MAST CELLS: BRIDGING HOST-MICROORGANISM INTERACTIONS

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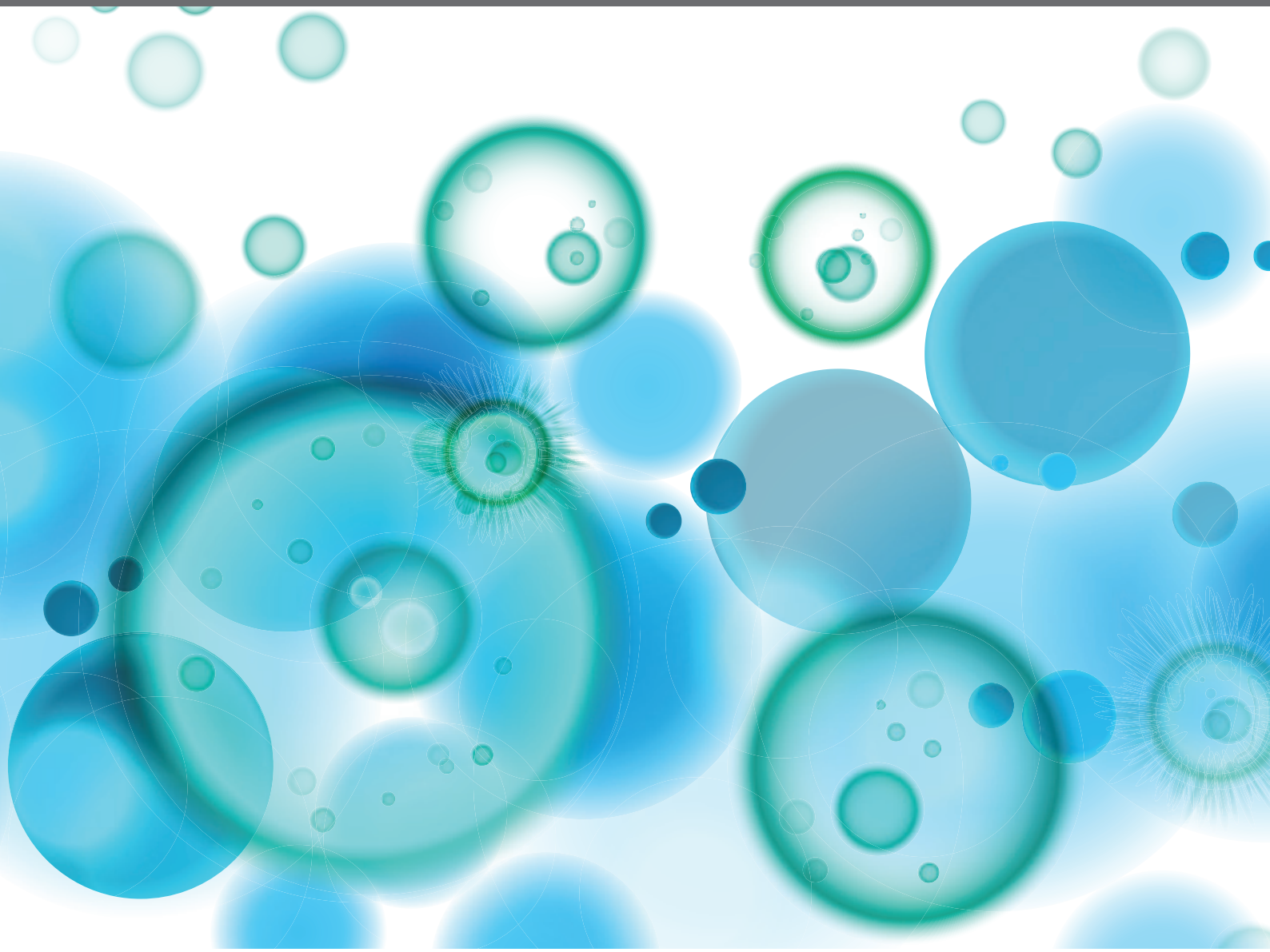

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MAST CELLS: BRIDGING HOST-MICROORGANISM INTERACTIONS

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EDITORIAL
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Editorial: Mast Cells: Bridging Host-Microorganism Interactions

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Editorial on the Research Topic

Mast Cells: Bridging Host-Microorganism Interactions

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Mast cells (MCs) evolved as part of the innate immune system more than 500 million years ago to respond to varying signals from microorganisms that establish different interactions with the host, ranging from mutualistic to parasitic. This Research Topic illustrates how MCs influence the immune system's relationship with different microorganisms and how these MC responses modulate subsequent host-microorganism interplay.

In a comprehensive overview, Jiménez et al. describe the biology and major functions of MCs with a focus on our current understanding of their interaction with microorganisms, ranging from viruses to parasites. They discuss the importance of MC mechanisms to induce protection against infection and they also illustrate the damage associated with exacerbated MC activation during different infectious diseases.

Yu et al. discuss the importance of MC activation during fungal infections. They review experimental evidence of the role of MCs during infections with the fungi *Candida albicans*, *Aspergillus fumigatus*, and *Sporothrix schenkii*.

Because of their strategic location, MCs are sentinels at epithelial interfaces and are first responders in host-microbiome interactions. MCs primarily interact with microbes *via* innate immune receptors, such as Toll-Like Receptors (TLRs). As Soria-Castro et al. describe, during infection with *Listeria monocytogenes*, MCs responses are mediated through TLR2 activation. However, they can also respond when TLR2 is blocked, using other innate immune receptors.

Draberova et al. describe alternate pathways of MC activation during infection, focusing on Gram-positive bacterial exotoxins that promote cholesterol-dependent cellular lysis. At low concentrations, these exotoxins cause pore formation in the membrane of MCs leading to the activation of cell signaling pathways that induce MC degranulation and production of several inflammatory mediators. Interestingly, other molecules that can modify MC membrane integrity can mimic this activation pathway.

However, the significance of MCs in host defense is more evident when we see MCs also modulate the acquired immune system. Palma et al. illustrate how MCs modulate B-cell responses. Their review describes the critical impacts of MCs on B cells using information from both clinical

and laboratory studies. They also discuss the implications of these findings on host responses to infections.

Antibody responses can modulate the responses of MCs to microorganisms, which is illustrated by Mamontov et al. during influenza virus infection, turning the capacity of MCs to recognize viruses into a damaging response. This study elegantly shows that virus-specific non-neutralizing antibodies can interact with MCs upon infection with the avian influenza virus. It is suggested that this antibody-mediated MC response, which includes significant histamine release, could potentially cause side effects when vaccinated subjects are infected with the influenza virus.

A second level of MC interactions with the adaptive immune response is evident during T cell activation following viral infections. Employing MC deficient mice, Hackler et al. reveal the critical role played by MCs in promoting adequate dendritic cell activation and the induction of specific CD8+ T-cell responses that limit lymphocytic choriomeningitis virus infections.

The ability of MCs to participate in the control of bacterial infections can be exploited by employing distinct polypeptides that target specific molecules on MCs. For example, Amponnawarat et al. show that murepavadin, a lipopolysaccharide transport protein D (LptD)- binding host defense peptidomimetic antimicrobial peptide that targets multi-drug resistant *Pseudomonas aureginosa*, can activate MCs *in vitro* through Mas-Related G Protein-Coupled Receptor member X2 (MRGPRX2), inducing degranulation and cytokine production. Moreover, *in vivo*, they revealed that murepavadin increases vascular permeability in mice.

Finally, Yeh et al. reveal that MC interactions with microorganisms can be a bidirectional response, where interactions with microbiota impact MC distribution in the lungs. They show that mice captured in the wild exhibit high numbers of MCs in the lung parenchyma compared to conventional pathogen-free laboratory animals. Remarkably, when laboratory mice are bred in conditions that mimic the environment in the wild, they exhibit increased numbers of MCs in the lungs.

In conclusion, this Research Topic updates the current state of knowledge regarding MC crosstalk with mutualistic and parasitic microorganisms and how this interaction impacts innate and adaptive immune response modulating host homeostasis. The role of MCs clearly depends on the nature of its interactions with specific microorganisms, as these interactions can promote either protection or damage to the host. Purposeful modulation of some of these interactions could represent new therapeutic targets and novel strategies to combat infectious diseases.

AUTHOR CONTRIBUTIONS

All authors made a substantial, direct, and intellectual contribution to this work and approved it for publication.

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MINI REVIEW
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The Emerging Role of Mast Cells in Response to Fungal Infection

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Mast cells (MCs) have been considered as the core effector cells of allergic diseases. However, there are evidence suggesting that MCs are involved in the mechanisms of fungal infection. MCs are mostly located in the border between host and environment and thus may have easy contact with the external environmental pathogens. These cells express receptors which can recognize pathogen-associated molecular patterns such as Toll-like receptors (TLR2/4) and C-type Lectins receptors (Dectin-1/2). Currently, more and more data indicate that MCs can be interacted with some fungi (*Candida albicans*, *Aspergillus fumigatus* and *Sporothrix schenckii*). It is demonstrated that MCs can enhance immunity through triggered degranulation, secretion of cytokines and chemokines, neutrophil recruitment, or provision of extracellular DNA traps in response to the stimulation by fungi. In contrast, the involvement of MCs in some immune responses may lead to more severe symptoms, such as intestinal barrier function loss, development of allergic bronchial pulmonary aspergillosis and increased area of inflammatory in *S. schenckii* infection. This suggests that MCs and their relevant signaling pathways are potential treatment regimens to prevent the clinically unwanted consequences. However, it is not yet possible to make definitive statements about the role of MCs during fungal infection and/or pathomechanisms of fungal diseases. In our article, we aim to review the function of MCs in fungal infections from molecular mechanism to signaling pathways, and illustrate the role of MCs in some common host-fungi interactions.

Keywords: mast cell, fungi, pathogen-associated molecular patterns, *Candida albicans*, *Aspergillus fumigatus*, *Sporothrix schenckii*

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INTRODUCTION

Fungal infections are a serious health problem all over the world. Currently, more than 300 million people suffer from severe fungal infections, and an estimated over 1.5 million people die from deep fungal infections each year (1). With broad-spectrum antibiotics used and the increase of immunodeficiency disease such as acquired immune deficiency syndrome, pathogenic fungi opportunistic infections showed a trend of rising sharply (2–4). Although early diagnosis and the use of antifungal drugs have a certain positive effect on the prevention and control of fungal

infection, there are still some unsolved problems in clinical practice, such as insufficient evidence of some etiology and drug resistance.

Both innate immunity and adaptive immunity play an indispensable role in anti-fungal infection. Mast cells (MCs) are one of the most important innate immune cells, which can initiate and regulate innate and adaptive immunity. Traditionally, MCs have been referred as the key effector cells of allergic diseases, such as urticaria, allergic rhinitis and bronchial asthma (5–7). MCs are mostly located in the border between host and environment such as skin, gastrointestinal and respiratory mucosa and thus may have easy contact with the external environmental pathogens (8). In addition, MCs express a variety of different pattern recognition receptors (PRRs) on their cell surface to detect bacterial, viral, fungal or parasitic components known as pathogen-associated molecular patterns (PAMPs) (9) such as β-glucan, mannan and lipopolysaccharide. Due to their strategic location and the wide variety of PRRs they express, many researchers have attempted to explore the potential immune relationship between MCs and fungal infection (9–11). Therefore, we will review the role of MCs in fungal infections from molecular mechanism to signaling pathways, and illustrate the role of MCs in some common fungal infections (e.g., *Candida albicans* (*C. albicans*), *Aspergillus fumigatus* (*A. fumigatus*) and *Sporothrix schenckii* (*S. schenckii*)) to light new ideas for prevention and treatment.

FUNGI RECOGNITION RECEPTORS ON MCs

MCs can directly recognize PAMPs *via* their PRRs and then be activated. PRRs have comprised mainly five families including Toll-like receptors (TLRs), C-type lectin-like receptors (CLRs), Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I) like receptors (RLRs) and absent-in-melanoma (AIM)-like receptors (ALRs) (12). Current studies have shown that TLRs and CLRs are main PRRs in host anti-fungal infection. The fungal wall is composed of many layers. Any component of the cell wall could be a potential PAMP. It is reported that the main molecules that trigger the immune response are chitin, β-glucan, mannan and others (13).

Toll-Like Receptors (TLRs)

The TLR family is an important group of receptors that recognize invading microorganisms through innate immunity. A total of 13 members of the TLRs family have been reported, 10 of which are associated with human including TLR1-10 (14). TLRs are distributed on a variety of cells including macrophages, dendritic cells and MCs. Mouse connective tissue-type MCs (CTMC) express TLR2, TLR4, and TLR5 on cell surface membrane, whereas TLR3, TLR7, and TLR9 are present both on the cell membrane and intracellularly (15). TLRs can recognize and be activated by different PAMPs. Compared with bone marrow-derived MCs (BMMCs) from wild type mice C57BL/6, a decrease in *C. albicans* phagocytosis and nitric oxide (NO) production were detected in TLR2$^{-/-}$ mice (16). Whereas mannan, extracted from *Saccharomyces cerevisiae*, directly activates murine CTMC to initiate the proinflammatory response *via* TLR4 (17). When pretreated with TAK242, a small-molecule-specific inhibitor of TLR4 signaling, CTMC significantly decreased the generation of cysLT and reactive oxygen species (ROS) as compared to nontreated MCs in response to mannan (17). In summary, TLR2 and TLR4 are the main members of TLRs on MCs with potential to bind different PAMPs on the surface of fungi and induce subsequent host immune response.

C-Type Lectin-Like Receptors (CLRs)

CLRs are a family of calcium dependent receptors that bind to carbohydrates. A total of 17 members have been reported. Lectin activity of these receptors depends on conserved carbohydrate recognition domains. CLRs include Dectin-1, Dectin-2, macrophage-inducible C-type lectin (Mincle), intercellular adhesion molecule (ICAM-3) and dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) (18). Studies on CLR expression and immune function in MCs are still limited. Dectin-1 is the most commonly studied receptor in CLRs, which exists in both human and murine MCs. Dectin-1 can recognize β-glucans and trigger a variety of cellular responses such as phagocytosis and the production of cytokines. Zymosan, a β-glucan derived from yeast of *Saccharomyces cerevisiae*, for example, induced mature mice peritoneal MCs migration and pro-inflammatory expression *via* Dectin-1 (19). Similar results were observed in human cord blood-derived mast cells (CBMCs). When incubated with zymosan, CBMCs significantly decreased the level of leukotriene (LT) B_4 and C_4 in the presence of inhibitors of Dectin-1 (20). Some studies have shown that Dectin-2 can activate FcRγ chain to recognize α-mannose and induce immune response in dendritic cells (21), macrophages (22), and neutrophils (23), which leads to upregulation of TNF-α and IL-1ra secretion (24). Host Dectin-2 preferentially binds to hyphae form of *C. albicans, Microsporum audouinii* and *Trichophyton rubrum* (24), which is essential for inducing Th1/Th17 immune responses (25). The expression and immune function of Dectin-2 of MCs remains to be further studied.

SIGNALING PATHWAYS OF MCs AGAINST FUNGI

TLR Signaling Pathway

TLR signaling pathway can be mainly divided into two different mechanisms, namely Myeloid differentiation 88 (MyD88) -dependent pathway and MyD88-independent pathway. The MyD88 dependent pathway is used by all TLRs except TLR3 (26). MyD88$^{-/-}$ mice showed an increase of susceptibility to *C. albicans* infection and the production of proinflammatory cytokines such as TNF-α, IFN-γ and IL-12p70 could not be detected from antigen-stimulated splenocytes (27). Upon

activation, MyD88 might trigger several different intracellular signaling pathways such as nuclear factor kappa-B (NF-κB), mitogen activated protein kinases (MAPK including extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK)) and phosphatidylinositol-3 kinase (PI3K), leading to the release of inflammatory cytokines and other inflammatory mediators. IgE-sensitized BMMCs from both TLR2 and MyD88 gene-deleted mice showed a decrease of IL-6 production in comparison of wild type BMMCs (28). Furthermore, a high concentration of *Malassezia sympodialis* extract inhibited phosphorylation of ERK1/2 and the release of IL-6 in IgE-sensitized BMMCs, indicating that the ERK1/2 pathway may regulate release of IL-6 (28). Except for ERK1/2, other molecules which involved MyD88-dependent pathways for TLR activation have been less investigated in MCs against fungi. PI3K pathway has been reported to be involved in lipopolysaccharide stimulation of murine BMMCs to produce IL-6 and TNF-α (29). In other cells, such as oral epithelial cells, all *Candida* species were able to activate the NF-κB and c-Jun pathway and only *Candida dubliniensis* and *C. albicans* hyphae can trigger MAPK/MKP1/c-Fos pathway (30).

CLR Signaling Pathway

CLR signaling pathway can be divided into tyrosine kinase Syk-dependent and -independent signaling pathways. Both signaling pathways eventually activated NF-κB, and produced cytokines and chemokines such as IL-6, IL-23, TNF-α, macrophage inflammatory protein and CXCL12, which affected differentiation of Th1 and Th17 cells. Traditionally, CLRs (e.g., Dectin-1, Dectin-2) are coupled with Syk to further activate NF-κB pathway by activating caspase recruitment domain protein 9 (CARD9) in Syk-dependent signaling pathway. CARD9 was expressed on the surface of MCs, but its expression level was slightly lower than that of macrophages, dendritic cells and neutrophils. It was found that LTC4 and LTB4 were released from human MCs co-cultured with zymosan, suggesting that the expression of LTC4 and LTB4 was mediated by Dectin-1/Syk pathway (20). In Syk-independent signaling pathway, activated CLRs (e.g., Dectin-1 and DC-SIGN) can recruit GTPase Ras proteins which subsequently activate serine/threonine-protein kinase Raf-1. This results in Raf-1 mediated phosphorylation of the p65 subunit of NF-κB, which leads to the production of cytokines (31). In addition, the interaction between PRR can be more effective in the control of fungal infection. Studies have been proved that Dectin-1 and TLR2 collaborate to enhance the role of MAPK pathway resulting in an increase of cytokines (32).

MCs AND FUNGI

MCs and *C. albicans* Infection

C. albicans is one of the most common dimorphic fungus colonizing mucosal surfaces such as gastrointestinal tract,oral-nasal cavity and skin. Vulvovaginal candidiasis is one of the most common forms of candidiasis. When host immunity is suppressed or damaged, *C. albicans* can result in severe invasive diseases. Invasive candidiasis is the most common critical care-associated fungal infection in patients hospitalized in intensive care units, with mortality rates between 40% and 55% (33).

MCs play a positive role in the defense mechanism against *C. albicans* infections, including phagocytosis and killing yeasts in the extracellular environment (34). Both yeast and hyphae of *C. albicans* can induce the degranulation of BMMCs and lead to the production of different cytokines and chemokines (CCL3 and CCL4) that regulate immune response (35). In response to the stimulation by yeast cells, BMMCs showed increased production of IL-6 and IL-β that could not be induced by hyphae (35). *C. albicans* hyphae hide their β-glucan residues beneath a cover of mannoproteins on the cell walls, thus escaping Dectin-1-mediated phagocytosis and stimulating different cytokines production (36). Dectin-1, but not TLR2, is involved in the release of TNF-α through NF-κB/Syk pathway (35). The production of ROS and NO enhanced the ability to defense against *C. albicans* by MCs. When challenged with *C. albicans*, deletion of TLR2 or Dectin-1 significantly reduced NO levels, in comparison with wild type BMMCs. Whether Dectin-1 participates in the production of ROS remains controversial (16, 36).

