as FimH-expressing isolates of *E. coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae* attached to MCs after 1 h of infection. In contrast, *E. coli* strains without the virulence factor showed a 5-fold lower adherence. This binding was dependent on CD48 in combination with caveolae (lipid raft invaginations of the cell membrane)[179]. It was further shown that the bound bacteria were phagocytosed and killed by ROS and acidification of the phagocytic vacuole similar as in macrophages[68]. In a follow up study, it was shown that BMMCs can phagocytose *S.*Tm and *E. coli* and present a T-cell antigen expressed by the bacteria to T-cell hybridoma via MHC-I[74]. In addition, BMMCs secreted TNF after infection with *E. coli* which increased in FimH possessing mutants[180]. BMMCs were also shown to phagocytose *Bordetella pertussis* and present the antigen to primary T-cells. In addition, MCs released TNF, IL-6 and low levels of IL-4 but not IL-10[181]. The *H. pylori* toxin VacA did not induce BMMC degranulate, but promoted the release of CCL2, TNF, IL-6, IL-1β, IL-10 and IL-13[182]. BMMCs can also express LL-37 when stimulated with LPS or LTA, which was required for killing of *Streptococcus faecalis*[183].

Listeriolysin O from *L. monocytogenes* and live bacteria induced degranulation of BMMCs and promoted TNF release[184]. This strong response was later shown to be independent of TLR2/4 while bacteria lacking the pore-forming toxins were only sensed by the TLRs, leading to a comparatively weak response[185]. BMMCs exposed to *S. pyogenes* responded with formation of extracellular traps, thereby reducing bacterial viability[186]. However, since streptolysin induces a sublytic activation of BMMCs and ultimately induces cell death[187], a clear distinction of cell death and extracellular traps may be difficult. In response to *H. pylori* antigen challenge, BMMCs responded with TNF, CXCL1 and CCL4 release while LPS only induced TNF[188]. In an influential study by Dietrich et al. (2010), *S*.Tm was shown to be unable to invade BMMCs and therefore only TLR- but not interferon signaling was induced[58]. Further, analogous to listeriolysin and streptolysin from *S. pyogenes*, *S. equi* induced IL-6, TNF, CCL2, IL-13 and many more cytokines in BMMCs[37], which was later shown to depend on sublytic membrane stress attributable to streptolysin S[187] (**Paper I**). Further studies demonstrated the induction of members of the A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)[189] and granzyme D[190]. BMMCs secrete IL-4 upon infection by *Francisella tularensis* in a TLR2-dependent manner[191]. Scheb-Wetzel et al. (2014) showed that BMMCs exhibit a strong response against live *E. faecalis*, involving the formation of extracellular traps, killing bacteria by the cathelicidin LL-37 release through degranulation. This effect was mediated by MyD88 and TLR2[192]. *S. aureus* depends on hemolysin-mediated upregulation of β1 integrin (CD29) in order to internalize into BMMCs[193]. Upon *P. gingivalis* challenge, IL-6 and TNF was secreted[194]. *B. burgdorferi* induced secretion of IL-6 and CCL2[195]. BMMCs degranulate and release IL-6 and CCL2 upon exposure to *S. pneumoniae* dependent on pneumolysin[196]. *Brucella abortus* induces secretion of IL-1β, IL-6, TNF and CCL2-4 in BMMCs[197]. Lastly, *Chlamydia trachomatis* was demonstrated to trigger TLR2-mediated IL-6 secretion[198].

Overall, BMMCs can be activated by bacteria by many pathways. Here it is noteworthy, that they do express several TLR receptors, including TLR2, 4 and 6 and respond to classical TLR ligands, a feature that is not present in all cell lines.

Mouse peritoneal mast cells and peritoneal cell-derived mast cells

Compared to rat peritoneal MCs, the mouse counterpart (PMCs) is a more rarely used model in investigations of bacterial infection. However, the establishment of peritoneal cell-derived MCs (PCMCs), which can proliferate in culture, has led to some recent insights. PCMCs are fully mature MCs in contrast to BMMCs which display an immature phenotype[199].

PMCs were shown to degranulate upon binding of *E. coli* expressing FimH[68] and secrete IL-6 in response to LPS stimulation. However, no VEGF was secreted in response to live *E. coli* or LPS[200]. PMCs can also react to immune complexes of *L. monocytogenes* in mouse serum with IL-6 secretion. This was shown to depend on the costimulatory binding of C1q or type 1 collagen to integrin α2β1 and the binding of *Listeria*'s internalin B to the c-met receptor. It did however not depend on the FcγR or other complement components[201]. Importantly, neither heat-killed bacteria, nor the TLR2 and TLR4 agonists Pam3CSK4 and LPS, promoted IL-6 secretion after 1 h. In addition, *Anaplasma phagocytophilum* binds to PCMs[202]. PCMCs infected with *S. aureus* launched transcription of various inflammatory factors including IL-3, IL-13, TNF with confirmed secretion, as well as CCL3, VEGFA and IL-6[76]. Following these observations, Johnzon et al. (2016) showed that VEGF is secreted in high amounts upon infection with *S. aureus* after 6 h but not 2 h of co-cultivation, while the bacterial cell wall components LPS, LTA, PGN, Pam3CSK4, or a combination of these, did not induce VEGF transcription[77]. Further, heat-killed *S. aureus* or conditioned media failed to induce a response and the effect was not dependent on MyD88, NFAT and only partially on NFκB[77]. This is in line with our own observations that *S. equi* can induce secretion of TNF, IL-6 and CCL2 in PCMCs only when producing streptolysin S, which indicates the role of TLRs is less important in the immune responses of MCs towards bacteria[187]. Besides, it was also demonstrated that PCMCs upregulate granzyme D transcription in response to *S. equi*. Intriguingly, we observed recently that PCMCs react to *S.*Tm similar to BMMCs with secretion of IL-6 as well as transcription of *Nr4a3*, *Tnf* and *Il1b*[60].

In summary, PCMCs are very similar to BMMCs in their responses to bacteria. Since the standard BMMC culture protocol with IL-3 and SCF results in connective tissue MC-like BMMCs, although immature[204], such similarities appear to be reasonable.

Human cord blood-derived mast cells

Analogous to the generation of MCs from progenitors in mouse or rat bone marrow, mature human MCs can be obtained by the culture of $CD133^+/CD34^+$ progenitor cells from cord blood, peripheral blood or bone marrow. CBMCs are widely used in human viral infections but also utilized in studies of bacterial interplay with MCs.

CBMCs are an early model of studying the interaction of bacteria and human primary MCs. Arock et al. (1998) characterized the interplay between CBMCs in human serum and clinical isolates of *S. aureus*, *S. faecium*, *K. pneumoniae* and *Citrobacter freundii* as well as *E. coli* strains with a plasmid for FimH expression[205]. All bacteria except $FimH^-$ *E. coli* bound to MCs (*K. pneumoniae* weakly). Interestingly, while strong binders induced a high TNF secretion, the weak binders triggered a 20-30-fold lower secretion. Internalization was observed for *S. faecium*, while killing was demonstrated for bound *S. aureus* or FimH$^+$ *E. coli*[205]. Lin et al. (1999) demonstrated later that "some" FimH$^+$ *E. coli* can be internalized by CBMCs[115]. In addition, CBMCs express MHCII, CD80 and CD117 and could efficiently present the superantigens SEB and toxic shock syndrome toxin 1 (TSST1) to T-cell hybridomas[115]. CBMCs can secrete IL-6 in response to *P. aeruginosa,* mediated by protein kinase C α (PKCα)[123]. It was also shown that *P. aeruginosa,* and to a smaller degree LPS but not *L. pneumophila,* could induce CCL20 secretion in CBMCs[122]. *P. aeruginosa* also induced secretion of GM-CSF. Intriguingly, Varadaradjalou et al. (2003) showed that priming with IL-4 and presence of fetal bovine serum (FBS) is necessary for CBMC TNF secretion in response to LPS from *E. coli* but not to PGN from *S. aureus*[207]. The authors showed that CBMCs transcribe and possess TLR2 and TLR4 protein in cell lysates, but their surface expression was low to undetectable. However, blocking TLR4 abolished the response to LPS, and blocking TLR2 the response PGN, indicating the function of those receptors on the cell surface. The effect of serum was solely dependent on the presence of soluble CD14. Although LPS did not promote histamine release from CBMCs, PGN induced degranulation in a dose-dependent manner. IL-4-primed CBMCs also secreted IL-5, IL-10 and IL-13 in response to both bacterial components. Further, recombinant *P. aeruginosa* endotoxin A as well as live bacteria caused apoptosis in CBMCs, mediated by caspase 3[124,125]. Interestingly, *S. aureus* was shown to bind to CBMCs with ca. 30% of bacteria binding and ca. 1.3% being internalized without being killed[208]. When using heat-killed bacteria, internalization was reduced by ~30%. Uptake of bacteria by CBMCs could be inhibited to 80% by using cytochalasin D. Analogous to their findings in LAD2 cells, Imajo et al. (2009) demonstrated that LTA can enhance the uptake of opsonized *S. aureus* in CBMCs[132]. While no secretion of IL-1β, Lymphotoxin α (formerly TNF-β) or IL-5 in response to *S. aureus* was seen, CBMCs reacted with the release of TNF, CXCL8 (both increased by LTA), IFN-γ, IL-2, IL-4, IL-10 (all decreased) and IL-12p (unaffected by LTA)[132]. Haidl et al. (2011) demonstrated that CBMC express NOD2 and NLRP3 and secrete IL-1β, IL-6, IL-10, CXCL8 but no IL-5 or IL-12p upon incubation with PGN (ligand to TLR2 and nod-like receptors (NLRs)) and Pam_3CSK_4 (TLR1/2 ligand), while MDP (NOD2, NRLP3 ligand) and murabutide (NOD2 ligand) did not induce cytokine secretion[209]. However, CBMC activation in response to Pam_3CSK_4 was enhanced when combined with MDP or murabutide. While no degranulation was observed in any condition, LTC_4 was secreted in a similar pattern as the cytokines. Intriguingly, IL-6 secretion was solely higher in response to NOD2/NLRP3 stimulation compared to PGN, which was mediated by the involvement of cAMP response element-binding protein (CREB), in addition to the NF-κB response common among all other induced cytokines. When inhibiting this pathway, IL-6 secretion was reduced dramatically, while IL-1β production remained unaffected. However, when inhibiting IKK-2, the release of both cytokines was decreased[209]. Mayavannan et al. (2023) discovered in a comprehensive study of *C. trachomatis* interaction with CBMCs that the bacteria are taken up but no increase of intracellular bacteria was observed until 48 h[198]. CBMCs responded in a TLR2- and intracellular receptor-dependent secretion of inflammatory cytokines, including TNF, IL-6 and GM-CSF. *C. trachomatis* did not affect viability or induced degranulation[198].

Human peripheral blood-derived mast cells

Analogous to CBMCs, peripheral blood progenitors can be cultured in the presence of IL-3, IL-6 and SCF to give rise to a mature MC population. Peripheral blood is widely available and the resulting MCs are generally viewed as a good model for human MCs. Importantly, the tissue from where the progenitors are extracted (cord blood, peripheral blood etc.), the progenitor population chosen ($CD34^+$ or $CD133^+$) as well as the culturing conditions affect the MC phenotype[210,211].

PBMCs transcribe and translate TLR1-7 and TLR9, and secrete TNF upon PGN (TLR2 ligand) stimulation[129]. In addition, polyI:C (TLR3 ligand) induced IFN-α and CysLT while LPS (TLR4 ligand) induced TNF, IL-1β, GM-CSF, CysLT and IL-5 secretion. Flagellin (TLR5 ligand) induced TNF and IL-1β while CpG-A oligonucleotides (TLR9 ligand) promoted IFN-α, TNF and CysLT. However, Suurmond et al. (2015) did not find a significant induction of TNF in response to PGN, LPS, flagellin or FSL-1 (TLR6 ligand) and PGN did not induce GM-CSF[212]. Besides, the group showed that IgE + antigen stimulation acted synergistically with triggering of different TLRs. In addition, LTA can enhance the uptake of opsonized *S. aureus*, as well as increase the amount of secreted CXCL8[132]. IL-1β, TNF , IFN-γ, IL-2, IL-12p70, IL-5 and IL-10 were either not secreted or secreted at amounts very close to the detection limit, while the secretion of IL-4 was not affected. When infected with two strains of *Aggregatibacter actinomycetemcomitans*, or *Chlamydia penumoniae*, PBMCs increased the transcription of CXCL8 for both bacteria but TNF and CCL2 only on infection with *C. pneumoniae*[213]. In addition, cytokine secretion was shown for all cytokines above when PBMCs were infected with *C. pneumoniae* with an increase proportional to the MOI of 1-10. While there was no change for PBMC activation when *A. actinomycetemcomitans* were killed by UV light, dead *C. pneumoniae* induced lower levels of CXCL8 and TNF than did the live bacteria. No bacteria caused IL-10 or TGF-β1 secretion or LDH release. Notably, CXCL8 and TNF gene transcription could also be induced when co-cultivating PBMCs with infected macrophages or their supernatant. In addition, the *A. actinomycetemcomitans* strain AT445b, which possesses fimbriae, did not induce a stronger response than JB2 which lacks fimbriae[213].