Human MC immune responses towards *C. albicans* can be mainly divided into three phases. Human mast cell line HMC-1 degranulate in an early and direct response to fungal encounter, thus reducing the viability of *C. albicans* by 30% (37). Next, infected cell line HMC-1 release pro-inflammatory cytokines (e.g. IL-8) to recruit neutrophils, followed by the release of anti-inflammatory mediators, such as IL-16 and IL-1ra (37). MCs extracellular traps will be formed to contribute to fungal physical restriction, but not killing activity, as there was no significant difference in fungal viability in the presence or absence of nuclease (37). *C. albicans* can cause MCs death by different mechanisms, including the release of MC extracellular traps and rupture by the growth of internalized *C. albicans* hyphae, leading to a temporary immune response (37).

Although secretion of monocyte chemotactic protein 1 was not observed in infected MCs (37), they can produce short-lived soluble mediators to improve the crawling of tissue-resident macrophages and induce migration to *C. albicans* infection (38). Uninfected MCs were found to restrict macrophage phagocytosis of *C. albicans*, which is the result of maintaining a balance between the host and the fungus (38).

MCs exhibited dual effects in response to *C. albicans*, which is dependent on their protease content and site of *C. albicans* colonization. In mice, two types of MCs have been described as CTMCs, which originate from fetal liver progenitors and are mainly located in stromal tissue, and mucosal MCs (MMCs), which originate in the bone marrow and reside in the intestine and lung (39). Two similar types of MCs are also observed in humans (40). Different MCs types regulated by cytokine production (e.g. TGF-β, IL-9, IL-10) can discriminate between the fungal morphotypes, thereby promoting local inflammation or protective tolerance. Some studies have found that MMCs contribute to barrier function loss in leaky gut models (39) or higher sensitization against food antigen (41) in response to yeast

stimulation. Whereas CTMCs induce local protective tolerance to infection by release of anti-inflammatory cytokine in response to hyphae (39). MCs promoting either inflammatory dysbiosis or tolerance were also observed in vulvovaginal candidiasis (42).

MCs and *A. fumigatus* Infection

A. fumigatus is the most frequent opportunistic pathogenic species of the genus *Aspergillus* by far, responsible for about 90% of the cases of *Aspergillus* diseases (43). *A. fumigatus* is abundant in the environment, releasing spores (conidia) which are inhaled. After inhalation, patients with lung function or immune defenses impaired are unable to eliminate conidia which will germinate into hyphae that colonize the airways. *A. fumigatus* hyphae will penetrate pulmonary tissues causing invasive aspergillosis, the most severe disease caused by *Aspergillus* spp (44). *A. fumigatus* is known to contain 23 antigenic components that can induce the production of specific IgE (45). Classically, antigen-specific IgE which cross links FcεRI can trigger MCs degranulation and increase IL-5 release (46). IL-5 is one of the important cytokines that can promote eosinophils migration to inflammation site, which are associated with severe asthma and allergic bronchial pulmonary aspergillosis (47, 48).With further studied, investigators found that mature *A. fumigatus* hyphae can directly stimulate MCs to degranulate in the absence of IgE (49). When cultivated with heat-killed *A. fumigatus* spores, HMC-1 cell line was activated to degranulation and release IL-5 (50). Taken together, the activation of MCs induced by *A. fumigatus* may be the initiator of the whole sensitization process. The studies concerning the signaling pathway of immune response of MCs in *A. fumigatus* infection are scarce. In dendritic cells, TLR2/MyD88 signaling pathway has been proved to regulate immune response to *A. fumigatus* conidia, which results in a development of Th2 response (51).

MCs and *S. schenckii* Infection

S. schenckii is a common biphasic fungal pathogen. The incidence of sporotrichosis is increasing and has become an urgent global health problem (52). The World Health Organization classifies sporotrichosis as one of the Neglected Tropical Diseases which place a significant burden on individuals, families and society (53).

Studies have shown that MCs play a non-protective role in response to *S. schenckii* infection. In our previous study, we observed that MC-deficient C57BL/6-Kit^{W}/Kit$^{W/v}$ and MC-deficient Cpa3-Cre/Mcl-1$^{fl/fl}$ (Hello Kitty, HK) mice had more serious inflammation symptoms with elevation of TNF-α and IL-6 in comparison with wild type mice (54). Rat peritoneal MCs can be activated by *S. schenckii* yeasts and release IL-6 and TNF-α *via* ERK pathway without degranulation (55, 56).

DISCUSSION

MCs are recognized as important effector in response to fungal infection. Their impacts can be protective and non-protective, depending on the pathogen and invasion site. Fungal recognition receptors commonly studied on MCs are TLR2/4 and Dectin-1.Upon activated by TLR/MyD88 or Syk pathway, MCs can produce specific cytokines and chemokines which can elicit a direct temporary immune response and recruit some neutrophils to the infection sites. MCs activation can enhance the immune response against infection. In contrast, the involvement of MCs in some immune responses may lead to more severe symptoms, such as intestinal barrier function loss, development of allergic bronchial pulmonary aspergillosis and increased area of inflammatory in S. schenckii infection, which suggests that MCs and their singling pathways are potential treatment regimens to prevent the clinically unwanted consequences. However, up to now, we still have a relatively poor understanding of the mechanism of MCs in response to common fungal infection. Many of the key signaling pathways involved in mediating selective cytokine and chemokine responses from MCs remain unknown. In the future, more *in vitro* and *in vivo* studies are urgently needed to further explore the role of MCs in response to fungal infection.

AUTHOR CONTRIBUTIONS

MY: substantial contributions to draft the manuscript. X-tS: drafted the article. BL: drafted the article. T-tL: participated in material collection. S-lL: participated in material collection. Z-tZ: substantial contributions to conception and reviewed the article critically for important intellectual content. All authors contributed to the article and approved the submitted version.

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Mast Cells Modulate Antigen-Specific CD8$^+$ T Cell Activation During LCMV Infection

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Mast cells (MCs), strategically localized at mucosal surfaces, provide first-line defense against pathogens and shape innate and adaptive immune responses. Recent studies have shown that MCs are involved in pathogenic responses to several viruses including herpes simplex viruses, dengue virus, vaccinia virus and influenza virus. However, the underlying mechanisms of MCs in the activation of CD8$^+$ T cells during viral infections are not fully understood. Therefore, we investigate the role of MCs in the development of virus-specific CD8$^+$ T cell responses using the well-characterized murine lymphocytic choriomeningitis virus (LCMV) model and the transgenic MasTRECK mice that contain the human diphtheria toxin receptor as an inducible MC-deficient model. Here, we report that MCs are essential for the activation and expansion of virus-specific CD8$^+$ T cells. After MC depletion and subsequent intradermal LCMV infection, the CD8$^+$ T cell effector phenotype and antiviral cytokine production were impaired at the peak of infection (day 8 p.i.). Importantly, MC-deficient mice were unable to control the infection and exhibited significantly higher viral loads in the spleen and in the ear draining lymph nodes compared to that of wild type control mice. In the absence of MCs, dendritic cell (DC) activation was impaired upon LCMV infection. In addition, type-I interferon (IFN) levels in the serum and in the spleen of MC-deficient mice were reduced during the first days of infection. Interestingly, depletion of MCs after intradermal LCMV infection did not impair virus-specific CD8$^+$ T cell expansion, activation or antiviral cytokine production. In summary, our results indicate that MCs play a pivotal role in the activation and antiviral functions of CD8$^+$ T cells through proper DC activation. A better understanding of the impact of MCs on CD8$^+$ T cell responses is mandatory to improve antiviral immune responses.

Keywords: mast cells, CD8$^+$ T cells, viral infection, immunosuppression, dendritic cells

INTRODUCTION

Mast cells (MCs) are long-lived immune cells distributed throughout nearly all tissues and particularly close to the skin and mucosa (1). MCs can quickly respond to invading pathogens and initiate immune responses due to their location and the expression of a wide spectrum of pattern recognition receptors (2–4). In addition, MCs sense stress and tissue damage *via* receptors of danger-associated molecular patterns (5–7). Furthermore, MCs can release a plethora of immune mediators including cytokines, chemokines, proteases, and antimicrobial peptides, which allow them to activate both immune and non-immune cells (8, 9). Thus, MCs can be considered a bridge between innate and adaptive immune responses (10). Several studies have shown that MCs play a protective role during bacterial, fungal and parasitic infections (11–16). In addition, increasing evidence using experimental infection models in mouse and human cell lines have revealed novel insights into the role of MCs in viral infections.

MCs can directly sense viruses (2) and can also be activated by inflammatory mediators produced during viral infections (17). Depending on the mechanism of viral recognition, MCs release immune mediators through degranulation or *de novo* cytokine and chemokine production (18). MCs have shown to modulate the course of cytomegalovirus, vaccinia virus, influenza virus, epstein barr virus and dengue virus infections (19–22). Moreover, MCs mediate the recruitment of short-lived effector $CD8^+$ T cells into the lung in a CCL-5 dependent manner after cytomegalovirus infection (19). Antimicrobial peptides produced by MCs such as cathelicidin exert antiviral properties against vaccinia virus infection as shown by increased viral loads in infected MC-deficient mice compared to infected wild type animals (23). MCs not only mediate recruitment of cytotoxic cells such as NK cells, NKT cells, $CD8^+$ T and γδ T cells (24, 25) but also contribute to viral clearance after dengue virus infection (26). Similarly, MC-deficient mice exhibited increased clinical severity and mortality with elevated virus titers compared to wild type mice after a HSV-2 infection (27).

Recent studies show that DC-MC interactions have a strong impact in the modulation of DC migration, activation and function (28–30). In addition, molecular transfers of major histocompatibility complex class II (MHCII) proteins between MCs and DCs enhanced T cell priming efficiency (31). MCs not only induce DC migration but also enhance DC maturation *in vitro*, antigen uptake, and cross-presentation (28, 32). In addition to direct MC-DC communication, a recent study show that MC granules and exosomes are able to promote DC maturation (33). MCs have been shown to induce the activation and migration of antigen-presenting cells from the skin. MC-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ or TNF(-/-) mice showed significantly reduced migration of airway DCs to local LNs 24 h after intranasal challenge with FITC-OVA in a model of contact hypersensitivity to FITC (34). In addition, activated mast cells were shown to alter the pulmonary micromilieu and induce antigen uptake, activation and migration of DCs (35).

Despite the increasing evidence for the critical role of MCs in immune responses and their protective role in viral infections, the underlying mechanisms are still not completely understood. Here, we report that MCs are crucial for the activation, expansion and function of virus-specific $CD8^+$ T cells. Accordingly, MC-deficient mice were not able to control the infection and exhibited high viral loads in the spleen and in the ear draining lymph nodes (ear-dLNs). In the absence of MCs, DC activation was impaired and type-I IFN levels were reduced. Furthermore, MC-deficient mice exhibited diminished chemokine concentrations that led to decreased recruitment of DCs to secondary lymphoid organs. Thus, our findings indicate that MCs are essential for the development of antigen-specific $CD8^+$ T cells responses during viral infections.

MATERIALS AND METHODS

Mice

C57BL/6 (WT) and MasTRECK mice on C57BL/6 background (36) were bred in the animal facility at Charité, Berlin under specific pathogen-free conditions. C57BL/6 (WT) were used for peritoneal mast cell and DC isolation. For mast cell depletion, C57BL/6 (WT) and MasTRECK mice received 250 ng of diphtheria toxin intraperitoneally during five consecutive days. All animal experiments were performed at the Charité, Berlin in accordance with the German law for animal protection and approved by the Landesamt für Gesundheit und Soziales of Berlin (LaGeSO approval number G0078/17).

Virus, Measurement of Viral Titers and Inoculation of Mice

LCMV-WE strain was propagated on L-929 cells. LCMV stocks and viral titers in the spleen and the ear-dLNs were titrated by standard immunofocus assays on MC57G cells as described previously (37). In brief, MC57G cells were plated with organ homogenates or virus stock dilutions and subsequently overlaid with 2% methylcellulose. After 48 h of incubation at 37°C, the confluent monolayer of cells was fixed with 4 % formaldehyde, permeabilized with 1 % Triton X-100 (v/v) and stained with antibodies against LCMV nucleoprotein (VL-4). After a secondary staining step with peroxidase-conjugated anti-rat IgG antibody (Jackson), foci were developed by 20 min incubation with OPD substrate (0.1 mol/L Na_2HPO_4, 0.5 mol/L citric acid, 0.03 % H_2O_2, and 20 mg o-phenylenediamine dihydrochloride). Mice were intradermally infected on the ventral side of the ear pinna with $9*10^4$ PFU of LCMV-WE in 10 µL PBS.

Preparation of Ear Cell Suspensions

Ears were splitted in two layers, cut into small pieces and subsequently placed into fleshly prepared digestion medium (915 µL RPMI, 40 µL FCS (4 %), 0.5 µL DNase (40 µg/mL) (Roche), 35 µL Liberase™ (10 mg/mL) (Roche) and 10 µL hyaluronidase (50 mg/mL). After incubation (60 min, 37°C, 1400 rpm) undigested tissue was removed using a 30 µm cell strainer and the cells were washed (10 min, 4°C, 300 g) in PBS/BSA (0.5 % w/v). Afterwards the single cell suspension was used for flow cytometry staining.

Flow Cytometry Analysis

Surface receptor staining and intracellular cytokine staining procedures have been described previously (38). The LCMV-specific CD8+ T cell response to the dominant glycoprotein-derived epitope GP33 and the nucleoprotein-derived epitope NP396 were assessed by MHC class I tetramer staining as described previously (39).

Surface receptor staining was performed in single cell suspensions of ears, ear-dLNs and the spleen after homogenization using mechanical disruption through a 70 µm cell strainer. FACS analysis included surface marker stainings for anti-CD4 (GK1.5), anti-CD45 (30-F11), anti-KLRG1, anti-CD127 (IL-7R), anti-CD62L, anti-CXCR3 (CXCR3-173), anti-CCR2 (SA203G11), anti-MHCII (M5/114.15.2), anti-CD11c (N418), anti-CD11b (M1/70), anti-CD8 (53-6.7), anti-CD80 (16-10A1), anti-CD86 (GL1), anti-Ly6C (HK1.4), anti-Ly6G (1A8) anti-B220 (RA3-6B2), anti-F4/80 (BM8) and anti-Siglec H (551) (BioLegend). Zombie-Aqua (BioLegend) was used as a live/dead discrimination marker. Rat IgG1 (R3-34) and rat IgG2a (R35-95) isotype control antibodies (BD Biosciences) were used at the same concentrations as the respective cytokine antibodies.

For the analysis of intracellular cytokines, cells were restimulated with GP33 (10^{-6} mol/L) and NP396 (10^{-6} mol/L) (Neosystem) for infected animals or with PMA (5 ng/ml) and ionomycin (500 ng/ml; Sigma) for uninfected mice. 5 µg/mL brefeldin A (Sigma-Aldrich) was added after 30 minutes. After 3 h, surface marker stainings were performed and cells were subsequently fixed with 2 % formaldehyde (Merck). Later on, cells were stained with the following rat anti-mouse cytokine antibodies or isotype control antibodies in permeabilization buffer containing 0.05% saponin (Sigma-Aldrich): anti-IFN-γ (XMG1.2), anti-TNF-α (MP6-XT22) and anti-IL-10 (JES5-16E3) in permeabilization buffer containing 0.05 % saponin (Sigma-Aldrich). Rat IgG1 (R3-34) and rat IgG2a (R35-95) isotype control antibodies (BD Biosciences) were used at the same concentrations as the respective cytokine antibodies. Flow cytometry analysis was performed in BD FACS Canto II. The gating strategy for flow cytometry analysis of immune subsets in the spleen, ear-dLNs and ears is shown in **Figure S1**. After gating on live cells and subsequently on $CD45^+$ cell, neutrophils ($Ly6G^+$ $CD11b^+$), macrophages ($F4/80^+$ $CD11b^+$) DC, ($MCHII^+$ $CD11c^+$) and inflammatory monocyte ($LyC6^+$ $CD11b^+$) were gated. For $CD8^+$ T cells subsets, cells were gated on $CD8^+$ T cells and subsequently, on KLRG-1^+ IL-$7R^-$ or $CD44^+$ $CD62L^-$ or $CXCL3^+$ for activated $CD8^+$ T cells, GP33-$Tetramer^+$ and NP396-$Tetramer^+$ for LCMV-specific $CD8^+$ T cells and IFN-γ^+ and IL-10^+ for cytokine production (**Figure S2**). For lymphocytic DCs, analysis gates were set on $CD8^+CD11b^-$ cells (**Figure S3A**) and for pDC, after excluding $CD3^+$ $CD19^+$ and $CD11b^+$ cells were gated on $B220^+$ and lastly on Siglec-H^+ (**Figure S3B**).

LCMV Infection Model In Vitro

For peritoneal MC isolation, the abdominal skin of mice was washed with 70 % ethanol. The peritoneum was exposed by a 1-cm midline abdominal incision, and 4.0 mL of sterile, pyrogen-free, 0.9 % NaCl and 4.0 mL of air were injected into the peritoneal cavity via a 22-gauge needle. The abdomen was massaged gently for ∼3 min and the peritoneal fluid was recovered via a 22-gauge needle. Peritoneal MCs show a purity ≥ 95% (depicted by FcεRI+ c-Kit+) after isolation in the flow cytometry analysis (**Figure S4A**). Subsequently, peritoneal MCs were then wash with PBS, infected with LCMV-WE at MOI 5 and cultured in RPMI 1640 plus GlutaMax-I supplemented with 10 % (vol/vol) FCS (Gibco; Life Technologies), penicillin (100 U/mL; Gibco; Life Technologies), streptomycin (100 µg/mL; Gibco; Life Technologies), and β-mercaptoethanol (50 ng/mL; Sigma-Aldrich) for 24h. Cells were harvested and stained for flow cytometry analysis. Supernatants were collected for cytokine and chemokine detection.

Cytokine and Chemokine Analysis in Supernatants

The concentrations of cytokines and chemokines were determined in supernatants of 2x10e5 splenocytes and cells from ear draining lymph nodes cultured in a 96 U-bottom well plate in 200 µL RPMI complete medium for 24 h and using a magnetic bead based multiplex ELISA (LEGENDplex™-BioLegend) and the chemokine 26-Plex Mouse ProcartaPlex™ Panel 1 (Invitrogen) according manufacturer's instructions.

Immunohistochemistry

Paraffin sections (5 µm) were deparaffinized as follows: 2 x 10 min in xylol, 2 x 3 min in absolute ethanol, 2 x 3 min in 96 % ethanol, 1 x 3 min in 70 % ethanol, 3 x 5 min in deionized water and 3 x 5 s in TBS buffer (Tris-Base (7,4 mmol/L), Tris-HCl (43,5 mmol/L), NaCl (150 mmol/L), pH = 7.5). For MC staining, the sections were incubated with Avidin-FITC (BioLegend) for 15 min in the dark. After washing (3 x 3 min with TBS) the sections were embedded with Fluoromount-G™ containing DAPI (Thermo Fisher Scientific) and dried for 24 h.

Statistical Analysis

GraphPad Prism (v8.0) software was used for data analysis. Statistical significance was determined by Student's t-test (unpaired two-tailed) for all figures when not indicated different. More than two groups were compared via one-way ANOVA with Bonferroni's post test for multiple comparisons. P = 0.01 to 0.05 was considered statistically significant (*), p = 0.001 to 0.01 as very significant (**), and p < 0.001 (***) as extremely significant, ns, not significant.

RESULTS

Mast Cells Are Crucial for Immune Cell Recruitment to the Site of LCMV Infection

In order to investigate the role of MCs in the development of virus-specific CD8+ T cell responses, we used transgenic MasTRECK mice that contain the human diphtheria toxin (DT) receptor under the control of an intronic enhancer that is essential for *Il4* gene transcription in MCs but not in other cell types (36). After five days of DT i.p. application into both wildtype (WT) and MasTRECK mice, MCs were depleted in

the skin of MasTRECK mice but not in that of WT mice (**Figure 1A**). The frequencies of different immune cell subsets in the spleen, ear draining lymph nodes (ear-dLNs) and ears were comparable between uninfected WT and MasTRECK mice after MC depletion (**Figures S1** and **S5A–E**). Basophils are concomitantly depleted with MC after DT administration and are therefore absent in MasTRECK mice during the course of LCMV infection including the last time point of analysis (day 8 p.i.) (36). One day after the last DT treatment, WT and MasTRECK mice were intradermally infected with LCMV on the ventral side of the ear pinna (**Figure 1B**). WT mice displayed a significant increase in ear thickness from day 6 to day 8 post infection compared to infected MasTRECK mice (**Figure 1C**). Furthermore, frequencies of haematopoietic CD45+ cells in the ear of WT mice were markedly increased compared to that of infected MasTRECK mice at the peak of infection (day 8 p.i.) (**Figure 1D**). In addition, infected MasTRECK mice displayed significantly reduced frequencies and absolute numbers of neutrophils, macrophages, DCs, and inflammatory monocytes compared to that of infected WT mice, suggesting that MCs are crucial for the recruitment of immune cells to the site of LCMV infection (**Figures 1E–H**).

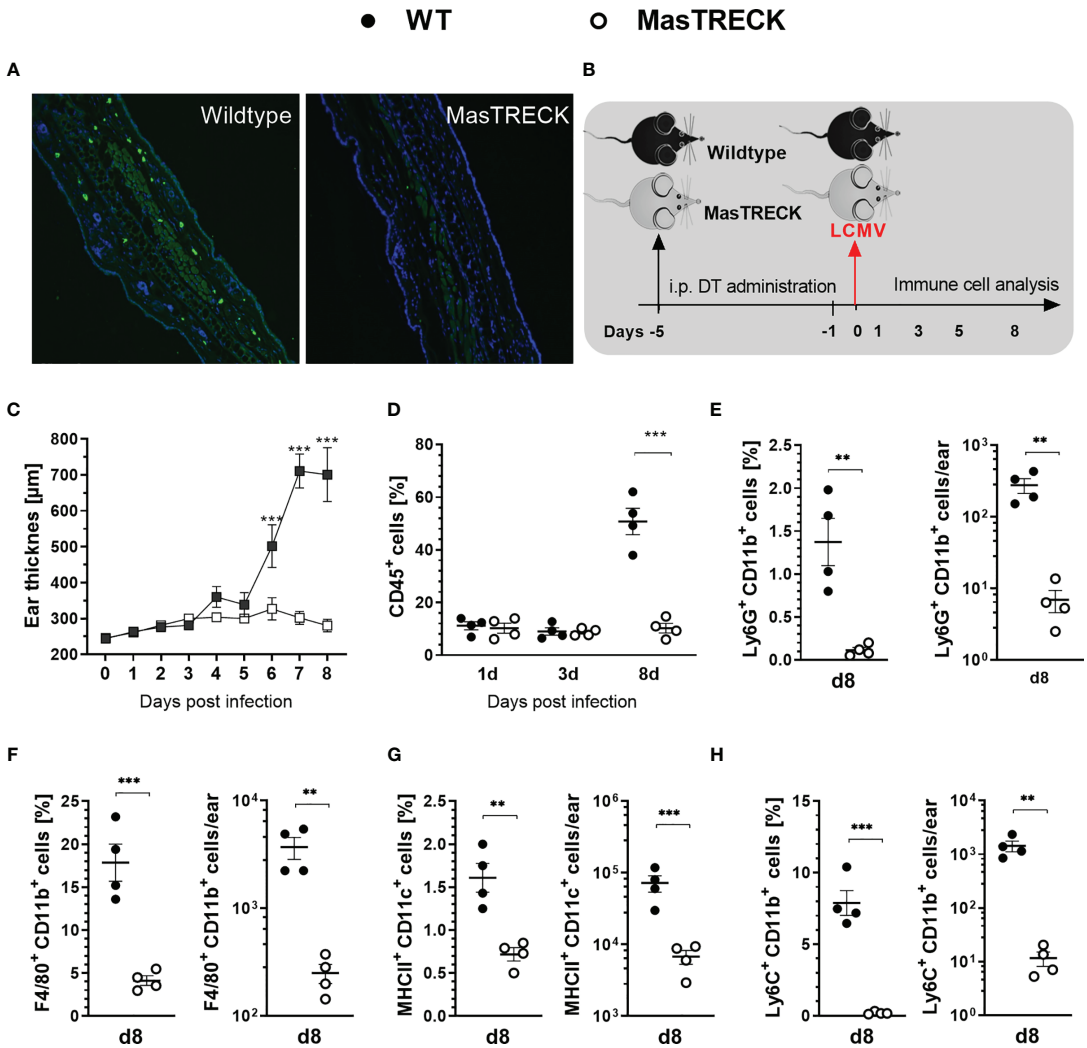

FIGURE 1 | Mast cells are crucial for immune cell recruitment to the site of LCMV infection. **(A)** Immunohistochemistry analysis of paraffin ear sections using Avidin-FITC and DAPI to assess MCs and cell nuclei, respectively after DT treatment. **(B)** Schematic experimental layout to analyze immune responses in intraperitoneally treated WT and MasTRECK mice for 5 consecutive days with 250 ng DT followed one day after by an intradermal LCMV infection into the ventral side of the ear pinna. **(C)** Ear thickness daily measured using a caliper during 8 days after LCMV infection. **(D)** Frequencies of CD45+ cells in the ear of WT and MasTRECK mice on day 1, 3 and 8 post LCMV infection analyzed by flow cytometry. Frequencies and absolute numbers of **(E)** neutrophils (Ly6G+CD11b+), **(F)** macrophages (F4/80+CD11b+), **(G)** DCs (MHCII+CD11c+) and **(H)** inflammatory monocytes (Ly6C+CD11b+) in the ear of WT and MasTRECK mice assessed by flow cytometry on day 8 post LCMV. All experiments were performed at least twice, and each experimental group included n ≥ 4. Data are representative and expressed as mean ± SEM. Statistically significant differences are analyzed by *t*- test and indicated as follows: **p < 0.01, ***p < 0.001.

Mast Cells Are Key Players in the Expansion and Recruitment of Virus-Specific CD8+ T Cells

We then examined LCMV-specific CD8+ T cell responses in WT and MasTRECK mice treated with DT for 5 days followed by intradermal infection with LCMV on the ventral side of the ear pinna (**Figure 1B**). Frequencies of total CD8+ T cells (**Figure 2A**) and CD8+ T cells specific for the dominant LCMV glycoprotein epitope GP33 and nucleoprotein epitope NP396 were markedly reduced in the ear-dLNs of MasTRECK mice compared to that of WT animals at day 5 post infection (**Figures 2B, C**). At this early time point, virtually no virus-specific CD8+ T cells were observed in the spleen of both groups of infected animals (**Figure S6**). At the peak of infection, on day 8, frequencies and absolute cell numbers of CD8+ T cells (**Figure 2D**) as well as of GP33- and NP396-tetramer+ CD8+ T cells were markedly reduced in the spleen of MasTRECK mice compared to that of WT animals (**Figures 2E, F**). Accordingly, GP33- and NP396-tetramer+ CD8+ T cell frequencies and absolute numbers were also diminished in the infected ear of MasTRECK mice compared to that of WT animals at day 8 post infection (**Figures 2G–I**). Taken together, these data indicate that the expansion of virus-specific CD8+ T cells in the secondary lymph organs and their recruitment to the site of the infection are impaired in the absence of MCs.

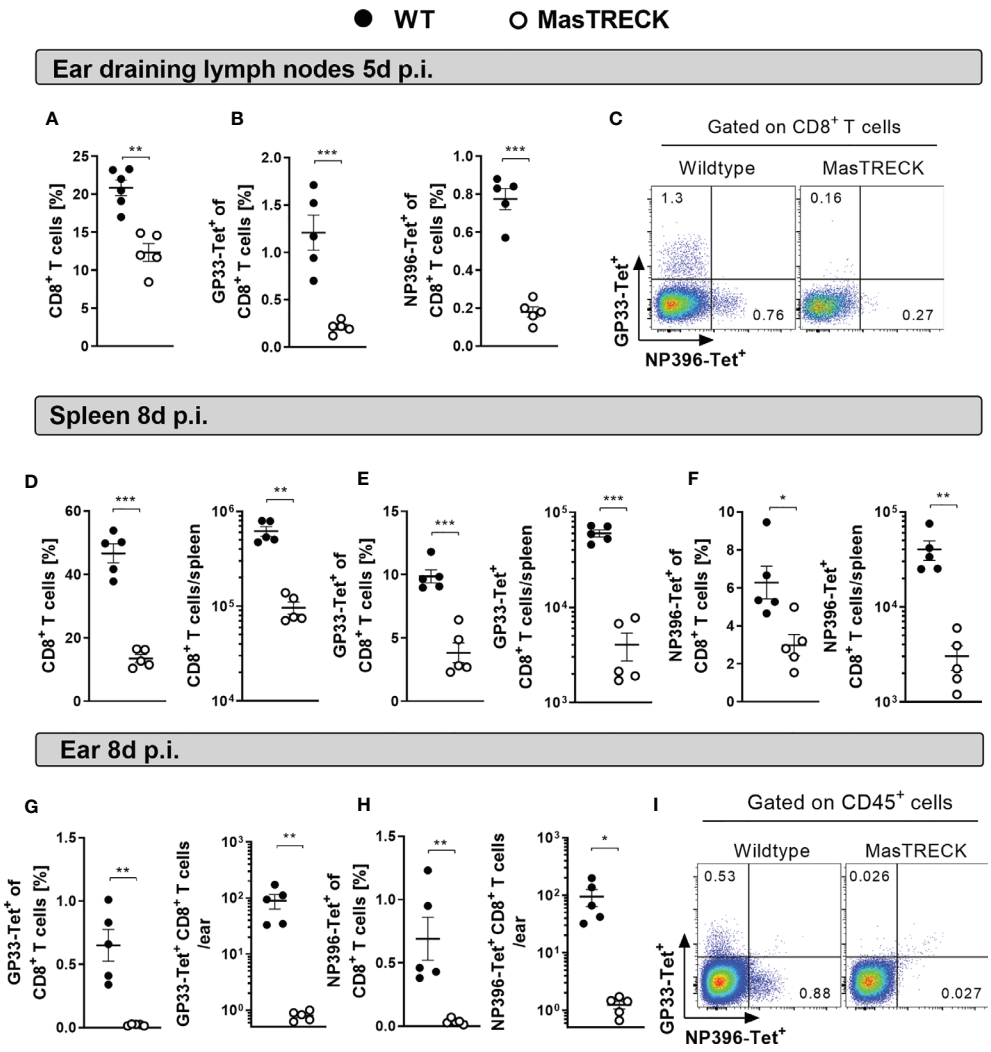

FIGURE 2 | Mast cells are key players in the expansion and recruitment of virus-specific CD8+ T cells. **(A)** Frequencies of CD8+ T cells, **(B)** GP33-Tetramer+ and NP396-Tetramer+ CD8+ T cells in the ear-dLN as well as **(C)** representative FACS plots from WT and MasTRECK mice at day 5 post intradermal LCMV infection. Frequencies and absolute numbers of **(D)** CD8+ T cells, **(E)** GP33-Tet+ and **(F)** NP396-Tet+ CD8+ T cells in the spleen of WT and MasTRECK mice at day 8 post LCMV infection. Frequencies and absolute numbers of **(G)** GP33-Tet+ and **(H)** NP396-Tet+ cells in the ear of WT and MasTRECK mice as well as **(I)** representative FACS plots at day 8 post LCMV infection analyzed by flow cytometry. All experiments were performed at least twice, and each experimental group included n ≥ 4. Data are representative and expressed as mean ± SEM. Statistically significant differences are analyzed by *t*- test and indicated as follows: *p < 0.05, **p < 0.01, ***p < 0.001.

Mast Cell Deficient Mice Show Impaired CD8+ T Cell Effector Phenotype and Antiviral Cytokine Production After Infection

We then characterized the phenotype and function of CD8+ T cells of WT and MasTRECK mice in the ear-dLN at day 5 post infection and in the spleen at the peak of LCMV infection (**Figure S2**). Uninfected WT and MasTRECK mice displayed similar frequencies of naïve ($CD44^-$ $CD62L^+$) as well as activated $CD8^+$ T cells ($CD44^+$ $CD62L^-$) (**Figure S7A**) as well as comparable frequencies of IFN-γ–producing $CD8^+$ T cells in the spleen (**Figure S7B**). However, infected MasTRECK mice displayed markedly reduced frequencies and absolute numbers of short-lived effector $CD8^+$ T cells, depicted by KLRG-1^+ IL-$7R^-$ expression, compared to that of infected WT animals in the ear-dLNs (**Figure S8A**) and in the spleen (**Figure 3A** and **Figure S9A**). Furthermore, we observed that not only total $CD8^+$ T cells showed an impaired effector phenotype, but also the few GP33-tetramer$^+$ $CD8^+$ T cells found in the spleen of infected MasTRECK mice displayed markedly reduced KLRG-1 expression compared to that of GP33-tetramer$^+$ $CD8^+$ T cells of WT infected mice (**Figure 3B**). In addition, $CD8^+$ T cells that expressed CXCR3, shown to be up-regulated on the surface of activated $CD8^+$ T cells and important for their recruitment to

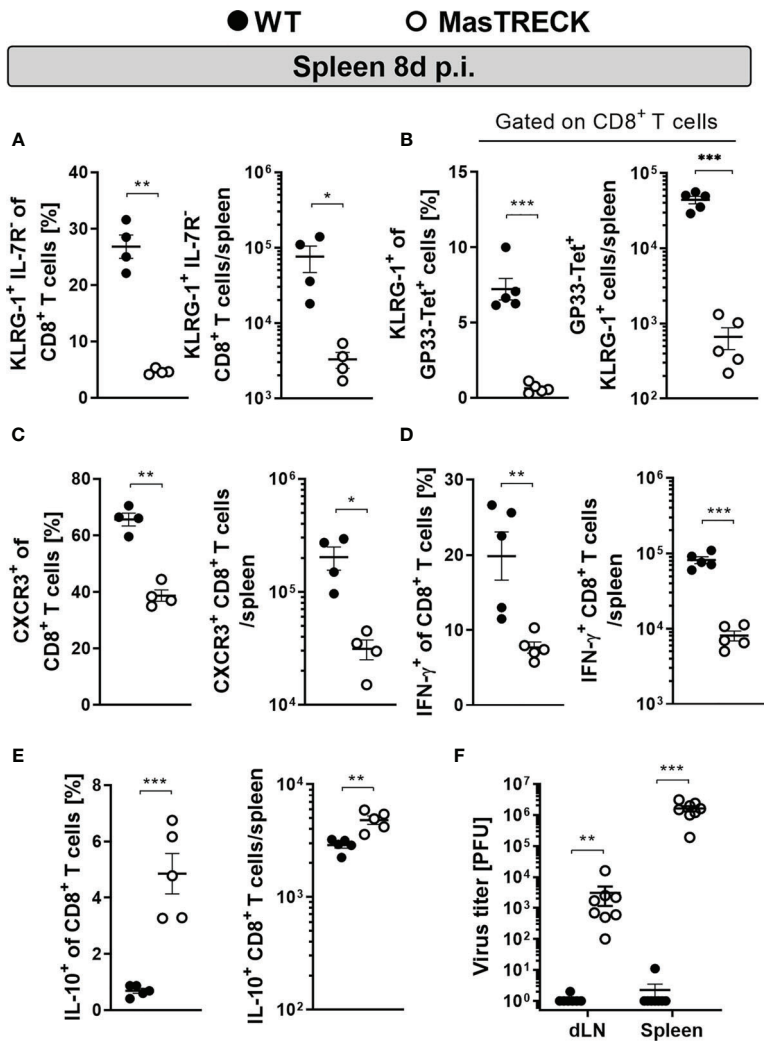

FIGURE 3 | Mast cell deficient mice show impaired $CD8^+$ T cell effector phenotype and antiviral cytokine production after infection. Frequencies and absolute numbers of **(A)** KLRG1^+ IL-$7R^-$, **(B)** KLRG1^+ gated on GP33-Tet$^+$ $CD8^+$ T cells and **(C)** CXCR3^+ $CD8^+$ T cells in the spleen of WT and MasTRECK mice at day 8 post intradermal LCMV infection. Frequency and absolute number of **(D)** IFN-γ– and **(E)** IL-10–producing $CD8^+$ T cells in the spleen after *ex vivo* restimulation with GP33 and NP396 peptides of WT and MasTRECK mice at day 8 post LCMV infection analyzed by flow cytometry. **(F)** Virus titers in ear-dLNs and spleen of WT and MasTRECK mice assessed by plaque assay at day 8 post intradermal LCMV infection. All experiments were performed at least twice, and each experimental group included $n \geq 4$. Data are representative and expressed as mean ± SEM. Statistically significant differences are analyzed by *t*- test and indicated as follows: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

antigen-rich areas of the spleen (40) were diminished in the ear-dLNs (**Figure S8B**) and in the spleen (**Figure 3C**) of infected MasTRECK mice compared to that of infected WT animals. Furthermore, infected MastTRECK mice had markedly reduced frequencies and absolute numbers of IFN-γ–producing $CD8^+$ T cells after GP33 and NP396 peptide restimulation *ex vivo* in ear-dLN (**Figure S8C**) and in the spleen (**Figure 3D** and **Figure S9B**) compared to that of WT animals. In contrast, the frequency and the absolute cell number of $CD8^+$ T cells producing the anti-inflammatory cytokine, IL-10 were increased in ear-dLNs (**Figure S8D**) and in the spleen (**Figure 3E**) of infected MasTRECK mice compared to that of WT mice.