Oksaharju et al. (2011) also investigated the transcriptional changes of PBMCs induced by the probiotic bacteria *Lactobacillus rhamnosus* GG and

Lc705, *Propionibacterium freudenreichii* ssp. *shermanii* JS (PJS) and *Bifidobacterium animalis* ssp. *lactis* Bb12 (Bb12)[214]. While after 3 h no significant transcriptional changes were observed, the group showed that, after 24 h, both Lactobacilli and *Bifidobacterium* induced the upregulation of cytokines (e.g., TNF, IL1β, CXCL8, IL10, CCL2), innate immune receptors (TLR1, TLR6, NOD2), CASP3 and CASP8. In addition, the Lactobacilli caused downregulated expression of genes involved in the cell cycle. The strain LC705 had the strongest effect on TLR1 and NOD2 while the strain Bp12 induced the strongest upregulation of IL1β and TNF. Intriguingly, LC705 induced downregulated expression of several FcεRI receptor components. Overall, the JPS strain induced only very weak overall effects on gene expression. It is noteworthy that the bacteria were added to PBMCs in medium containing antibiotics (penicillin and streptomycin)[214].

When targeting TLRs by different agonists, Suurmond et al. (2015) found that PBMCs respond with CXCL8 secretion to all tested stimuli including PGN and LPS while PGN failed to induce GM-CSF or IL-10[212]. It is noteworthy that the response to flagellin was limited to CXCL8 induction. Neither PGN nor LPS induced TNF or CCL3 expression. Intriguingly, stimulation with IgE + antigen enhanced the response of TLRs to their agonists[212]. The opposite effect was observed when PBMCs where treated with cysteinyl leukotrienes, which caused decreased transcription of the TLR1-7 genes, depending on the type of leukotriene, but no effects on the expression of TLR8 or TLR9[135]. LTC_4 was shown to cause decreased surface expression of TLR2 and TLR6, but not TLR3 and TLR4. While overall, PBMCs reacted to PGN, LTA and Pam3CSK4 with TNF-secretion, no response was observed to LPS. In addition, LTC4 only decreased the response to PGN (TLR2).

PBMCs were shown to degranulate upon challenge with *L. monocytogenes* or *E. coli* but not in response to *S. pneumoniae* and *S. aureus*[215]. This pattern was reflected by secretion of CXCL8 and CCL2 while *S. aureus* also induced a prostaglandin response. *S. penumoniae,* on the other hand, induced strong ROS production in PBMCs. It is noteworthy that effects were observed in narrow experimental conditions of MOI 25 and 2 h of bacterial challenge, which might not be appropriate to compare different bacteria with each other.

Induced pluripotent stem cell (iPSC) mast cells

Over the last years, iPSCs have revolutionized various fields of research. The ability to gain pluripotent patient-specific stem cells which can be differentiated into a wide array of cells applying noninvasive procedures such as the use of skin fibroblasts from patients enable new dimensions in cell biology. Subsequently, several groups reported the generation of iPSC-derived MCs[216–218]. Future findings will reveal if MCs from iPSC will have an impact on our understanding of the interaction of MCs with bacteria.

Mast cells derived from human tissue

Fully mature MCs can be isolated from theoretical every tissue known to contain MCs, to study their phenotype.

Among the first to study the effect of bacterial antigens on tissue-derived MCs were Brzezińska-Błaszczyk et al. (1988) who utilized the mesentery of gall bladders and adenoids from patients. Both mesenteric and adenoid MCs released histamine in response to a mix of *S. aureus* protein[219]. Bischoff et al., (1999) showed that "mucosal MCs" inside human intestinal tissue as well as those isolated from tissues, express TNF[220]. Moreover, TNF was increased by cultivation of isolated cells with LPS or a conglomerate of gut bacteria such as *E. coli*, *Klebsiella oxytoca* and *Stenotrophomonas maltophilia* and it was indicated that MCs are among the strongest producers of intestinal TNF[220]. Following up on these observations, Bischoff's group showed the constitutive expression of a wide array of other cytokines by intestinal MCs, including IL-1β and IL-6, while also observing a suppressive role of IL-4 on MCs [220,221]. However, infection with their bacterial conglomerate only further induced TNF expression while not affecting other cytokines[221]. Genovese et al. (2000) isolated MCs from the heart of patients and found that these release histamine in response to dead *S. aureus*, harboring protein A on their cell wall as well as soluble protein A on its own, while strains not expressing protein A did not evoke a reaction. This was depending on protein A binding to surface-bound IgE on MCs, and this mode of stimulation evoked similar histamine and LTC_4 release as in MCs activated with Anti-IgE[222]. The same response pattern was observed to dead *Peptostreptococcus magnus* and its superantigen protein L. The effect of *S. pneumoniae* on lung MCs isolated from macroscopically normal lung from lung cancer patients was studied by Cruse et al. (2010). Lung MCs reacted to pneumolysin-bearing *S. penumoniae* by secretion of LTC_4 and killing of bacteria, while no reaction could be observed for a strain lacking pneumolysin. Intriguingly, sublytic concentrations of pneumolysin alone led to the same response in the lung MCs and no histamine or cytokines were triggered in any setting[127]. Further, the tick-borne pathogen *A. phagocytophilum* was shown to efficiently bind to and invade MCs derived from human skin[202].

Generalizable mechanism(s) for mast cell sensing of bacteria and effector mechanisms

Since the current literature on the interaction between MCs and bacteria is heterogenous and rife with contradictions, it is widely believed that MC activation depends on the MC model, bacterial species, or even the specific strain (reviewed in [59]). However, generalizable models for how MCs respond to bacterial insult are required for disease prediction and treatment.