In line with the impaired effector phenotype and defective cytokine production displayed by their virus-specific $CD8^+$ T cells, MasTRECK mice were unable to control LCMV infection and exhibited significantly higher viral loads in the spleen and ear-dLNs at the peak of infection compared to that of infected WT mice (**Figure 3F**). These findings collectively suggest that after intradermal LCMV infection, MCs are essential for antigen-specific $CD8^+$ T cell effector differentiation, antiviral cytokine production and viral clearance at the peak of infection.

Dendritic Cell Activation Is Impaired in Mast Cell Deficient Mice After LCMV Infection

The initiation of antigen-specific $CD8^+$ T cell responses requires the interaction of naive $CD8^+$ T cells with mature DCs (41). Particularly, $CD8^+$ DCs have been shown to be crucial in the initiation of $CD8^+$ T cell responses after LCMV infection (42, 43) and also appeared to dominate cytotoxic T cell priming after skin infection (44). Since the virus-specific $CD8^+$ T cell immune response was strongly impaired in infected MasTRECK mice at the peak of the infection, we hypothesize that the absence of MCs hindered the proper activation of $CD8^+$ DCs at early time points after LCMV infection. Therefore, we examined the frequency of $CD8^+$ DCs ($CD8^+MHCII^+CD11c^+$) cells and their costimulatory molecule expression on in the spleen at day 1 and 3 post infection (**Figure S3A**). Infected MasTRECK mice exhibited reduced frequencies and absolute cell numbers of $CD8^+$ DCs compared to that of infected WT mice (**Figure 4A**). In addition, the mean fluorescent intensity of the costimulatory molecules CD80 and CD86 expressed on $CD8^+$ DCs was significantly lower in infected MasTRECK mice compared to that of WT mice at day 3 p.i.in the spleen (**Figure 4B**). Similarly, infected MasTRECK mice exhibited reduced frequencies and absolute cell numbers of $CD8^+$ DCs as well as reduced mean fluorescent intensity of CD86 in the ear-dLNs compared to that of infected WT mice (**Figures S10A, B**).

Splenic macrophages also play an important role in the activation of $CD8^+$ T cells as well as in the control of viral load upon LCMV infection (45). Interestingly, infected MasTRECK mice exhibited reduced frequencies of $F4/80^+$ $CD11b^+$ cells (**Figure 4C**) and the mean fluorescent intensity of their costimulatory molecules CD80 but not CD86 was also significantly lower compared to that of WT mice at day 3 p.i. in the spleen (**Figure 4D**).

$CD8^+$ T cell activation is not only driven by properly activated DCs but also by the presence of pro-inflammatory cytokines such as type-I IFNs (46). Therefore, we assessed the frequency and activation of plasmacytoid DCs (pDCs) (**Figure S3B**) known to be the major producers of type-I IFNs (47). pDCs frequencies and absolute numbers were similar in the spleen of infected MasTRECK mice compared to that of WT animals at day 1 post infection but increased at day 3 p.i. (**Figure 4E**). Furthermore, the mean fluorescent intensity of CD86 on pDCs was significantly lower in infected MasTRECK mice compared to that of WT mice (**Figure 4F**). Interestingly, levels of IFN-α were also significantly reduced in the serum and in spleen homogenates of infected MasTRECK mice at day 3-post infection (**Figure 4G**). Taken together, these data indicate that the maturation of both classical and plasmacytoid DCs as well as the production of IFN-α after intradermal LCMV infection are impaired in the absence of MCs.

Mast Cell Deficient Mice Display Decreased Chemokine Levels at the Peak of the Infection

MCs produce several cytokines and chemokines upon activation and during viral infections (48, 49). Chemokines such as CCL3 and CCL4 are produced by human cord blood-derived mast cells (CBMCs) infected with mammalian reovirus (50) and play a key role in T cell-DC interactions involved in the generation of immune responses (51). We hypothesized that MC deficiency induces a modified chemokine milieu that alters $CD8^+$ T cell and DC recruitment and activation. Indeed, CCL3, CCL4 and CXCL10 concentrations were decreased in ear-dLNs (**Figure 5A**) and spleen homogenates (**Figure 5B**) from infected MasTRECK mice compared to that of WT mice at day 8 p.i. Few frequencies of isolated peritoneal MCs (**Figure S4A**) were directly infected *in vitro* with LCMV at MOI 5 (**Figure S4B**). However, high levels of CXCL1 and CCL2 were detected in the supernatant of infected peritoneal MCs compared to uninfected counterparts after 24 hours p.i. (**Figures S4C, D**). Interestingly, infected MasTRECK mice displayed significantly lower concentrations of CCL2 in the spleen compared to that of infected WT mice at day 3 post LCMV infection (**Figure 5C**). CCR2, the receptor of CCL2 has been reported to play a critical role in the recruitment of DCs (52) and $CD8^+$ T cells during viral infections (53). Accordingly, infected MasTRECK mice exhibited lower frequencies of $CCR2^+$ DCs and $CCR2^+$ $CD8^+$ T cells in the spleen compared to that of WT mice at day 3 post LCMV infection (**Figures 5D, E**). These results show that the chemokine milieu in the secondary lymphoid organs is altered in the absence of MCs.

Mast Cell Depletion After LCMV Infection Does Not Impair Virus-Specific $CD8^+$ T Cell Responses

Our data suggested that the presence of MCs is important for proper DC maturation and recruitment at early time points after intradermal LCMV infection, which in turn is essential for proper effector differentiation, and antiviral cytokine

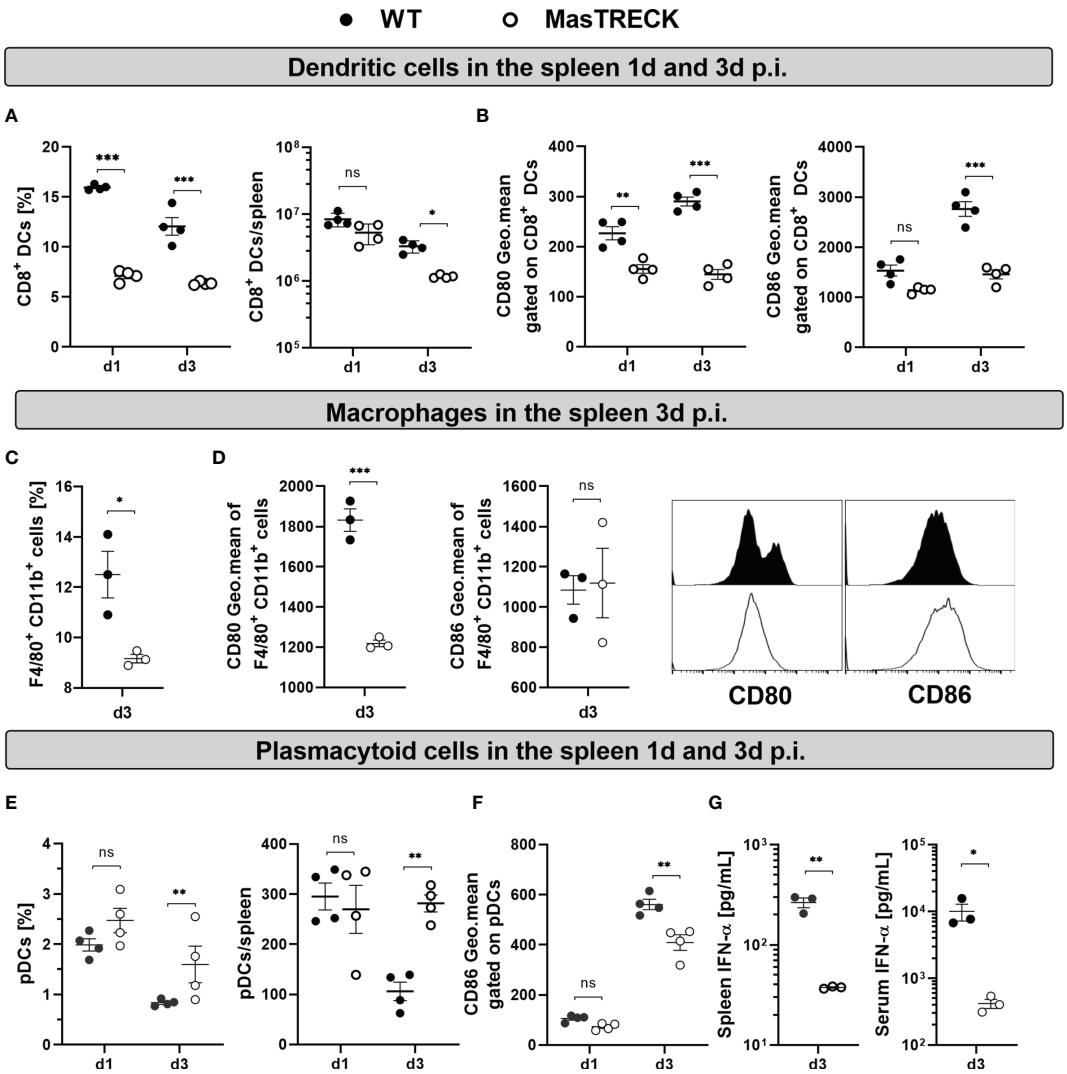

FIGURE 4 | Dendritic cell activation is impaired in mast cell deficient mice after LCMV infection. **(A)** Frequency and absolute number of $CD8^+$ DCs in the spleen of WT and MasTRECK mice on day 1 and 3 post intradermal LCMV infection. **(B)** Expression levels of CD80 and CD86 (geometric mean of fluorescence intensity) gated on $CD8^+$ DCs in the spleen of WT and MasTRECK mice at day 1 and 3 post infection. **(C)** Frequency of splenic macrophages ($F4/80^+$ $CD11b^+$) and **(D)** expression levels of CD80 and CD86 (geometric mean of fluorescence intensity) gated on macrophages as well as representative histograms in the spleen of WT and MasTRECK mice at day 3 post infection. **(E)** Frequency and absolute number of pDCs in the spleen of WT and MasTRECK mice at day 1 and 3 post infection. **(F)** Expression levels of CD86 (geometric mean of fluorescence intensity) gated on pDCs in the spleen of WT and MasTRECK mice at day 1 and 3 post infection. **(G)** IFN-α concentration in the spleen and serum of WT and MasTRECK mice at day 3 post LCMV infection assessed by magnetic beads multiplex ELISA. All experiments were performed at least twice, and each experimental group included $n \geq 3$. Data are representative and expressed as mean ± SEM. Statistically significant differences are analyzed by *t*- test and indicated as follows: ns = not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

production of antigen-specific $CD8^+$ T cells as well as viral clearance. Therefore, we investigated the impact of MC depletion on the phenotype and the function of virus-specific $CD8^+$ T cells at a later time point after LCMV infection. For this, WT and MasTRECK mice were initially infected with LCMV on the ventral side of the ear pinna, and DT treatment followed between day 4 and day 8 post infection (**Figure 6A**). Interestingly, the conditional depletion of mast cells at a later time point after infection completely reverted the suppression of LCMV-specific $CD8^+$ T cell responses observed when MCs were depleted before the infection. No difference in the ear thickness was observed between infected WT and MasTRECK mice (**Figure 6B**). In addition, absolute cell numbers of $CD8^+$ T cells as well as GP33- and NP396-tetramer$^+$ $CD8^+$ T cells in the spleen of WT and MasTRECK mice were comparable at the peak of infection (**Figures 6C, D**). Furthermore, similar frequencies of $KLRG1^+$ $CD8^+$ T cells were observed in the spleen of both groups of infected animals (**Figure 6E**). Moreover, absolute cell numbers of IFN-γ– and IL-10–producing $CD8^+$ T cells in the spleen of infected

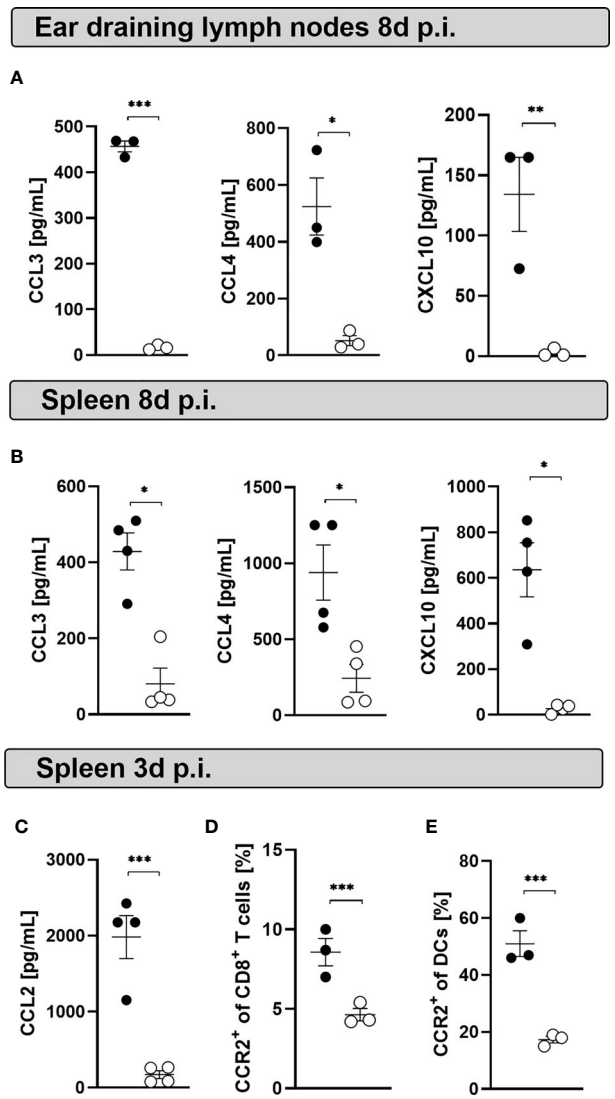

FIGURE 5 | Mast cell deficient mice display decreased chemokine levels at the peak of the infection. Concentrations of CCL3, CCL4 and CXCL10 in the supernatants of cultured cells from **(A)** ear-dLNs and from the **(B)** spleen of WT and MasTRECK mice at day 8 post infection. **(C)** Concentrations of CCL2 in supernatants of cultured splenocytes of WT and MasTRECK mice at day 3 post infection assessed by the chemokine 26-Plex Mouse ProcartaPlex™ Panel 1. **(D)** Frequencies of $CCR2^+$ $CD8^+$ T cells and **(E)** $CCR2^+$ DCs in the spleen of WT and MasTRECK mice at day 3 post infection analyzed by flow cytometry. All experiments were performed at least twice, and each experimental group included $n \geq 3$. Data are representative and expressed as mean ± SEM. Statistically significant differences are analyzed by *t*- test and indicated as follows: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

MasTRECK mice after GP33 and NP396 peptide restimulation *ex vivo* were similar to that of infected WT mice (**Figures 6F, G**). As expected, both infected WT and MasTRECK mice were able to control LCMV infection and no viral loads were detected in the spleen and ear-dLNs at day 9 p.i. (**Figure 6H**). In summary, our data demonstrate that the presence of MCs at the beginning of the intradermal infection is crucial for the expansion, activation and antiviral cytokine production of virus-specific $CD8^+$ T cells.

DISCUSSION

In recent years, there is increasing evidence that MCs are key players of both innate and adaptive immune responses (1). MCs have been particularly known for their pivotal role in allergic type-I reactions (54). However, several studies have demonstrated that MC orchestrate the development of immune responses due to their strategic location, their ability to sense pathogens and danger as well as their capacity to directly and indirectly modify the activation and function of other immune cells (55). Furthermore, studies show MC influence on the cellular immune response to viruses (17). In a mouse model of Newcastle virus infection, recruitment of $CD8^+$ T cells to the site of infection was dependent on the presence of MCs (56). MCs are equipped with a plethora of immune mediators that influence the migration, activation and function of granulocytes, DCs, macrophages, NK, NKT and T cells (24, 26, 48). We used transgenic MasTRECK mice that do not harbor any additional alterations of other immune cell subsets and it is an inducible model that allows us to deplete MCs with the administration of DT at different time points before and after LCMV infection. Although, basophils are also depleted after DT treatment, we do not envision any direct effects of basophils on antigen-specific $CD8^+$ T cell responses upon LCMV infection known to induce a strong Th-1 immune response. Here, we report that MCs are crucial for the proper activation of DCs, splenic macrophages and pDCs during the first days of intradermal LCMV infection. The absence of MCs at the beginning of the intradermal LCMV infection led to an impaired activation, expansion and function of $CD8^+$ T cells in infected mice.