One topic of controversy within the current literature is the role of PRRs and especially TLRs in bacterial infection. One needs to keep in mind that certain PRRs may be expressed in (primary) cell lines but are not active *in vivo*. In addition, ligand-receptor binding does not necessarily lead to activation. For example, there are several studies showing that commensal bacteria suppress TLR activation in the gut and other tissues. Nevertheless, it can be postulated that cellular MC models mostly respond to bacterial cell wall components in some way if TLRs are expressed. On the bacterial side, it needs to be considered that bacteria are more than bags of LPS or PGN. It is unlikely that only a single type of molecule promotes a specific phenotype. PRR responses derive from a combination of specific ligands expressed by a bacterium which may carry modifications (smooth/rough LPS, or a capsule which shields cell wall ligands from detection). In addition, PRRs may or may not be expressed or active in the given window in time and space where the bacterium and the MC meet. One of the most studied bacterial cell wall molecules for MC activation is LPS. While LPS can activate various kinds of MCs[164,207], bacteria containing LPS do not necessarily do so, and can even inhibit MC activation mediated by other stimuli[223]. TLR ligands alone do not seem to trigger MC activation to the same extent as bacterial virulence factors or IgE-mediated activation. A much more potent form of MC activation is mediated by pore-forming toxins. When observing the response of human intestinal tissue and MCs isolated from the latter to S. *flexneri* and various *E. coli* strains[224], and the responses of BMMCs to *L. monocytogenes*[184], *S. pneumoniae*[196] and *S. equi*[187], MCs respond very weakly to most bacteria via TLRs. In all these cases, only lytic or sublytic concentrations by the bacterial toxin hemolysin, listeriolysin O, pneumolysin or streptolysin S mounted a powerful proinflammatory response, in strong contrast to bacteria lacking pore-forming toxins. The role that these toxins play *in vivo* and the relative potency to TLR-, or other PRR-dependent, MC activation remains a matter of future elucidation. One example can be found in the framework of atopic dermatitis, where δ-toxin expressing *S. aureus* were responsible for MC activation[225]. Interestingly, pore-forming toxin-mediated MC activation is not exclusive to bacteria. Noteworthy examples of this include candidalysin of *Candida albicans*[226]. Another emerging mechanism for the bacteria-mediated activation of host cells is the concept of effector-triggered immunity (reviewed in [227,228]). Many enterobacteria harbor needle-like TTSS to inject effector proteins into host cells or deliver virulence factors in other ways. The ultimate goal is to influence host signaling to force phagocytosis of the bacterium inside the host cell, suppression or activation of immune responses as well as inhibiting cell death pathways. However, there are many guards in place inside the host cell to sense manipulations by such bacterial effectors. In addition to effector-triggered immunity, another paradigm shift in infection biology is the concept of innate immune activation by infection infidelities, involving for example accidental leakage of cell wall components from bacteria inside host cells[229].

Once the bacteria are recognized, MCs have a repertoire of response mechanisms. These include different forms of cell death, extra- and intracellular bacterial killing, phagocytosis of bacteria, degranulation, leukotriene release or cytokine release. Some of the above strategies are mutually exclusive or lead to different outcomes for the environment[59]. Apoptotic cell deaths, for instance, is unlikely to lead to the release of granule components into the environment[230], whereas lytic cell death likely does[187].

If bacteria are actively internalized by MCs or passively being invaded by S. *aureus* or *S.*Tm is difficult to assess and requires non-virulent bacteria as control. Heat-killed bacteria may not be taken up although live bacteria would be. Bacterial uptake into MCs is especially frequently reported in the literature when bacteria were opsonized with MC-species compatible serum (human serum for human MCs, mouse serum for mouse MCs)[132,178]. During the process of opsonization, foreign particles are covered with serum-derived proteins such as C3b or antibodies (usually IgG). Instead of the innate immune system sensing of bacteria directly, the antibodies or serum-derived proteins bind to Fc receptors or complement receptors, respectively, which trigger distinct signaling pathways inside the MC, leading to different outcomes[132,178]. It seems plausible that actively invading bacteria, which create their own intracellular niche and a strategy to survive, are less in danger of being killed by intracellular MC mechanisms, especially compared to those which are already opsonized. In addition, it can be predicted that MC pathways are highly similar towards opsonized bacteria, independent of the bacterial strain or species. To the best of my knowledge, the only bacterium *in vivo* being found inside MCs to a quantifiable amount in previous studies is *S. aureus* in nasal polyp MCs[231]. Hence, it has been proposed that *S. aureus* is in fact the only bacterium being able to invade MCs[232]. However, we find that *S.*Tm can invade both BMMCs, PCMCs and LUVA cells (**Paper III),** and most likely also other MC tissue culture models. This is likely also the case with similar invasive bacteria unrestricted to a specific cell type such as *S. flexneri*. Active uptake by MCs of non-opsonized bacteria is a rarer process. So far, this was shown for $FimH^+$ *E.* coli as mentioned above and in one study for cord blood derived MCs[205] and in BMMCs for the non-virulent competent *E. coli* BL21[233], *P. aeruginosa*[234] and *A. actinomycetemcomitans*[235]. The ability of MCs to kill intracellular and extracellular bacteria by use of their granule compounds which contain defensins such as cathelicidins/LL-37, is likely to be a general mechanism. However, the resulting outcome by an infection can, again, be influenced by the bacterial pathogen, utilizing proteinases or inhibitors to degrade the defensin[236]. Autophagy was also suggested as a strategy to combat intracytosolic bacteria[234]. How much MCs contribute to bacterial killing *in vivo* remains to be determined. One important factor in the efficiency of eradicating bacteria is the MOI, which describes the number of bacteria per host cell in an infection experiment. Bacterial killing is much more likely to be seen when using MOIs < 1, where the MC are in the majority, as compared to at higher MOIs, where

MCs are highly outnumbered by bacteria[117,192]. Another mechanism of antibacterial defense are METs, for example seen in BMMCs[186,192]. Those were to date not shown in animals upon bacterial infection but observed for several MC tissue culture models. Their relevance in physiologically native infection models remain to be demonstrated. Lastly, returning to the role of MCs in adaptive immunity, the role of IgE is mostly ignored in *in vitro* infection settings when not addressed directly. *In vivo*, all MCs likely always harbor surface-bound IgE, while this is not the case during most tissue culture infection experiments.

Immunomodulatory mechanism of the gut microbiota

In contrast to intestinal pathogens which promote a powerful immune response and massive increase of mucosal MC numbers in the gut, a wide body of literature suggests a suppressive role of the commensal microbiota on MCs in the intestinal mucosa. This includes *Lactobacillus*, certain Enterobacteriaceae and *Enterococcus* on MMCs. Commensals generally appear to only trigger a subtle inflammatory response in the gut. Moreover, they are needed during development to obtain a functioning population of MCs in the intestines. In addition, bacterial products from *Lactobacillus helveticus* present fermented milk, but not the bacterium itself, slightly enhanced numbers of MMCs and other immune cells in the intestine via induction of calcineurin[237]. Although the presence of a gut microbiota was observed to have a clear effect on MMCs in human flora-associated piglets, the composition of the microbiota does not necessarily change MC numbers in different parts of the intestine, as long as they are free of major pathogens[238]. However, piglets inoculated with human microbiota differed from those obtaining pig flora in intestinal morphology and the further immune landscape. Wang et al. (2017) demonstrated that feeding live bacteria (probiotics) to animals with already present microbiota is still contributing to a beneficial outcome[239]. For instance, rabbits fed with *Lactobacillus casei* or *Lactobacillus zeae* increased the number of MCs in caecum while no influence on MCs in duodenum or jejunum was seen, but the incidence of diarrhea was reduced in comparison to a normal control diet.

The art of mast cell tissue culture

I believe that the choice of MC model and culture conditions affect the interaction of bacteria and MCs, and can be a confounding experimental factor too infrequently discussed. Hence, I dedicate a chapter to the topic. Since the general methods are included in the manuscripts, I will provide some additional background and personal insights for cell culture in infection biology.

While BMMCs are widely used, their method of culture varies, and their phenotype is mostly assessed by flow cytometry of cells stained for CD117 (c-kit) and FcεRI. However, transcripts, expression of other surface receptors and even the protease types and levels expressed may vary in different culture conditions. Every BMMC culture starts with the mouse sacrificed for bone marrow. During my projects, I did not observe differences in BMMCs derived from donor mice of different gender or age (6-20 weeks) in the readouts I performed. To the best of my knowledge, the influence of the microbiota and animal facility on BMMC phenotype has not been studied so far.

Following the donor selection, the researcher has a small variety of different bone marrow extraction methods. While some groups only use the tibia as source[240,241], others use both, femur and tibia[242]. In addition, bone marrow cells may be dissociated by passing cells through needles and/or applying cell strainers to remove clumps[240,243]. The presence of tissue residues in the bone marrow culture may have an effect on the culture conditions.

During bone marrow culture, BMMCs and their progenitors will be selected from bone marrow by three different enrichment strategies: (1) the presence of MC specific growth factors, (2) the removal of attached cells during passaging and (3) long culture time to enrich for long-lived cells. Changes of protocol in any of these steps will likely change the yield of BMMC and/or their phenotype.

BMMC are commonly cultured in DMEM[187,242,244], IMDM[245] or RPMI media[246] with 10% FBS, 4.5 g/l glucose and a source of L-glutamine (direct or GlutaMAX). Optional components include 2-mercaptoethanol, pyruvate, and/or non-essential amino acids. Those conditions could result in slightly different BMMC phenotypes, but to date and to the best of my knowledge, no study comparing basal or complete medium culture conditions in BMMC establishment and growth exist. Instead, phenotypical differentiation is commonly induced with niche-specific cytokines. To select for MC differentiation in mouse bone marrow, IL-3 or SCF needs to be present. Either cytokine alone is sufficient to generate >90% of CD117$^+$ FcεRI$^+$ BMMCs which express Mcpt6 and Cpa3[247]. Both cytokines in combination with IL-4 shifts the MCs into a CTMC-like phenotype and enhances Mcpt5 promoter activity[248]. Supplying TGF-β will change the BMMC phenotype to mucosal-like MCs expressing Mcpt1 and Mcpt2 while downregulating the proteases above, which can be further supported by the addition of IL-9 and higher SCF levels[244,249]. For all MC-dependent growth factors, recombinant cytokines or conditioned media can be used. Also, a combination of both is applied by some groups[204]. Although results are highly comparable when using the same growth factor concentrations, those should be assessed regularly in conditioned medium.

When selecting suspension cells, I noticed that different cell culture-treated flasks perform better than others. Bone marrow cells attach, for example better on Saarstedt flasks than on those from VWR. Bone marrow cultured in tissue culture-treated VWR flasks led to a BMMC population after 4 weeks which was barely proliferating anymore. On another note, quantitatively, the cytokine response of different BMMC cultures can differ under equal conditions. Hence, when comparing WT BMMC to BMMC from different mutant mice, the experiments should ideally be performed on more than one BMMC culture per genotype. Lastly, the genetic background of the mice can affect the expression of cytokines and other factors[246].

Present investigations

Aims

The current literature on the interaction of MCs and bacteria is based on a large variety of cell culture models from humans and other animals. This resulted in a lack of general mechanisms or postulates of how MCs are activated by bacteria. Therefore, our research aimed to identify and explore generalizable response pathways which extend beyond bacterial strain and species-specific MC responses. In my work, I used two classes of bacteria with distinct virulence factors. While *S. equi* is a gram-positive bacterium that infects horses and activates MCs by until this point poorly defined virulence factors, *S.*Tm is a gram-negative intracellular pathogen which invades host cells by their TTSS and modulates the MC intracellular signaling. *S. equi* was chosen as a tool for MC activation due to its diverse set of virulence factors, shared with many other Streptococci. *S.*Tm causes disease across a variety of humans and other animals, but prior to our studies it had remained unclear if *S.*Tm can actively invade MCs, and what responses extracellular versus invasive bacteria would elicit.

The specific aims were:

Paper I
To uncover which virulence factor(s) of *S. equi* is/are involved in the powerful MC activation seen during MC/*S. equi* co-culture and understanding the mechanism behind it.

Paper II
To explore if *S.*Tm can activate MCs, if this activation involves invasion or endocytic uptake and what role the TTSS plays in the interaction between MCs and *S.*Tm.