The role of MCs in T cell expansion has been mostly associated with the ability of MCs to modulate DC migration to the dLNs (57, 58). In recent years, studies have revealed strong interactions between MCs and DCs that subsequently modulate their activation and their functionality. MC activation not only promotes DC migration (59) but also induces DC activation that in turn is critical for optimal $CD8^+$ T cell activation (28). Accordingly, we show that in the absence of MCs, the frequency and absolute number of $CD8^+$ DCs as well as their costimulatory molecules CD80 and CD86 were decreased during the first three days after intradermal LCMV infection. Subsequently, impaired DC activation led to the generation of defective virus-specific $CD8^+$ T cells that displayed a dysfunctional effector phenotype characterized by low KLRG-1, low CXCR3 expression, reduced IFN-γ and high IL-10 production at the peak of infection that hindered the control of the viral clearance at the peak of the infection. In addition, the presence of MCs were also important for the proper activation of splenic macrophages. The presence of MCs at the beginning of the intradermal infection was crucial for the generation of optimal antigen-specific $CD8^+$ T cells since MC depletion at day

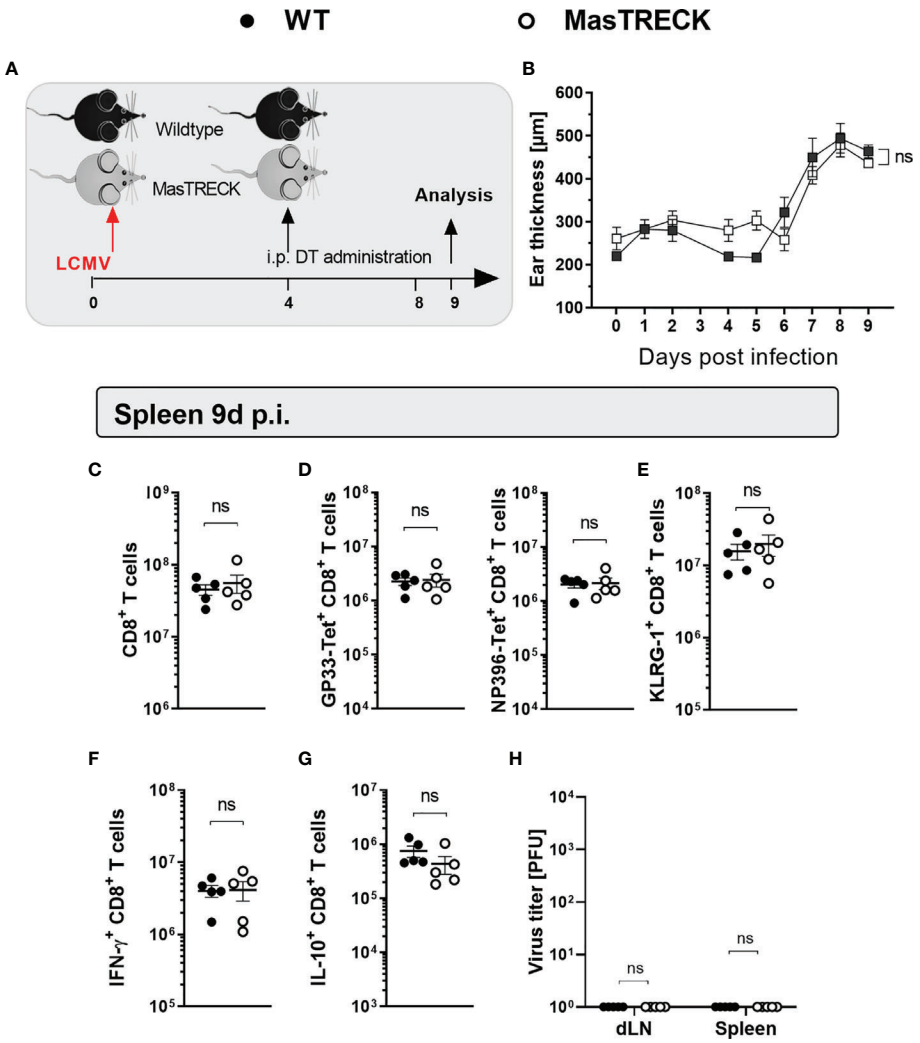

FIGURE 6 | Mast cell depletion after LCMV infection does not impair virus-specific CD8+ T cell responses. **(A)** Schematic experimental layout to analyze virus-specific $CD8^+$ T cell responses in WT and MasTRECK mice that were first infected with LCMV into the ventral side of the ear pinna and subsequently treated with 250 ng DT i.p. for 5 consecutive days between days 4-8 p.i. **(B)** Ear thickness daily measured using a caliper during 9 days after LCMV infection. **(C)** Absolute numbers of $CD8^+$ T cells, **(D)** GP33-Tet^+ and NP396-Tet^+ $CD8^+$ T cells as well as **(E)** $KLRG1^+$ $CD8^+$ T cells in the spleen of WT and MasTRECK mice at day 9 post infection. **(F)** Absolute numbers of IFN-γ– and **(G)** IL-10–producing $CD8^+$ T cells in the spleen of WT and MasTRECK mice after *ex vivo* restimulation with GP33 and NP396 peptides at day 9 post infection assessed by flow cytometry. **(H)** Virus titers in ear-dLN and spleen of WT and MasTRECK mice assessed by plaque assay at day 9 post intradermal LCMV infection. All experiments were performed at least twice, and each experimental group included n ≥ 4. Data are representative and expressed as mean ± SD. Statistically significant differences are analyzed by *t*-test and indicated as follows: ns = not significant.

4 post infection did not impair the phenotype or antiviral cytokine production of virus-specific $CD8^+$ T cells and infected mice were able to control the infection.

One of the most challenging aspects for the role of MCs in the development of virus-specific T cell responses is the spatial separation between peripheral MCs and the $CD8^+$ T cells in the spleen. However, MCs are not only located in the connective and mucosal tissue, they are also distributed around blood vessels and in close proximity to perivascular DCs (33). In addition, MCs can modulate $CD8^+$ T cell responses over a distance, and signals from MCs can reach the spleen *via* the bloodstream, e.g. in the context of degranulation or *via* the release of the exosomes (60). Recent studies indicate that MCs can exert long distance effects through MC granule trafficking *via* lymphatic vessels and active shuttling of MC granules by DCs (61). Moreover, MCs can modulate T cell activation through exosomes that harbor inflammatory mediators (62, 63). CCL3 and CCL4 have been shown to facilitate T cell-DC interactions (51). In our study, the levels of CCL2, CCL3, CCL4 and CXCL10 were reduced in infected MC-deficient mice. We used peritoneal MCs as a surrogate for skin-MCs. Although very few frequencies of LCMV infected peritoneal MC were observed *in vitro* after

24 h p.i., high production of CCL2 and CXCL1 was detected. MCs could sense LCMV particles and produce chemokines such as CCL2 as previously reported (26). Furthermore, frequencies of $CD8^+$ T cells–expressing CXCR3, the receptor for CXCL10, and of $CD8^+$ T cells–expressing CCR2, the receptor for CCL2, were reduced in MC-deficient mice.

Collectively, our results indicate that after intradermal LCMV infection, MCs promote optimal $CD8^+$ DC and pDC activation leading to the generation of a proper proinflammatory cytokine and chemokine milieu essential for the activation of antigen-specific $CD8^+$ T cells that are crucial to achieve the control of the viral infection.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Animal protocols were performed in accordance with the German law for animal protection and the institutional guidelines of the Charité Berlin. The animal study was reviewed and approved by Landesamt fuer Gesundheit und Soziales LAGeSo G0078/17.

AUTHOR CONTRIBUTIONS

FS, MMu, and MMa designed the research. YH and MMu performed the research. YH and MMu analyzed the data. YH, ML, and MMu performed the visualization, preparation, and presentation of the data. YH and MMu wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.688347/full#supplementary-material

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TLR2 Regulates Mast Cell IL-6 and IL-13 Production During *Listeria monocytogenes* Infection

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Listeria monocytogenes (L.m) is efficiently controlled by several cells of the innate immunity, including the Mast Cell (MC). MC is activated by L.m inducing its degranulation, cytokine production and microbicidal mechanisms. TLR2 is required for the optimal control of L.m infection by different cells of the immune system. However, little is known about the MC receptors involved in recognizing this bacterium and whether these interactions mediate MC activation. In this study, we analyzed whether TLR2 is involved in mediating different MC activation responses during L.m infection. We found that despite MC were infected with L.m, they were able to clear the bacterial load. In addition, MC degranulated and produced ROS, TNF-α, IL-1β, IL-6, IL-13 and MCP-1 in response to bacterial infection. Interestingly, L.m induced the activation of signaling proteins: ERK, p38 and NF-κB. When TLR2 was blocked, L.m endocytosis, bactericidal activity, ROS production and mast cell degranulation were not affected. Interestingly, only IL-6 and IL-13 production were affected when TLR2 was inhibited in response to L.m infection. Furthermore, p38 activation depended on TLR2, but not ERK or NF-κB activation. These results indicate that TLR2 mediates only some MC activation pathways during L.m infection, mainly those related to IL-6 and IL-13 production.

Keywords: mast cell, *Listeria monocytogenes*, toll like receptor-2, IL-6, IL-13, p38, MAPK, LLO

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INTRODUCTION

Classically, mast cells (MC) are associated with type I hypersensitivity reactions (1). However, growing evidence has placed them as initiators of the inflammatory process against several infectious agents, including bacteria (2). Due to their strategic location in mucosal epithelia, skin, and connective tissue, they can respond immediately to the signals derived from mutualistic and pathogenic bacteria, adapting their response accordingly to maintain host homeostasis (3). In this way, MC are provided with at least one member of each of the Pattern Recognition Receptor (PRR) families. These include Toll-Like Receptors (TLR), C-type Lectin Receptors (CLR), NOD-Like Receptors (NLRs), RIG-Like Receptors (RLRs), and Scavenger Receptors (4). When MC are activated, they release many preformed mediators found in their granules, through cell degranulation. Furthermore, they can also synthesize *de novo* molecules such as inflammatory mediators derived from arachidonic acid, reactive oxygen species (ROS) as well as cytokines and chemokines (5). Several studies have shown that MC are involved in the immune response to pathogenic bacteria, including: *Pseudomonas aeruginosa* (6), *Klebsiella pneumoniae* (7), *Staphylococcus aureus* (8), *Mycobacterium tuberculosis* (9) and *Listeria monocytogenes* (L.m) (10), to mention only a few.

L.m is a Gram-positive, facultative intracellular bacteria and the causal agent of listeriosis, a foodborne disease with a high mortality rate (11). L.m presents tropism towards the gravid uterus and central nervous system (CNS), contributing to the most severe clinical manifestations (12). Although, these severe cases are rare in immunocompetent individuals, they increase in case of immunosuppression or immunodeficiencies (11). This implies that host immune response mechanisms are crucial to the containment of the bacteria, and its alteration can increase susceptibility to the infection (13).

Cells of the innate immune response play a crucial role in containing L.m infection, notably, macrophages, dendritic cells, neutrophils, NK cells and MC (14, 15). In experimental murine listeriosis models, MC have been shown to initiate the effector response against this bacterium, promoting recruitment of neutrophils and macrophages to the site of infection (10, 15). Furthermore, MC response to L.m includes intracellular infection (16), degranulation (16, 17), ROS production (18) and different cytokines and chemokines (15, 16). However, it is unclear which receptors mediates L.m activation by MC.

TLR2 is a transmembrane type I receptor, which contains a cytoplasmic TIR domain as well as an extracellular domain with leucine-rich repeats (19). TLR2 ligands include: Diacil-lipopeptides, lipoarabinomannan, lipoproteins, lipoteichoic acid (LTA), peptidoglycan (PGN), porins, phospholipomannan and zymosan (20). Once TRL2 binds to its ligand, it is dimerized with either TLR1 or TLR6 (21). When this occurs, TIR domain recruits the adaptive molecules TIRAP and MyD88 that lead to the activation of the transcription factor NF-κB and the mitogen-activated protein kinases (MAPK) that activate the transcription factor AP-1 (21, 22). Additionally, TLR2 activates the P13K-AKT signaling pathway (22)

Activation of TLR2 promotes diverse cellular functions such as phagocytic activity (23), bactericidal activity (24), ROS production (25), degranulation (26) and cytokine production (27). In addition, TLR2-deficient mice are more susceptible to L.m infection than wild type mice, which is consistent with poor control of the bacterial load on target organs (28). In addition, MyD88-deficient MC produce less IL-6 and MCP-1 in response to L.m (16). Considering that MC express TLR2 (29), we decided to dissect the activation mechanisms that are regulated by TLR2 during mast cell activation by *Listeria monocytogenes* infection.

MATERIAL AND METHODS

Bacteria Culture

L.m strain 1778+H 1b (ATCC 43249, USA, Manassas, VA, USA) was grown in brain heart infusion broth (BHI, BD-Difco, USA) for 18 h at 37°C with constant shaking at 112 xg. Bacterial cultures were washed with Hanks Balanced Saline Solution (HBSS) (Life Technologies, USA) and bacterial pellets resuspended in RPMI-1640 Glutamax (Life Technologies, USA) supplemented with 40% Fetal Bovine Serum (Life Technologies, USA) and frozen at -70 °C until use. Aliquots of L.m were serially diluted and plated in BHI agar at 37°C for 18-24 h. Bacterial numbers were determined by counting Colony-Forming Units (CFU).

Mast Cells

Bone Marrow-derived Mast Cell (BMMC) was obtained following the protocol described by (30). Briefly, bone marrow cells were obtained from femurs and tibias of 6-8-week-old C57BL/6 female mice. Cells were maintained in RPMI-1640 supplemented with 10% FBS, 5 μM β-mercaptoethanol (Life Technologies, USA) and 2% antibiotic and antimycotic (Sigma, USA) (complete RPMI 1640 medium) plus 10 ng/mL of murine recombinant IL-3 (Peprotech, USA) and 10 ng/mL of murine recombinant stem cell factor (Peprotech, USA)). Non-adherent cells were transferred to fresh culture medium twice a week for 6–9 weeks. The purity of BMMC was ≥90% assessed by flow cytometry after staining of CD117 (clone: 2B8, BioLegend, USA; 0.25 μg/mL) and FcεRI (clone: MAR-1, BioLegend, USA; 0.16 μg/mL) (**Supplementary Figure 1A**).

Peritoneum-derived mast cells (PMC), cells were obtained from peritoneal cavity of mice and cultured in complete RPMI-1640 medium plus IL-3 (30 ng/mL) and SCF (20 ng/mL). Non-adherent cells were transferred to fresh culture medium twice a week for 3–4 weeks. The purity of PMC was ≥90% assessed by flow cytometry (**Supplementary Figure 1C**).

All experiments followed institutional biosecurity and safety procedures. All animal experiments were approved by the Research Ethics Committee of the ENCB, IPN (ZOO-016-2019).

Toluidine Blue Staining

$2x10^5$ BMMC or $2x10^5$ PMC/0.25 mL of RPMI-1640 supplemented with 10% FBS and 5μM β-mercaptoethanol (complete medium) were plated in cytospin chambers, and then stained with toluidine blue for 10 minutes. Finally, slides

were air-dried and mounted with Entellan resin (Merck Millipore, USA) under a coverslip. Images were captured with a digital camera attached to a brightfield microscope (Zeiss Primo Star, Germany), and analyzed with Micro capture v7.9 software (**Supplementary Figure 1B, D**).

Viability Mast Cell Assay

$2.5X10^5$ BMMC/0.25 mL of complete medium were stimulated with L.m at different MOI (1:1, 10:1, 100:1) or stimulated with LLO (125, 250, 500, 1000 ng/mL) for 24 h. Then cells were washed with 1 mL of Annexin V binding Buffer 1X (Invitrogen, USA) and stained with 1 μg/mL Annexin V (BioLegend, USA) and 0.5 μg/mL propidium iodide (eBioscience, USA). After staining, cell viability was measured by flow cytometry (**Supplementary Figure 2**).

Degranulation Assay

The degranulation assay was carried out as described previously (31). Briefly, $2x10^5$ BMMC/0.25 mL of complete medium were incubated with L.m at different MOI for 90 minutes. Then were washed and stained with anti-CD107a (clone: 1D4B Biolegend, USA; 0.25 μg/mL) and anti-FcεRI. Staining was measured by flow cytometry.

β-Hexosaminidase release was performed as follows: $2x10^5$ BMMC/0.25 mL of HEPES-Tyrode Buffer (HBT) (130 mM NaCl, 5.5 mM glucose, 2.7 mM KCl, 1.0 mM $CaCl_2$ 2 H_2O, 0.1% [wt/vol] Bovine Serum Albumin (BSA), 12 mM HEPES, 0.45 mM NaH_2PO_4 $1H_2O$, pH 7.2) were stimulated with L.m at different MOI or stimulated with PMA (125 nM) plus Ionomicin (10 μM) for 90 minutes at 37°C. The supernatants were then recovered, and the cell pellet was lysed with 200 μL of 0.2% Triton X-100 in HBT. Both supernatants and cell lysates were incubated with 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich, USA; 1 mM in 200 mM Na Citrate Buffer pH 4.5) for 2 h at 37°C. The enzyme reaction was stopped by the addition of 100 μL of 200 mM Tris base, pH 10.7. The samples were analyzed in a fluorescence plate reader (SpectraMax M, USA) using excitation 356 nm and emission 450 nm. The percentage of release of β-Hexosaminidase is calculated using the formula: $\% \quad Release = \left[\frac{supernatan}{(supernatan+cellpellet)}\right] \times 100$.

Superoxide Anion(O_2^-) Production

$2x10^5$ BMMC were cultured in 0.25 mL of RPMI-1640 without phenol red (Life Technologies, USA) and then, stimulated with L.m at different MOI for 120 minutes. In the last 15 minutes of incubation 25 μL of a solution of p-nitro blue tetrazolium (NBT) (Sigma-Aldrich, USA) at 1 mg/mL were added. Then, cells were washed with PBS 1X (Life Technologies, USA) and fixed with absolute methanol. The formazan precipitates were dissolved by adding 54 μL of potassium hydroxide (KOH) 2mM and 46 μL of Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA). The samples were read at a wavelength of 620 nm in a plate reader (Multiskan EX, Thermo Scientific, USA).

Bacterial Load Assay

$2x10^5$ BMMC/0.25 mL of complete medium were incubated with L.m at different MOI for 2 h. At the end of incubation, HBSS supplemented with ampicillin at 200 ng/mL was added for 1 h. Afterwards, cells were washed and then transferred to complete medium supplemented with ampicillin at 200 ng/mL for 0 and 24 h. Then, cells were washed with HBSS and then lysed with sterile distilled water. Cell suspension were homogenized, and serial decimal dilutions were prepared in saline solution. Afterwards, 20μL of each dilution were plated on BHI agar at 37°C for 48 h, bacterial numbers were determined by counting CFU.

Cytokines Quantification

$2x10^5$ BMMC/0.25 mL of complete medium were stimulated with L.m at different MOI or stimulated with Recombinant Listeriolysin-O (LLO; RayBiotech, USA) at different concentration (125, 250, 500 and 1000 ng/mL) for 24h. Then, supernatants were collected for the detection of TNF-α, IL-1β, IL-6, MCP-1 (Biolegend, San Diego, CA., USA) and IL-13 (eBioscience, USA) by ELISA according to manufacturer's instructions.

Evaluation of Phosphorylated Proteins

$2x10^5$ BMMC/0.25 mL of complete medium were stimulated with L.m MOI 100:1 for 15 minutes to evaluate p-ERK 1/2, 30 minutes for p-p65 and 60 minutes for p-p38. Then, the cells were preserved with 250 μL of Fixation buffer (BD-Bioscience, USA) for 10 min/37°C. Subsequently, were washed with 1 mL of Stain Buffer (BD-Bioscience, USA) and permeabilized with 1mL of 0.5x Perm buffer IV (BD-Bioscience, USA) for 15 min at room temperature (RT) and protected from light. Then, the cells were washed with 1 mL of Stain Buffer. After blocking with 0.015 μg of anti-CD16/32 (Mouse BD Fc Block™, clone: 2.4G2. BD-Biosciences, USA) cells were stained with antibodies to p-ERK 1/2-PE (clone: 20A; 4 μL per tube), p-p65-PE (clone: K10-895.12.50; 4 μL per tube), p-p38-PE (Clone: 36/p38; 4 μL per tube) or isotype controls (Clone: MOPC-21; Mouse IgG_1, κ-PE or Clone: MCP-11; Mouse IgG_{2b}, κ-PE). (All from BD-Biosciences, USA) for 60 min at RT and protected from light. Finally, the cells were washed and resuspended in 0.15 mL of Stain Buffer and analyzed by flow cytometry. The times chosen for the detection of each phosphorylated protein were selected based on kinetic assay (**Supplementary Figure 4**).

Expression of TLR2

$2x10^5$ BMMC/0.1 mL of PBS 1X were marked with anti-TLR2-biotin (clone: 6C2, eBioscience, USA; 1 μg/0.1 mL) or isotype control-biotin (clone: eB149/10H5, eBioscience, USA; Rat IgG_{2b}, κ-biotin; 1 μg/0.1 mL) and stained with streptavidin-APC (BD-Bioscience, USA; 0.02 μg/mL), prior blocking with anti-CD16/32. Staining was measured by flow cytometry.

TLR2 Blocking Assays

$2x10^5$ BMMC/0.25 mL of complete medium were pre-incubated 30 minutes with 200 ng/mL of anti-TLR2 (clone: C9A12; IgG2a) or 200 ng/mL of isotype control (clone: T9C6; 1gG2a), both from Invivogen, USA. Afterwards, cells were stimulated with L.m MOI 100:1 and the bacterial load, O_2^- production, degranulation, cytokines production and evaluation of phosphorylated proteins assays were performed as indicated above. The

bacterial load was analyzed as endocytic activity and bactericidal activity, according to the following formula: endocytic activity = (CFU at 0 h/MOI added) *100 (%). Bactericidal activity = (100-(CFU at 24 h/CFU at 0 h) *100) (%) (32). To evaluate the efficiency of antibody-mediated TLR2 inhibition, $2x10^5$ BMMC/0.25 mL were pre-incubated with different concentrations of anti-TLR2 (12.5, 25, 50, 100 y 200 ng/mL) for 30 min and then stimulated with the TLR2 agonist: *Staphylococcus aureus* peptidoglycan (PGN) (Sigma-Aldrich, USA) at 10 μg/mL for 24 h. Then, supernatants were collected for the detection of IL-6 by ELISA (**Supplementary Figure 5**).

Flow Cytometry

All cell samples stained with fluorochrome-conjugated antibodies were acquired using FACSAria Fusion (BD Biosciences, USA) and analyzed with FlowJo software version 6.0 (FlowJo, LLC). Cell debris and doublets were excluded from the analysis.

Statistical Analysis

All statistical analyses were performed with SigmaPlot software version 14.0, from Systat Software, Inc., San Jose California USA, www.systatsoftware.com. Data normality was assessed by Kolmogorov-Smirnov with Lilliefors correction. Variables that followed normal distribution were plotted as mean ± standard error mean (s.e.m), represented as bars and analyzed with one way-analysis of variance (ANOVA) with Student-Newman-Keuls (SNK) post-hoc. While variables that did not follow normal distribution, semi-quantitative variables, normalized variables or percentages, were plotted as median + range, represented as boxes and analyzed with the Mann-Whitney test (comparisons between two groups) or Kruskal-Wallis test (comparisons between more than two groups). A value of $p < 0.05$ was considered to be significant.

RESULTS

Mast Cell Activation Concurs With the Control of *Listeria monocytogenes* Infection

Previous studies have noticed that MC can respond to L.m infection through degranulation, ROS production, internalization, and clearance of this bacterium (16–18). To corroborate the presence of these MC activation mechanisms during L.m infection, we incubated BMMC with different MOI of this bacterium to determine degranulation either through the surface expression of CD107a (**Figure 1A**) or β-hexosaminidase release (**Figure 1B**), ROS production with detection of O_2^- (**Figure 1C**) and internalization and clearance of L.m by evaluating intracellular CFU (**Figure 1D**). We found that degranulation and O_2^- production from BMMC occurred using L.m MOI 100:1 (**Figures 1A–C**). Also, we detected viable bacteria within BMMC at 0-hour post-infection (hpi) with the different MOI of L.m tested; however, we recovered small amounts of this bacterium at 24h, corresponding to a reduction of more than 3 logarithms for any MOI tested (**Figure 1D**). These results indicate that BMMC degranulate and produce ROS with high amounts of L.m and that BMMC are capable of internalizing and controlling L.m infection.

Listeria monocytogenes Induces Mast Cell Cytokine and Chemokine Release

Previous reports have shown that L.m induces the *de novo* synthesis of different cytokines and chemokines (16, 33). To further corroborate the MC response to L.m we determined the production of TNF-α, IL-1β, IL-6, IL-13 and MCP-1. We stimulated BMMC with different MOI of L.m and at 24 h we determined the levels of each mediator. We found that BMMC produced TNF-α with MOI 1:1, 10:1 and 100:1 of L.m (**Figure 2A**). While IL-1β production was only detected in BMMC incubated with MOI 100:1 of L.m (**Figure 2B**). Similarly, we found that this bacterium induced IL-6 (**Figure 2C**) and IL-13 (**Figure 2D**) only with MOI 100:1 of L.m in BMMC. While MCP-1 was induced with MOI 10:1 and 100:1 of L.m in BMMC (**Figure 2E**). Together, these results indicate that L.m induces *de novo* synthesis of cytokines and chemokines.

Listeria monocytogenes Induces the Activation of Signaling Pathways Associated With TLR Activation on Mast Cells

Since L.m promoted the activation of BMMC, we decided to evaluate if this was associated with the induction of some intracellular signals that are associated with TLR receptors (22). To this end, we incubated BMMC with L.m (MOI 100:1) and determined the phosphorylation of some signaling proteins by flow cytometry. Initially we evaluated the phosphorylation of ERK 1/2 (**Figure 3A**) and p38 (**Figure 3B**), molecules belonging to the MAPK signaling pathway and which have been related to the production of pro-inflammatory cytokines in macrophages infected with L.m (34, 35). Interestingly, we found that L.m induced phosphorylation of both signaling proteins in BMMC (**Figures 3A, B**). An important transcription factor in the production of pro-inflammatory cytokines and chemokines by macrophages infected with L.m is NF-κB (34). Therefore, we determined whether this transcription factor was activated in BMMC in response to L.m, by evaluating the phosphorylation of p65 subunit. Interestingly, we found that L.m induced phosphorylation of p65 in BMMC (**Figure 3C**). Together, these results indicate that L.m induces the activation in BMMC of cell signaling molecules that are associated with TLR activation.

TLR2 Is Not Involved in Mast Cell Degranulation, ROS Release, Endocytosis and *Listeria monocytogenes* Clearance

One of the main receptors found in various cells of the innate immune response that recognizes different components of the cell wall of Gram-positive bacteria, such as L.m, is TLR2 (20). The importance of macrophage TLR2 for phagocytosis and ROS

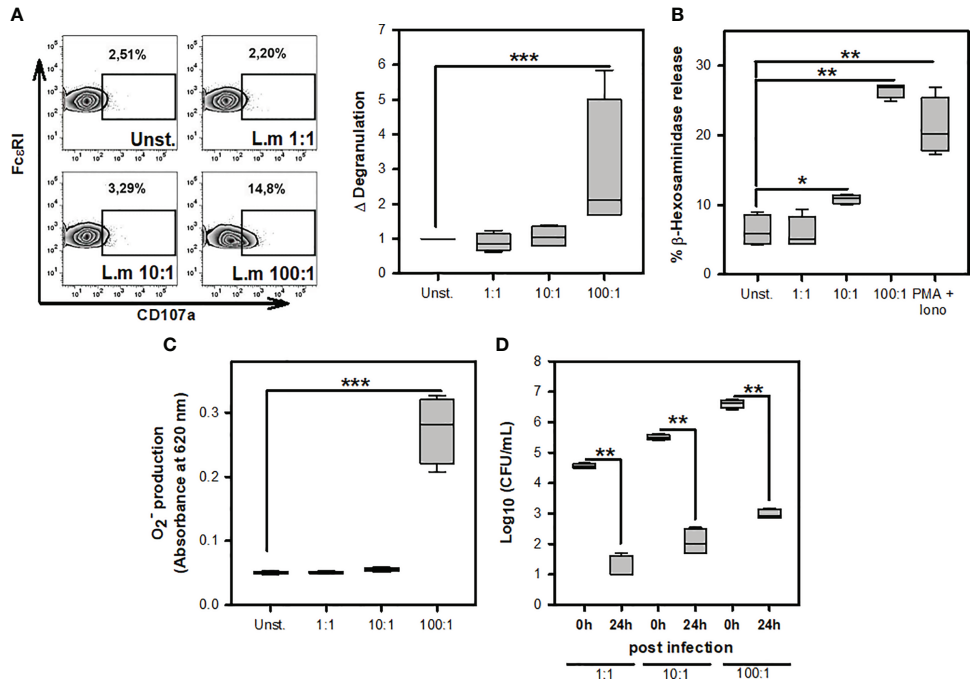

FIGURE 1 | Mast cell activation in response to *Listeria monocytogenes* infection. **(A)** 2.5X10^5 BMMC/0.25 mL of complete medium were stimulated with L.m for 90 minutes at the MOI indicated. BMMC degranulation was determined by flow cytometry. Left panel shows representative zebra-plots. Right panel shows the fold change in the percentage of FcεRI$^+$/CD107a$^+$ cells with respect to unstimulated MC. (sum of 4 independent experiments, n=4 per group; ***p<0.001; Kruskal-Wallis test). **(B)** 2.5X10^5 BMMC/0.25 mL of complete medium were stimulated with L.m at the MOI indicated or PMA (125 nM) plus Ionomycin (Iono; 10 μM) for 90 minutes. BMMC degranulation was determined through beta-hexosaminidase release as indicated in Material and methods. (sum of 4 independent experiments, n=4 per group; *p<0.05, **p<0.01 ***p<0.001; Kruskal-Wallis test). **(C)** 2.5X10^5 BMMC/0.25 mL of RPMI-1640 without phenol red were stimulated with L.m for 2 h at the MOI indicated. Afterwards, the O_2^-determination was performed by the NBT reduction assay. (sum of 4 independent experiments, n=4 per group; ***p<0.001; Kruskal-Wallis test). **(D)** 2.5X10^5 BMMC/0.25 mL of complete medium were infected with L.m for 2 h at the MOI indicated. Intracellular bacteria were quantified by CFU assay as indicated in Material and methods. (sum of 4 independent experiments, n=4 per group; **p<0.01; Mann-Whitney test).

production during infection with L.m has been demonstrated previously (36). Furthermore, BMMC TLR2 is known to participate in degranulation upon stimulation with peptidoglycan (PGN) (26). Based on these findings, we decided to evaluate whether TLR2 is involved in recognizing L.m and promoting the activation mechanisms in MC. Because some studies have suggested that human MCs do not express TLR (37), we evaluated the expression of this receptor on the surface of BMMC by flow cytometry. As reported previously (18, 22), this receptor was present in BMMC (**Figure 4A**). Afterwards, we pre-incubated BMMC with anti-TLR2 or isotype control for 30 minutes and then added to L.m (MOI 100:1). Then again, we evaluated cell degranulation through CD107a expression, ROS production with O_2^- detection, endocytic and bactericidal activity with intracellular CFU. We found that TLR2 blockade did not affect degranulation (**Figure 4B**), the levels of O_2^- produced (**Figure 4C**). We did not observe any change in the endocytic or bactericidal activity of BMMC incubated with L.m when TLR2 was blocked (**Figures 4D, E**). Altogether, our results indicate that MC TLR2 is not involved in the development of degranulation, ROS production, endocytosis or bacteria clearance during L.m infection.

Mast Cell TLR2 Participates in IL-6 and IL-13 Production During *Listeria monocytogenes* Infection

Previous studies have shown the importance of macrophage TLR2 activation for the secretion of IL-1β, IL-6, TNF-α, IFN-β during the infection with L.m (38, 39). Furthermore, MyD88-deficient BMMC are reduced in their ability to produce IL-6 and MCP-1 in response to L.m implying a role of TLR in MC response (16). Therefore, we decided to investigate whether this MC TLR2 could mediate *de novo* synthesis of the cytokines TNF-α, IL-1β, IL-6, IL-13 and MCP-1 in two different sources of MCs: peritoneal MCs (PMC) and bone-marrow-derived MCs (BMMC). To this end, we pre-incubated MCs with 200 ng/mL anti-TLR2, a dose that efficiently blocks the interaction of TLR-2 and its natural ligand (**Supplementary Figure 5**), or its respective isotype control for 30 minutes and then stimulated with L.m MOI 100:1 for 24 h. We found that blocking TLR2 in both PMC and BMMC did not affect the levels of TNF-α (**Figure 5A**), IL-1β (**Figure 5B**) or MCP-1 (**Figure 5E**). However, we observed that blocking this receptor significantly reduced the levels of IL-6 (**Figure 5C**) and IL-13 (**Figure 5D**) in both PMC and BMMC. These results show that MC TLR2 does not participate in the

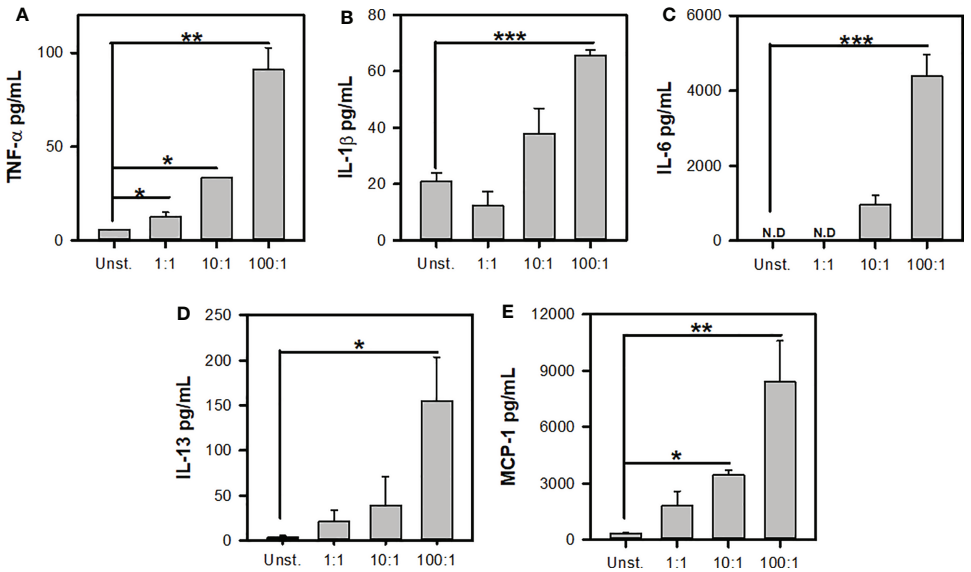

FIGURE 2 | *Listeria monocytogenes* induces *de novo* synthesis of cytokines by mast cells. 2.5x10^5 BMMC/0.25 mL of complete medium were stimulated with L.m for 24 h at the MOI indicated. Mediator levels were evaluated in culture supernatants by ELISA. **(A)** TNF-α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-13 and **(E)** MCP-1. (The figure shows the sum of 3 independent experiments, n=3 per group and for each mediator; *p<0.05; **p<0.01; ***p<0.001; N.D, Not detected; One Way ANOVA test).

production of TNF-α, IL-1β; and MCP-1; but regulates IL-6 and IL-13 production during L.m infection.

To discern which cell signaling pathway was regulated in MC by TLR2 during L.m infection, p38, ERK and p65 activation was evaluated after incubating MCs with anti-TLR2. Interestingly, we noticed that p38 phosphorylation was inhibited when TLR-2 was blocked, while ERK and p65 phosphorylation were not affected (**Figures 6A–C**). This result showed that p38 cell signaling is regulated by TLR2 in MCs during L.m infection.

Finally, previous reports indicate that L.m soluble products, like listeriolysin O, can activate MCs through increasing intracellular Ca^{2+} by altering endoplasmic reticulum independently of MCs receptors (17). To evaluate whether MC TLR2 was involved in LLO mediated activation, BMMCs were incubated with blocking antibodies to TLR-2 and stimulated with 1000 ng/mL LLO, a dose that did not induced MC apoptosis nor necrosis, but induced cytokine production (**Supplementary Figure 2** and **3**). We noticed that MC released similar amounts of TNF-α, IL-6, IL-13 and MCP-1 in response to LLO independently of TLR2 (**Figures 7A–D**). These results indicated that TLR2 was not necessary for MC activation by LLO.

Collectively, our data show that MC activation by TLR2 during L.m infection regulates IL-6 and IL-13 production and suggest that other receptors could be involved in mediating other mechanisms of MC activation (**Figure 8**).

DISCUSSION

MC are cells of the innate immune system that are key to the control of diverse infectious processes, since they promote the recruitment of other effector cells, through the release of cytokines and chemokines (2). In experimental murine listeriosis model, MC are crucial in controlling the infection by promoting recruitment of neutrophils to the site of infection (10). Therefore, this suggests that MC can recognize L.m and get activated. However, little is known about the MC receptors involved in recognizing the bacteria and whether these interactions mediate MC activation.

In this study, we confirmed that L.m activates MC by inducing cell degranulation and ROS production. In addition, L.m infects MC, but they control the intracellular bacterial load. Moreover, MC produce different cytokines in response to L.m. We also provide evidence that L.m promotes the activation of some intracellular signaling pathways in MC that are involved in TLR activation. Furthermore, we demonstrate that TLR2 is not involved in cell degranulation, ROS release or in the endocytosis and clearance of L.m. Similarly, MC TLR2 does not participate in the production of the inflammatory cytokines TNF-α, IL-1β and MCP-1; but it is crucial for the production of IL-6 and IL-13 in response to L.m infection.

MAPK pathway and NF-kB has been reported to be activated on macrophages after infection with L.m, and their expression are related with the production of different cytokines (34, 40, 41). In the case of MC, MAPK is induced in response to hemolysin-producing *Escherichia coli* (42), streptococcal exotoxin streptolysin O (43), and *E. coli* LPS (44); and NF-kB in response to *E. coli* LPS (29) and *Candida albicans* (45). Here, we show for the first time that L.m induces the activation of MAPK molecules (ERK and p38) and NF-kB in BMMC, indicating that these signaling pathways are active in these cells and could promote the cytokine and MCP-1 production.

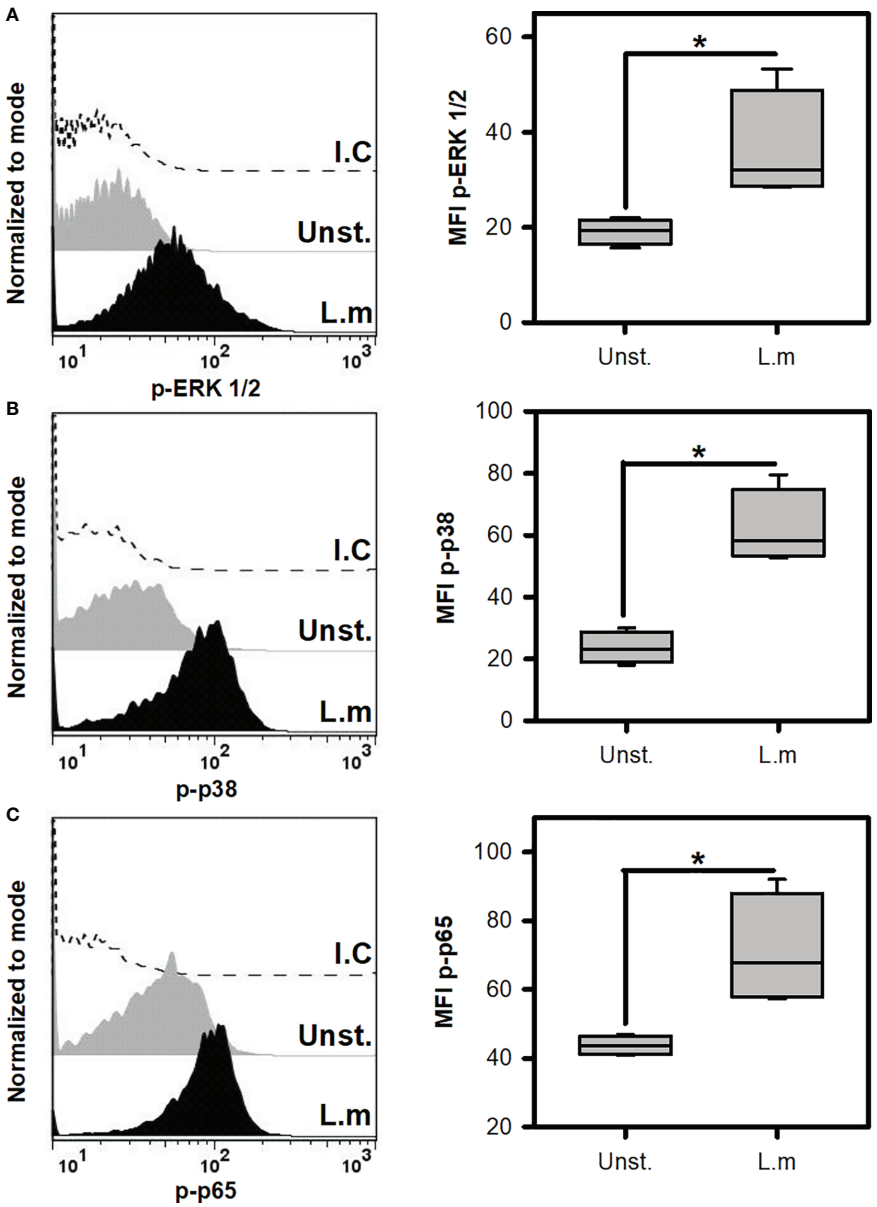

FIGURE 3 | *Listeria monocytogenes* induce the phosphorylation of cell signaling proteins involved in TLR activation in mast cells. 2.5x10^5 BMMC/0.25 mL of complete medium were stimulated with L.m at MOI 100:1 for different times to determine the phosphorylation of **(A)** ERK 1/2 at 15 minutes, **(B)** p38 at 60 minutes and **(C)** p65 at 30 minutes by flow cytometry. The graphs show the median fluorescence intensity (MFI). (Sum of 4 independent experiments, n=4 per group and for each phospho-protein. *p<0.05; Mann-Whitney test).