Paper III
To investigate if different MC cell models, belonging phenotypically to distinct tissue niches in connective tissue or mucosa, respond differently to infection by *S.*Tm.

Paper I: Streptococcal *sagA* activates a proinflammatory response in mast cells by a sublytic mechanism

Originally, the group of Gunnar Pejler established that BMMCs respond potently to the equine pathogen *S. equi* in a mostly contact-dependent matter, relying on TLR2 and TLR4[37]. While no degranulation was seen, BMMC responded with a strong immunomodulatory response including the secretion of cytokines such as IL-6, TNF and CCL2.

We adapted this infection setup to explore which virulence factors and virulence-associated genes of *S. equi* are responsible for the phenotype observed in BMMCs. The group of Andrew Waller supplied expertise and bacterial knockout strains, which comprised both single- and multi virulence gene knockouts [38]. Since we knew which cytokines are secreted by BMMCs in response to *S. equi*, we screened BMMCs infected with virulence factor knockout strains for IL-6, TNF and CCL2 secretion by ELISA[187]. While the contribution of many virulence-associated genes was assayed, only deletion of one gene led to a radical change of phenotype: *sagA* (streptolysin associated gene cluster a). BMMCs or PCMCs which were infected with *S. equi* lacking *sagA* (*S. equi*$^{\Delta sagA}$) showed none of the phenotypic changes caused by *S. equi*^{wt}. In fact, the only observed difference between untreated MCs and *S. equi*$^{\Delta sagA}$ was an aggregation of cells in cocultures with *S. equi*^{wt} but not *S. equi*$^{\Delta sagA}$, seen by live microscopy. Since *sagA* encodes for streptolysin S (reviewed in [250]), we wondered if cell lysis is involved in the MC response triggered by *S. equi*^{wt}. In contrast to *S. equi*$^{\Delta sagA}$, *wt S. equi* induced LDH release (lactate dehydrogenase, an intracellular enzyme which is released during lytic cell death) and killed the whole BMMC population from ca. 6 h onwards, observed by live microscopy using the fluorescence cell death exclusion dye propidium iodide. Intriguingly, powerful cytokine release was observed already at 3-4 h. This led us to believe that similar to other cytolytic virulence factors such as listeriolysin O from *L. monocytogenes*[184], hemolysin from *E. coli*[224] or streptolysin S from *S. pyogenes*[251], the pores formed in MCs and other immune cell membranes by the streptolysin S trigger activation before the membrane damage becomes fatal (reviewed in [252]). The downstream signaling of this sublytic activation was shown to depend on the MAPKs Erk and p38 but not on JNK. We were also able to phenocopy the cytokine response of BMMCs infected by *S. equi* with recombinant pneumolysin from *S. penumoniae*, and even with low levels of detergents such as saponin and digitonin.

Overall, we established a model of a general, bacterial species-independent, sublytic activation of MCs by *S. equi* and uncovered its potential role in a diversity of different MC activation scenarios.

Paper II: A two-step activation mechanism enables mast cells to differentiate their response between extracellular and invasive enterobacterial infection

The enteropathogen *S.*Tm invades mammalian host cells by binding and inserting its needle-like TTSS into the membrane of its target. Subsequently, this either leads to uptake of *S.*Tm by TTSS effector-driven membrane ruffling or discreet invasion[43]. Since we established in **Paper I** that MCs can be activated by membrane pores and the TTSS should produce those as a needle-like injectosome, we hypothesized that *S.*Tm triggers a similar response as *S. equi*. Initially, we infected BMMCs with *S.*Tm^{wt} and knockout strains lacking a TTSS (*S.*Tm$^{\Delta invG}$) or the four main effector proteins SipA, SopB, SopE and SopE2 (*S.*Tm$^{\Delta 4}$). If the TTSS by itself and its capacity for forming membrane pores through its translocon would be sufficient for MC activation, *S.*Tm^{wt} and *S.*Tm$^{\Delta 4}$ should induce a highly similar phenotype while *S.*Tm$^{\Delta invG}$ would fail to elicit a response. However, BMMCs secreted high amounts of IL-6 when challenged with *S.*Tm^{wt} but not with any of the mutants. With the rejection of our hypothesis, we learned that MCs are potently activated by *S.*Tm but respond in a fundamentally different way to *Salmonella* compared to the cytolysins of other bacteria. Intriguingly, we did not observe degranulation of MCs in response to *S.*Tm, but a strong cytokine response, independent of the presence of the SptP effector that was previously reported to inhibit MC activation[154].

*S.*Tm invades the epithelial barrier from the lumen and turns off virulence factors such as the TTSS gradually when reaching the deeper tissue layers where MCs often reside. To understand if the MC activation we observed during *in vitro* tissue culture could exist under physiological conditions *in vivo*, we stained caecum tissue sections from mice orally infected with *S.*Tm[55] for 2 and 42 days and corresponding controls. Importantly, we used an *Nramp*-sufficient mouse strain, with streptomycin pre-treatment which enables reproducible gut infection without systemic spread as seen in mice lacking *Nramp*[55]. We were able to see direct contact between *S.*Tm and avidin^{+} MCs, a migration of MCs towards the epithelial layer and a 2-fold increase in MC numbers in the inflammatory state. In addition, occasionally avidin positivity but no corresponding DAPI staining was seen in the lumen, possibly indicating dead MCs coming from regions beneath the epithelial monolayer. Following up on the initial experiments with BMMCs and the mutants above, we observed highly similar phenotypes in PCMCs and the human LUVA MC line as well as with two *S.*Tm isolates (SL1344 & 14028). Intriguingly, invasive *S.*Tm strains induced a distinct phenotype from non-invasive strains. While invasion or its associated effector proteins induced high levels of IL-6 and TNF as well as IL-13 and *Ifnb1*, non-invasive mutants triggered only low levels of IL-6. Transcript levels of *Il1b* and *Nlrp3* however were not higher in invaded MCs. These two distinct MC responses depended solely on TLR4 sensing of the non-invasive mutants compared to SopB-mediated Akt phosphorylation in combination with TLR4 sensing for the invasive strains. Importantly, we were not able to block uptake of *S.*Tm without disturbing the mechanism of effector proteins and we are therefore unable to determine if bacterial uptake needs to take place or if injection of SopB is a sufficient trigger. However, blocking Akt phosphorylation dampened MC activation without influencing invasion. Another important finding is the fact that while epithelial cells and macrophages die by inflammasome-mediated pyroptosis within a few hours, > 80% of the infected BMMCs survived for up to 24 h in culture. This could be explained by low expression of PRRs, inflammasomes and gasdermins in MCs and could have implications for MCs as long-term *S.*Tm reservoir in mice and humans.