TLR2 is one of the main receptors expressed in cells of the innate immune response. TLR2 is one of the main receptors expressed in cells of the innate immune response. Unlike TLR4, which recognizes ligands such as lipopolysaccharide (LPS) and mannuronic acid polymers from Gram-negative bacteria, some viral components and Damage-associated molecular patterns (DAMP) (46). TLR2 recognizes different ligands including diacyl-lipopeptides, lipoarabinomannan, lipoproteins, lipoteichoic acid (LTA), peptidoglycan (PGN), porins, phospholipomannan and zymosan (20). Although TLR4 also recognizes ligands from Gram-positive bacteria such as LTA (46) or LLO from L.m (47), TLR2 seems to play a more important role in the recognition of Gram-positive bacteria, such as L. m (20) and is expressed on MC, as shown here and by others (29, 48, 49). Interestingly, TLR2-deficient mice are susceptible to L.m infection (28). Although TLR2 has been involved in MC degranulation against *S. aureus* PGN (26), L.m seems not to be inducing this mechanism through TLR2. Therefore, our findings

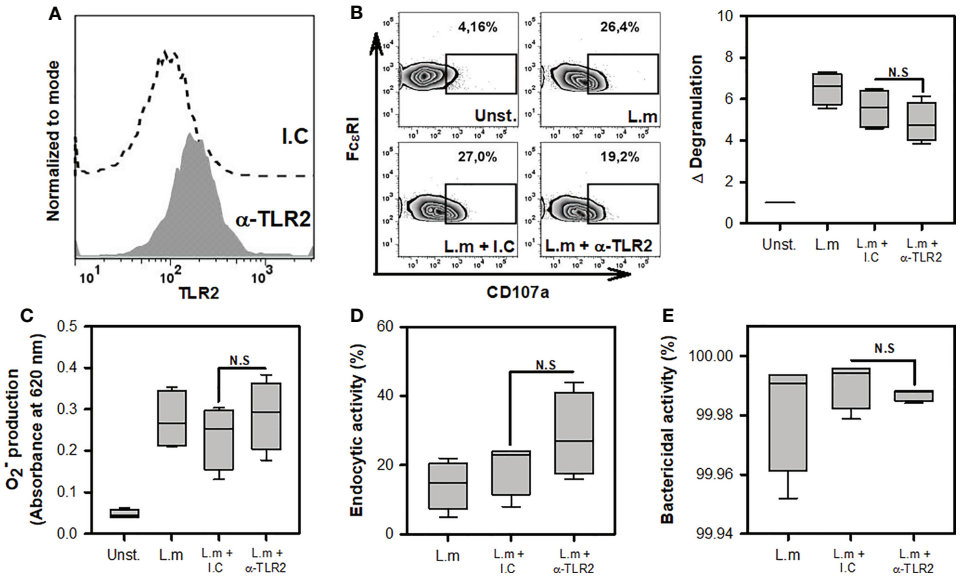

FIGURE 4 | Mast cell degranulation, ROS production, endocytosis and clearance of Listeria monocytogenes are TLR2-independent. **(A)**. 2.5x10^5 BMMC were marked with anti-TLR2-biotin (1 µg/0.1 mL) or biotinylated isotype control (I.C) (1 µg/0.1 mL) and stained with streptavidin-APC. Staining was evaluated by flow cytometry. **(B)** 2.5x10^5 BMMC/0.25 mL of complete medium were preincubated with anti-TLR2 for 30 minutes and then were stimulated with L.m (MOI 100:1) for 90 minutes. BMMC degranulation was determined by flow cytometry. Left panel shows representative zebra-plots. Right panel shows the fold change in the percentage of FcεRI+/CD107a+ cells with respect to unstimulated MC (sum of 4 independent experiments, n=4 per group; N.S, Not Significance; Kruskal-Wallis test). **(C)** 2.5x10^5 BMMC/0.25 mL of complete medium were preincubated with anti-TLR2 for 30 minutes. Afterwards, were stimulated with L.m (MOI 100:1) for 2 h. Then, the determination was performed by the NBT reduction assay (sum of 4 independent experiments, n=4 per group; N.S, Not Significance; Kruskal-Wallis test). **(D, E)** 2.5x10^5 BMMC/0.25 mL of complete medium were preincubated with anti-TLR2 for 30 minutes. Afterwards, were stimulated with L.m (MOI 100:1) for 2 h. Then the O_2^-intracellular bacteria were quantified by CFU assay. **(D)** Graphs show the percentage of endocytic activity (CFU at 0 h/MOI added x 100). **(E)** Graphs show the percentage of bactericidal activity (100-(CFU at 24 h/CFU at 0 h) x 100). (sum of 4 independent experiments, n=4 per group; N.S, Not Significance; Kruskal-Wallis test).

suggest that TLR2-independent interactions promote MC degranulation against L.m.

ROS production in L.m infected MC is associated with the release of MC extracellular traps (MCET), through a mechanism dependent on NADPH oxidase, MCET contribute to L.m extracellular clearance (18). Moreover, ROS production also promotes the intracellular clearance of internalized *E. coli* in MC endosomes (50). Furthermore, TLR2 promotes in L.m infected macrophages to the release of mitochondrial ROS associated with TNF-α, IL-1β and IL-6 synthesis *via* NF-kB and MAPK activation (36). However, our findings suggest that ROS release by L.m infected MC is not associated with TLR2- interactions.

Despite L.m endocytosis by macrophages has been shown be dependent of PI3K-AKT-Rac1 signaling pathway induced by TLR2 (23), we did not find any involvement of TLR2 in L.m endocytosis by MC. One possibility is that L.m entry does not involve TLR2 in MC. Additionally, we also noticed that MC TLR2 did not participate in L.m intracellular clearance, contrary to what is observed in TLR2-deficient MC infected with *F. tularensis* (24). This suggests that different signals may promote the activation of intrinsic microbicidal mechanisms in MC or that L.m virulence factors may promote the permanence of this bacterium within MC (16).

MC release a wide variety of cytokines and chemokines in response to L.m, including TNF-α, IL-1β, IL-6, IL-13 and MCP-1 (15, 16, 33). Interestingly, TNF-α or TNF-receptor deficient mice are highly susceptible to L.m infection (51–53). This could be associated with the TNF-α exacerbation of intracellular killing of L.m by macrophages through ROS and nitric oxide production (54, 55). On the other hand, antibody-mediated IL-1 receptor blockade affects neutrophil recruitment and macrophage activation in L.m-infected mice (56), which coincides with an increase in bacterial load in the spleen and liver (57). Similarly, mice deficient in MCP-1 are more susceptible to L.m infection, which correlates with poor recruitment of inflammatory monocytes (58). While IL-6 deficient mice are unable to mobilize neutrophils from the bone marrow into the blood circulation, making them highly susceptible to L.m infection (59). Finally, administration of IL-13 to mice infected with L.m favors infection control, which coincides with enhanced NK cells activation and an increase in serum IL-12 concentration (60). Therefore, these mediators produced by MC may contribute significantly to host defense against L. m infection.

In contrast to previous reports where it has been shown that TLR2-deficient MC exhibit a reduced production of TNF-α in response to *S. aureus* PGN (29) and *S. equi* (61), we found TNF-

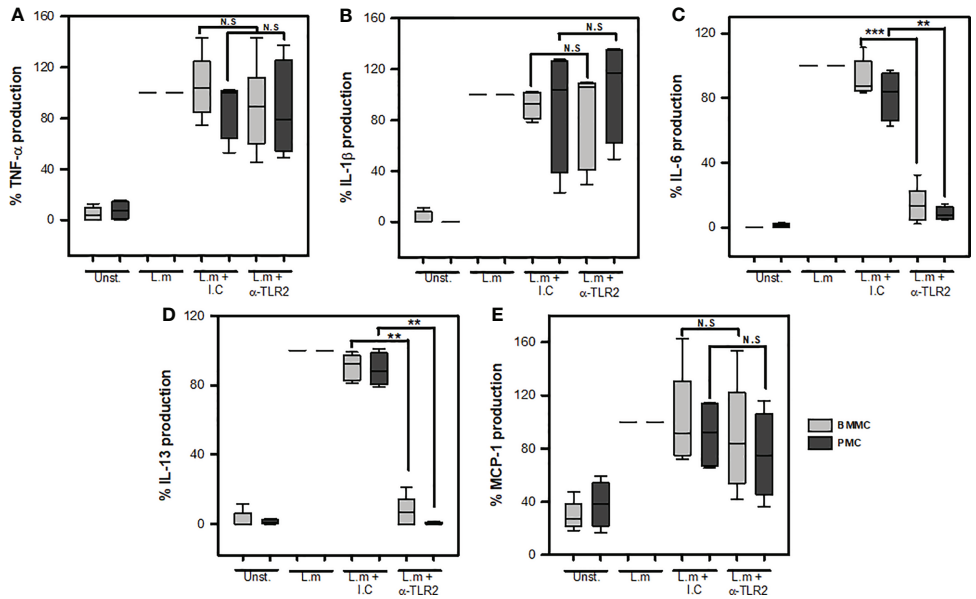

FIGURE 5 | Mast cells TLR2 is required for IL-6 and IL-13 production in response to *Listeria monocytogenes*. 2.5x10^5 BMMC or 2.5x10^5 PMC in 0.25 mL of complete medium were preincubated with anti-TLR2 for 30 minutes and then were stimulated with L.m (MOI 100:1) for 24 h. Mediators levels were evaluated in culture supernatants by ELISA. **(A)** TNF-α, **(B)** IL-1β, **(C)** IL-6, **(D)** IL-13 and **(E)** MCP-1. Data are expressed as percentage of cytokine production, considering as 100% the production induced for L.m only. (Sum of 6 independent experiments, n=6 per group and for each mediator for BMMC (light grey boxes); sum of 4 independent experiments, n=4 per group and for each mediator for PMC (dark grey boxes); N.D, Not detected; N.S, Not Significance; **p<0.01, ***p<0.001; Kruskal-Wallis test).

α production was independent to TLR2 signals. A possibility for this difference could rely on the ability of L.m to release the virulent factor LLO. Previous studies, showed that LLO increases intracellular calcium levels in MC, leading to activation of NFAT, a transcription factor that promotes TNF-α synthesis (17). Furthermore, we observed that BMMC produced significant amount of TNF-α after being stimulated with LLO (**Supplementary Figure 3**), and this production was not affected when TLR2 was blocked (**Figure 7A**). This suggest LLO-activated signaling pathway is more relevant than TLR2 for the production of TNF-α in MC.

Similarly, TLR2 was not involved in IL-1β production by L.m infected MC. This coincides with findings in MyD88 or TLR2/4 -deficient MC infected with L.m (33). IL-1β production in L.m-infected macrophages depends on both the recognition of L.m lipoproteins by TLR2 and the recognition of L.m PGN fragments by NOD1/NOD2 receptors intracellularly. In both cases, pro-IL-1β transcription through NF-kB is induced (62, 63). Therefore, we suggest that TLR2 is not mediating IL-1β production by L.m infected MC and that PGN of this bacterium may induce its production through their recognition by NOD1/NOD2. Furthermore, other interactions could mediate the production of this cytokine, such as integrin α1β2 (CD49b/CD29), which has been shown partially involved in IL-1β production by L.m stimulated MC (64).

We observed that TLR2 was not involved in MCP-1 production by L.m infected MC. Interestingly, TLR2 is important for the production of this chemokine by *S. equi* infected MC (61). This suggests that other PRR are involved in the production of MCP-1 in response to L.m. MCP-1 production is dependent on NF-κB activation (65). Therefore, different signaling pathways that converge in NF-κB may be involved in its production. The recognition of L.m PGN by NOD1/NOD2 receptors would contribute to the activation of NF-κB and thus the induction of MCP-1. Interestingly, MCP-1 production in MC infected with L.m, depends partially on the signaling carried by MyD88, E-cadherin-IntA interaction and LLO (16).

However, we observed that TLR2 participates in the production of IL-6 and IL-13 in MC infected with L.m. In fact, TLR2-deficient MC exhibit a reduced production of IL-6 in response to *S. aureus* PGN (26) and *S. equi* (61); and MyD88-deficient MC stimulated with *S. aureus* PGN and L.m are unable to produce IL-6 (16). Moreover, IL-13 production is TLR2-dependent in MC stimulated with bacterial pathogens such as *S. equi* (61) or with peptidoglycan from *S. aureus* (26). Therefore, we suggest that MC TLR2 may recognize the cell wall components of L.m and thus promote IL-6 and IL-13 synthesis. IL-6 expression is generally associated with NF-κB, MAPK/AP-1, C/EBP, CREB and PI3K/AKT activation (66–69) and interestingly, TLR2-MyD88 signaling leads to activation of NF-κB, MAPK/AP-1, PI3K/AKT (22, 70). Our results indicate L.m is inducing NF-κB, ERK and p38 activation. Interestingly, p38 was the only cell signaling pathway modulated by TLR2 which could be involved in IL-6 and IL-13 production, as has been described in BMMC stimulated with IL-33 (71). On the other hand, a recent study demonstrated that L.m induces ERK 1/2 phosphorylation in human epithelial

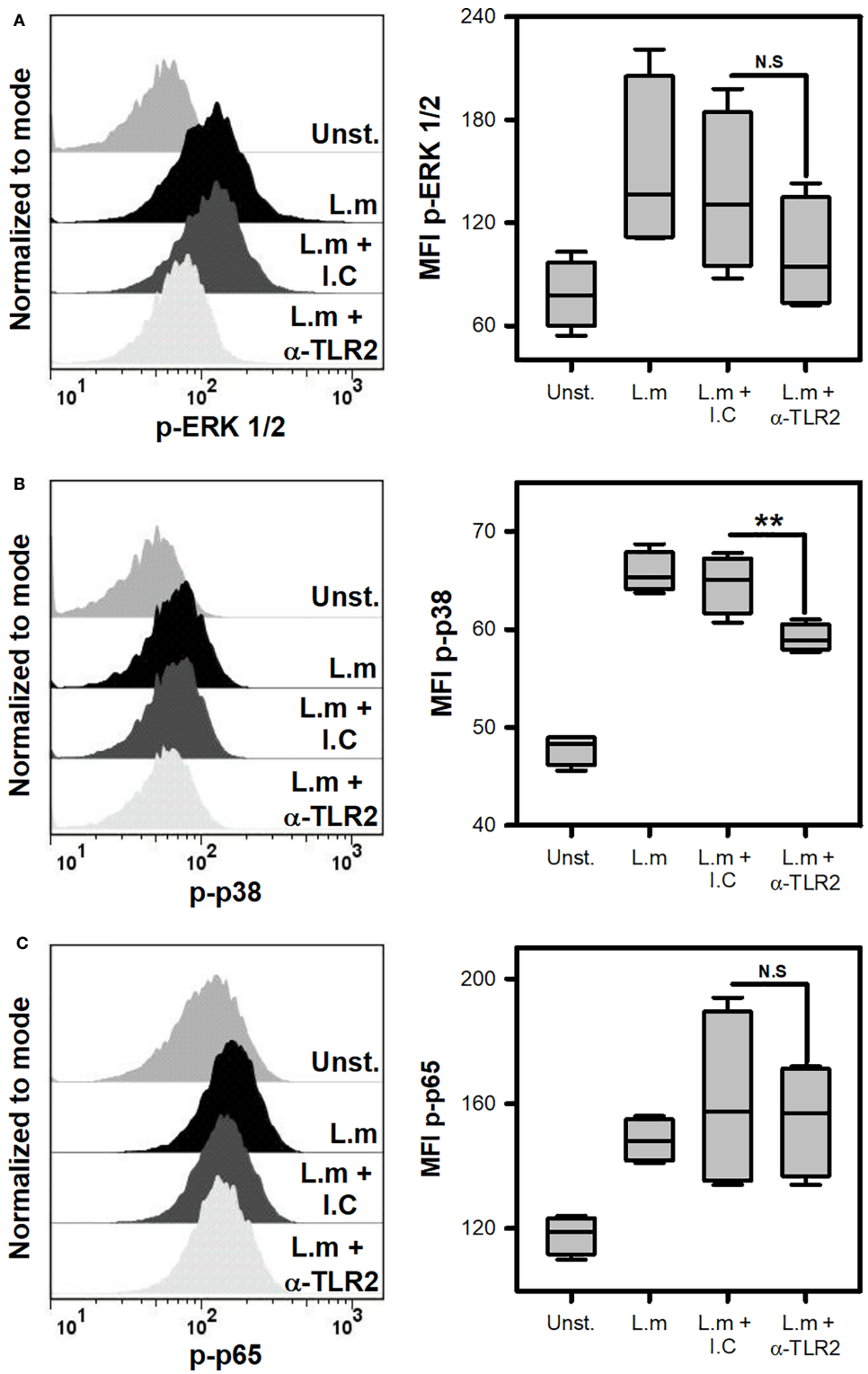

FIGURE 6 | Mast cells TLR2 regulates p38 activation in response to *Listeria monocytogenes*. 2.5x10^5 BMMC/0.25 mL of complete medium were preincubated with anti-TLR2 for 30 minutes and then were stimulated with L.m (MOI 100:1) for different times to determine the phosphorylation of **(A)** ERK 1/2 at 15 minutes, **(B)** p38 at 60 minutes and **(C)** p65 at 30 minutes by flow cytometry. The graphs show the median fluorescence intensity (MFI). (The figure shows the sum of 4 independent experiments, n=4 per group and for each phospho-protein. **p<0.01; N.S, Not Significance; Kruskal-Wallis test).