Overall, we demonstrated that MCs can not only be invaded and potently activated by *S.*Tm, but that this response also requires the dual factors of TLR4 sensing and SopB (and to a lesser extent SopE and SopE2) injection. This stands in contrast to the literature where MCs are believed to be silenced by *S.*Tm[154], lack interferon response and are unable to be invaded[58]. A likely explanation is the importance of growing *S.*Tm under virulence-inducing conditions which is required for TTSS expression and invasiveness.

Paper III: Mast Cell Subtype Features Impact the Interplay with Pathogenic *Salmonella* Typhimurium Bacteria

In **Paper II**, we established how *S.*Tm activates MCs in a two-step activation mechanism involving pattern recognition sensing via TLR4 and effector-triggered immunity[228] to distinguish invasive pathogens from bystanders. In this manuscript, we explored the intracellular interaction of *S.*Tm with MC subtypes in a variety of MC model systems and how MC activation and intracellular lifestyle differences may depend on each other. In mice, MCs can be separated into recruited bone marrow-derived MCs (Mucosal MCs, MMCs) and tissue-resident fetal-derived MCs (connective tissue MCs, CTMCs)[253]. While PCMCs are regarded as mature CTMC-like MCs, BMMCs are rather immature when cultured with IL-3 and/or SCF[204]. In this project we first generated CTMC-like "CT-BMMCs" and Mucosal-like "M-BMMCs" with respective cytokine cocktails. While transcripts overall reflected the expected phenotypes, M-BMMCs showed a strong difference to baseline "IL-3-BMMCs" in granule number and density, as well as in protease and TLR expression. We also generated BMMCs from knockout mice, lacking either the four main CTMC proteases Mcpt4, Mcpt5, Mcpt6 and CPA3, or lacking serglycin, the latter promoting the overall storage and integrity of MC granules. While percentages of *S.*Tm-invaded IL-3-BMMCs and CT-BMMCs were mostly similar throughout the genotypes, M-BMMCs showed reduced percentages of MCs harboring intracellular *S.*Tm. This was evident for the vacuolar population of *S.*Tm, but more dramatic for the cytosolic population. The observation contrasted $SG^{-/-}$ M-BMMCs, which harbored the highest levels of intracytosolic *S.*Tm in all tested BMMC types . We also observed intracellular bacteria fusing with or even inside MC granules by transmission electron microscopy. While different MC granule contents led to a vastly different intracellular fate of *S.*Tm inside MCs, strikingly, MC activation and cytokine output was dictated by TLR expression and entirely independent of *S.*Tm's intracellular niche. While baseline IL-3-BMMCs and CT-BMMCs, as well as PCMCs, responded via the previously discovered two-step activation mechanism, using TLR4 as priming response, human LUVA MCs, which lack TLR4, responded via a TLR2/6 axis in combination with TTSS activity recognition. In addition, M-BMMCs lacked response to non-invasive bacteria entirely, due to the lack of TLR2 and TLR4, hence phenocopying the TLR4-knockout IL-3-BMMCs from our previous study (**Paper II**). Overall, this study revealed for the first-time differences in the impact of MC subtype and granule content on enterobacterial infection, and places our "two-step MC activation model" in the context of MC subtype diversity.

Concluding remarks and future perspectives

This thesis contributes to our understanding of the mechanisms for how MCs are activated in cell culture and explored the interdependence between three layers of MC activation by bacteria: PRRs to sense extracellular bacteria, pore-forming toxins to trigger calcium-influx mediated stress responses and the TTSS and its effectors. Generally, there is always potential for deeper investigations: exploring other pore-forming toxins, more TTSS-harboring bacteria, a greater variety of MC models with a focus on primary human MCs, ideally from the native tissue environment where the bacterial infection in focus takes place. However, what I believe is most worthwhile in the current state of the literature, is the elucidation of the phenotype and role of MCs in their native tissue environment to bacterial infection. Besides models of sepsis and certain skin diseases, it is difficult to create a model which addresses a natural route of infection, involving pathways relevant to human biology and hence clinically applicable insights into the role of MCs in bacterial infections. A difficulty to address is the relative rarity of MCs in tissues, for example in the human intestines. Those rare numbers should not discourage the investigator, since MCs release highly concentrated localized effector proteins such as proteases, leukotrienes, and cytokines directly onto neighboring target cells. When combined with the striking longevity of MCs, this makes them a potent force in the tissue environment.

To study these powerful cells further, I suggest single cell or single nucleus based sequencing and spatial omics combined with either patient samples or experimental models, suited for human translation. Those methods are essential to address the MC heterogeneity in the tissues, their role in homeostasis and how they adapt to bacterial infection.

In vivo mouse models are an important tool to experimentally test those insights derived from human tissue and cell culture. Importantly, appropriate genetic backgrounds should be used. In the case of *S.*Tm infection, Nramp^{+} mice are to be preferred, since long term infection can be studied. For general infection setups where the lack of MCs is compared to WT mice, the method of MC knockout should be considered carefully to ensure removal of MCs in the targeted tissue environment with minimal off target effects.

Responses of MCs in tissue culture to bacteria or their ligands are largely explored. I see two opportunities to add insight into niche-specific MC phenotypes: generating iPSC-based tissue specific MC lines (1) and exploring the differences between chymase and tryptase expressing MCs vs. tryptase-only MCs, induced by TGF-β[254] (2).

Overall, the studies contained in this thesis pave the way for future experiments across human and animal mast cell biology. Afterall, as mast cells being tackled by multiple bacteria, hypothesis's being tackled by multiple studies increases the chances of successful infection – or the creation of knowledge.

Populärvetenskaplig sammanfattning

Mastceller tillhör till det medfödda immunförsvaret som bland annat skyddar kroppen från bakteriella infektionssjukdomar. Mastceller är inte bara utrustade med en mängd olika detektorer för att upptäcka främmande mikroorganismer, utan innehåller många defensiva ämnen för att motverka infektioner. På så sätt försvarar mastceller oss mot patogena bakterier som streptokocker eller *Salmonella*. Medan streptokocker försöker påverka mänskliga celler från utsidan med toxiner, försöker *Salmonella* tränga in i tarmens slemhinna och dess celler efter upptag via maten.

Trots årtionden av forskning är det fortfarande i stort sett oklart via vilka allmänna mekanismer mastceller aktiveras vid bakterieinfektion. I min doktorsavhandling behandlar jag just denna fråga, utifrån tre delstudier. I den första studien undersökte jag vilka virulensfaktorer av bakterien *Streptococcus equi* som utlöser ett immunsvar i mastceller. Intressant nog sker detta uteslutande genom toxinet streptolysin S, som orsakar små hål i det yttre skalet (cellmembranet) av celler och på så sätt leder till aktivering av mastcellerna. Vi kunde visa att denna mekanism även är verksam för pneumolysin, det liknande toxinet för pneumokocker, och även för det tvålliknande saponinet. I den andra studien undersökte vi sedan om *Salmonella*, som har en mini-injektionsnål, också bildar små hål i mastcellsmembranet som sedan aktiverar immuncellerna. Istället visade det sig att *Salmonella* aktiverar mastceller i en tvåstegsprocess där detektorer på det yttre skalet först upptäcker bakterierna och den andra signalen triggas av att *Salmonella* tränger in i cellerna. På så sätt kan mastceller skilja på ofarliga och farliga, ”invasiva” bakterier och skicka lämpliga signaler till tarmmiljön. I min tredje och sista studie odlade jag olika typer av mastceller och jämförde dem med avseende på deras svar på *Salmonella*. Jag och mina kollegor upptäckte att, till skillnad från mastceller som liknar dem som finns i bindväv, reagerar mastceller som liknar dem som man hittar i slemhinnor uteslutande på invasiva *Salmonella*. Denna skillnad beror på mastceller som liknar dem som finns i bindväv inte har detektorer för bakterier som finns utanför cellerna.

Sammanfattningsvis har jag med denna doktorsavhandling bidragit med viktig kunskap till den övergripande förståelsen av mastcellens roll vid bakterieinfektioner.

Populärwissenschaftliche Zusammenfassung

Unser angeborenes Immunsystem besteht aus vielen verschiedenen Bestandteilen, die es ermöglichen, den Körper unter anderem vor bakteriellen Infektionskrankheiten zu schützen. Sogenannte Mastzellen sind eines dieser Bestandteile, die mit einer Vielzahl an Detektoren zur Erkennung von Fremdkörpern ausgestattet sind, und ein Repertoire an Abwehrstoffen besitzen, um Infektionen entgegenzuwirken. Auf diese Weise können uns Mastzellen gegen verschiedene krankheitserregende Bakterien wie Streptokokken oder Salmonellen verteidigen. Beide Erreger schaden menschlichen Zellen auf verschiedene Weise. Während Streptokokken Giftstoffe ausscheiden, um von außen menschlichen Zellen zu beeinflussen, versuchen Salmonellen über die Nahrung in den Darm und seine Zellen einzudringen. Wie genau Mastzellen durch Streptokokken und Salmonellen aktiviert werden ist trotz jahrzehntelanger Forschung bis heute weitgehend unklar.

In meiner Doktorarbeit beschäftigte ich mich mit dieser Fragestellung, die auf drei Studien aufgebaut ist. In der ersten Studie untersuchte ich, welche Virulenzfaktoren des Bakteriums *Streptococcus equi* in Mastzellen eine Immunantwort hervorrufen. Interessanterweise geschieht dies ausschließlich durch das Toxin Streptolysin S, das winzige Löcher in der Außenhülle von Zellen verursacht und auf diese Weise zu einer Aktivierung der Mastzellen führt. Wir konnten nachweisen, dass dieser Mechanismus auch mit Pneumolysin, dem entsprechenden Toxin der Pneumokokken und sogar mit dem seifenartigen Saponin passiert, die ebenso kleine Löcher in die Mastzellmembran einfügen. In der zweiten Studie untersuchten wir dann ob Salmonellen, die keine Giftstoffe anwenden, jedoch über eine Miniatur Injektionsnadel (Typ-3-Sekretionssystem) verfügen, um in die Zellen der Darmumgebung einzudringen, auf eine ähnliche Art gegen Mastzellen wirken. Wir gingen davon aus, dass Salmonellen mit Hilfe ihrer Injektionsnadel, ebenso wie die Giftstoffe der Streptokokken, Poren in der Mastzellmembran formen, die die Immunzellen dann aktivieren. Stattdessen stellte sich jedoch heraus, dass Salmonellen Mastzellen in einem Zweischritt-Prozess aktivieren, in dem Detektoren auf der Außenhülle erst die Bakterien erkennen und das zweite Signal von Salmonellen ausgelöst wird, die in die Zellen eindringen. Auf diese Weise können Mastzellen zwischen harmlosen und gefährlichen, invasiven Bakterien unterscheiden, und ihrer Umgebung im Darm entsprechende Signale geben. In meiner dritten und letzten Studie kultivierte ich verschiedene Arten von Mastzellen und verglich sie auf ihre Reaktion auf Salmonellen. Dabei fanden ich und meine Kollegen heraus, dass im Gegensatz zu Mastzellen, die denen im Bindegewebe ähneln, jene der Schleimhautart, ausschließlich auf invasive Salmonellen reagieren. Dieser Unterschied beruht darauf, dass Schleimhautmastzellen keine Detektoren für Bakterien außerhalb der Zellen besitzen.

Zusammengefasst habe ich mit dieser Doktorarbeit wichtiges Wissen dem Gesamtverständnis beigesteuert, wie Mastzellen bakterielle Infektionen erkennen. Streptokokken nutzen Giftstoffe, die Poren in der Mastzellmembran formen. Salmonellen produzieren zwar auch kleine Löcher in der Membran, jedoch führen diese, vermutlich durch ihre begrenzte Anzahl, nicht zur Aktivierung der Mastzellen. Stattdessen erkennen die Immunzellen Salmonellen durch eine Kombination aus Rezeptoren an ihrer Oberfläche und durch die injizierten Proteine der *Salmonella* Injektionsnadel.

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“Was wir wissen, ist ein Tropfen, was wir nicht wissen, ein Ozean.” - Adam

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