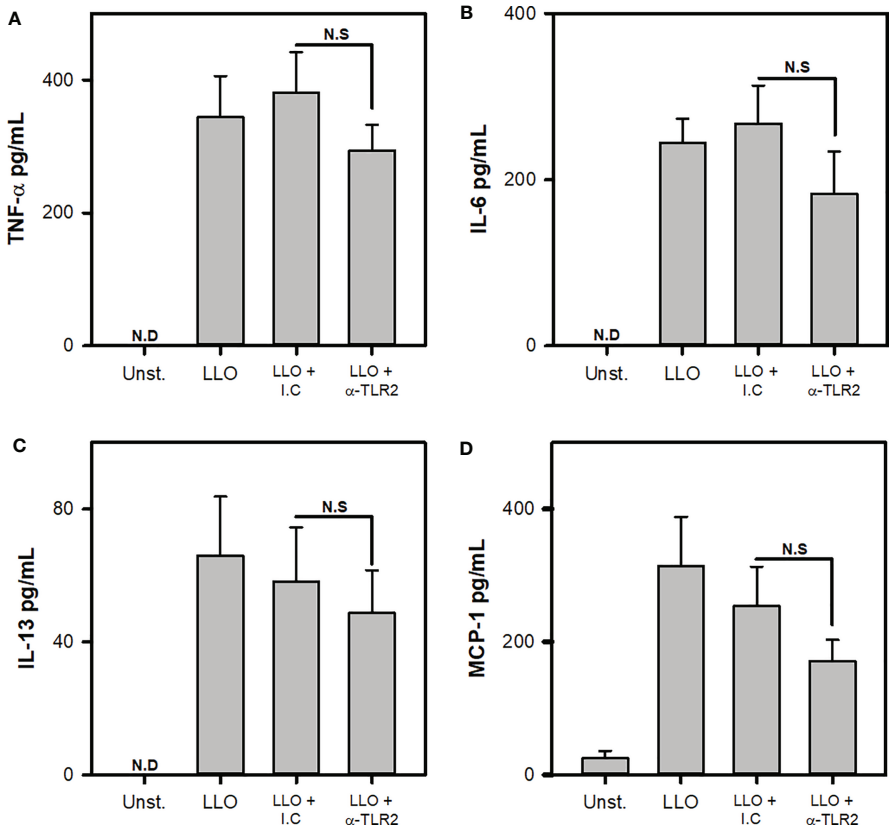

FIGURE 7 | Cytokine production by mast cells in response to Listeriolysin-O is independent of TLR2. 2.5x10^5 BMMC/0.25 mL of complete medium were preincubated with anti-TLR2 for 30 minutes and then were stimulated with 1000 ng/mL of Listeriolysin-O (LLO) for 24 h. Mediators levels were evaluated in culture supernatants by ELISA. **(A)** TNF-α, **(B)** IL-6, **(C)** IL-13 and **(D)** MCP-1. (Sum of 4 independent experiments, n=4 per group and for each mediator; N.D, Not detected; N.S, Not Significance; One Way ANOVA test).

Caco-2 cells, mediated mainly by LLO binding to cholesterol and subsequent pore formation. Interestingly, this effect correlates with the ability of L.m to invade and replicate into fibroblasts and macrophages (72). Therefore, we suggest that ERK 1/2 activation in L.m-stimulated MC may be LLO-dependent. However, it is unclear whether L.m takes advantage of this signaling pathway in MC to invade and replicate in them beyond promoting the induction of proinflammatory cytokines.

Interestingly, we found that LLO induced the release of IL-6 and IL-13 by MC, without requiring TLR2 signaling. This shows that multiple L.m pathogen-associated molecular patterns (PAMPs) lead to IL-6 and IL-13 production, indicating a vital role of these cytokines in the host immune response to L.m, and suggests that LLO could be recognized by MC through other receptors, with TLR4 being a potential candidate (47, 73).

IL-13 together with IL-4, IL-5 and IL-10 belong to the group of cytokines of the type 2 response profile, since they promote the induction of the humoral immune response by favoring the production of antibodies by B cells, diminish the cellular immune response and lead to an anti-inflammatory environment (74, 75). The type response profile 2 cytokines and other mediators produced by MC are able to direct, amplify and perpetuate the Th2 immune response in allergy, helminthic infection and in oral immunization models (76–80). Interestingly type 2 response has been recently associated as a relevant mechanism during bacterial infection, in particular during the skin infection with *S. aureus*, by favoring IgE production and effector mechanisms regulated by MC (81). Our results suggest that MC TLR2 could have a relevant role during the innate immune response to bacteria and promote an environment that favors type-2 immune response. However, it is unclear whether this response could be elicited by L.m. Our findings suggest that IL-13 production by MC responds to different L.m-activated signals, some independent of TLR2 (as is the case with LLO). This is surprising since previous studies have shown that LLO inhibits Th2-mediated reactions in murine models of allergic rhinitis (82). Thus, the functional role of IL-13 produced by MC in the context of L.m infection needs to be further explored.

In conclusion, our results show that L.m induces the activation of signaling pathways in mast cells, mainly related to cytokines synthesis. In addition, TLR2 participates in IL-6 and IL-13 production and p38 activation. While TNF-α, IL-1β,

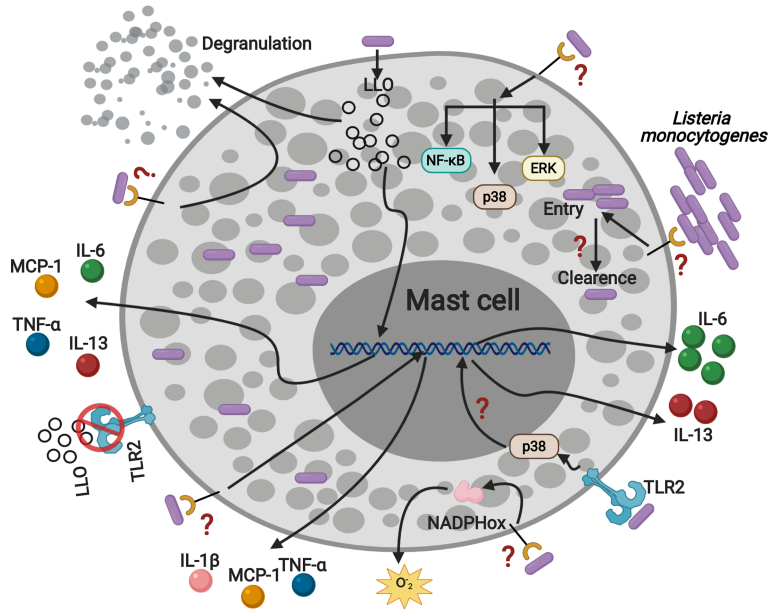

FIGURE 8 | *Listeria monocytogenes* activates mast cell through TLR2 inducing p38 activation, IL-6 and IL-13 production. MC are infected by L.m and they can eliminate the intracellular bacterial load. In addition, L.m infection activates several signaling proteins: ERK, p38 and NF-κB, which could drive MC to degranulate, ROS and cytokines production. Furthermore, the recognition of L.m by TLR2 promotes p38 activation and the production of IL-6 and IL-13. In addition, LLO induced the production of TNF-α, IL-6, IL-13 and MCP-1 independently of TLR2, indicating that other mechanisms or mast cell receptors are regulating the interaction with L.m. This figure was created with BioRender.com.

MCP-1 production, ROS release, cell degranulation, the endocytic and bactericidal activity of mast cells, as well as ERK and NF-κB activation are TLR2-independent mechanisms. Therefore, we demonstrate that mast cell TLR2 plays a crucial role in regulating the synthesis of IL-6 and IL-13 during *Listeria monocytogenes* infection in MC.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Research Ethics Committee of the ENCB, IPN.

AUTHOR CONTRIBUTIONS

Experimental design and analysis: RS-C, AA-D, GR-L, MC-N, YM-P, AG-S, VA-J, JY-P, RM-F, AD-F, SE-P, SP-T, AC-B, and RC-S. Experimental performing: RS-C, AA-D, GR-L, YM-P, and AG-S. Manuscript preparation: RS-C, GR-L, MC-N, VA-J, JY-P, RM-F, and RC-S. Funding: AC-B and RC-S. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.650779/full#supplementary-material

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REVIEW
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Responses of Mast Cells to Pathogens: Beneficial and Detrimental Roles

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Mast cells (MCs) are strategically located in tissues close to the external environment, being one of the first immune cells to interact with invading pathogens. They are long living effector cells equipped with different receptors that allow microbial recognition. Once activated, MCs release numerous biologically active mediators in the site of pathogen contact, which induce vascular endothelium modification, inflammation development and extracellular matrix remodeling. Efficient and direct antimicrobial mechanisms of MCs involve phagocytosis with oxidative and non-oxidative microbial destruction, extracellular trap formation, and the release of antimicrobial substances. MCs also contribute to host defense through the attraction and activation of phagocytic and inflammatory cells, shaping the innate and adaptive immune responses. However, as part of their response to pathogens and under an impaired, sustained, or systemic activation, MCs may contribute to tissue damage. This review will focus on the current knowledge about direct and indirect contribution of MCs to pathogen clearance. Antimicrobial mechanisms of MCs are addressed with special attention to signaling pathways involved and molecular weapons implicated. The role of MCs in a dysregulated host response that can increase morbidity and mortality is also reviewed and discussed, highlighting the complexity of MCs biology in the context of host-pathogen interactions.

Keywords: mast cells, phagocytosis, extracellular traps, mast cell mediators, pathology development participation, signaling pathways

INTRODUCTION

Described by Paul Ehrlich in 1878 and widely studied in the context of allergy, the mast cells (MCs) are cellular components of the immune system that perform crucial functions in innate and adaptive immune responses (1). MCs contain cytoplasmic granules that store a plethora of preformed mediators, such as heparin, histamine and enzymes, mainly chymase, tryptase and carboxypeptidase A, which are released upon cell activation. Depending on the stimulus, MCs can also *de novo* synthesize eicosanoids, such as leukotrienes (LTs), prostaglandins (PGs) and

platelet activation factor, as well as a wide variety of cytokines, chemokines, and growth factors (2). Several of these compounds prompt vasodilation, an increase in vascular permeability and recruitment of inflammatory cells during the allergic process and the antimicrobial response.

Different experimental models are used to study MC biology and its participation in physiological and pathological processes (**Figure 1**). *In vitro* studies of MCs are predominantly performed using MCs isolated from the peritoneal cavity of mice and rats (3–5), or rodent or human MCs obtained by cultures from bone marrow progenitors (BMMC), umbilical cord blood progenitors (CBMC) or embryonic stem cells (6–9). Immortalized MC lines from rodent (RBL-2H3, MC-9) and human (HMC-1, LAD2) origin have been also developed and are commonly used (5, 10, 11).

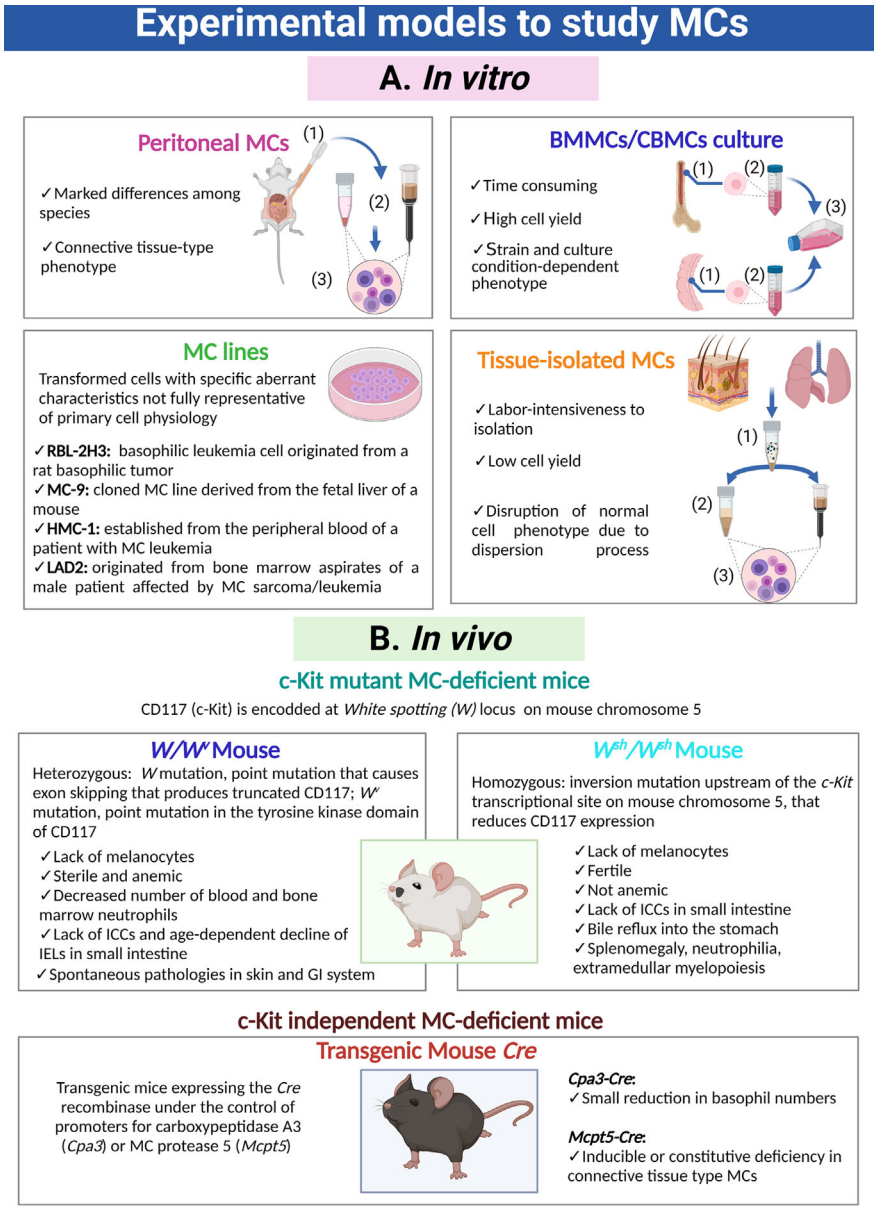

FIGURE 1 | Cellular and animal models utilized to investigate MCs. **(A)** Distinct MC preparations and cultures used for *in vitro* approaches. Purification of peritoneal MCs requires (1) peritoneal lavages, (2) purification through density gradients or magnetic beads coupled to specific antibodies and (3) final recovery of cells. Generation of bone marrow-derived MCs or cord blood-derived MCs requires (1) the isolation and disruption of the primary organ, (2) purification of immature precursors and (3) culture of those precursors for a prolonged period of time in the presence of specific cytokines and growth factors. Isolation of tissue-resident MCs is a process that requires (1) fragmentation of the organ and gentle enzymatic digestion, (2) purification of MCs utilizing density gradients, cell sorting or magnetic beads coupled to specific antibodies and (3) recovery of MCs. **(B)** Main animal models to analyze the role of MCs *in vivo*, indicating their phenotypic abnormalities. MC, mast cell; ICCs, interstitial cells of Cajal; IELs, intraepithelial lymphocytes TCRγδ; GI, gastro-intestinal.

In addition, MCs can be isolated from peripheral tissues through enzymatic digestion and enrichment processes (12). MC transcriptome changes depending on the tissue from which cells are obtained or whether they are or not subjected to culture conditions (13, 14). In this sense, the identification of tissue-specific expressed genes arises the possibility to study individual cell population within the tissue, circumventing the necessity of extensive MC purification (13, 14). *In vivo* studies of MCs were detonated with the discovery of c-Kit mutant MC-deficient mice (most used are W/W^v, W^{sh}/W^{sh}) and the development of c-Kit independent MC-deficient mice strains (*Cpa3-Cre* and *Mcpt5-Cre*) (15–19). These animal models permit to evaluate the role of MCs in particular conditions, since they can be reconstituted by adoptive transfer of cultured MCs obtained from congenic wild-type or transgenic or knock-out mice (20). Each experimental approach has its own limitations to consider when interpreting or extrapolating the results (**Figure 1**).

ORIGIN, LOCATION, HETEROGENEITY, AND PHYSIOLOGICAL FUNCTIONS

Early observations led to consider MCs as components of connective tissue derived from undifferentiated mesenchymal cells. The hematopoietic origin of MCs in mice and humans was demonstrated in 1977 and 1994, respectively, when it was shown that these cells were derived from bone marrow (BM) progenitor cells (21, 22). Recently, the use of hematopoietic fate mapping tools in mice revealed that MCs initially derive from yolk sac precursors in the embryo but are progressively replaced by definitive MCs at later stages of development (23). During embryogenesis, early erythro-myeloid progenitors (EMP)-derived MCs firstly populate most tissues, but are later replaced in most connective tissues by late EMP-derived MCs with exception of adipose tissue and pleural cavity; finally, fetal hematopoietic stem cells (HSC)-derived MCs populate the mucosa (24). After birth, these embryonic MCs continue their development into mature MCs. While evidence support that mucosal MCs depend on adult HSCs for their replacement, connective MCs do not. Specifically, MC progenitors in skin expand locally to form clonal colonies and mature MCs are self-maintained independent of BM, except during the inflammatory process in which there is an influx of new BM-progenitors that proliferate to form new colonies (25). In humans, a single MC-committed progenitor derived directly from the pluripotent stem cell $CD34^+$, c-Kit^+ was described (26). This progenitor was sensitive to stem cell factor (SCF), the ligand of c-Kit receptor, and can be detected in BM, peripheral blood, and peripheral tissues (27). In mice, three MC-committed progenitors were described, two of them in BM which were derived directly either from a multipotent progenitor or from a common myeloid progenitor, and the other one in the spleen (28). The MC-committed progenitors circulate in the vascular system as immature progenitor cells and complete their maturation when homing within tissues and are exposed to the influence of characteristic factors of each tissue. In humans, in response to several cytokines such as interleukin (IL)-3, IL-4, IL-9 and IL-10, they stop expressing CD34 and the IL-3Rα chain (CD123) and begin expressing higher levels of the high-affinity receptor for IgE (FcεRI) and c-Kit (29–32). Besides ILs, SCF derived from tissue-resident stromal cells also regulate MC differentiation, maturation, and survival (33). The importance of the tissue microenvironment in MC maturation is evidenced when MCs are transferred from one anatomical site to another, as they change their phenotype (20, 34).

MCs reside near to blood vessels and nerve endings in almost all vascularized tissues, being especially abundant in the skin and the mucosal tissues, which are sites exposed to the external environment and the gateway of pathogens (35). Mature MCs constitute a very heterogeneous cell population both in humans and rodents, showing differences in number, distribution, type of expressed proteases, proteoglycans and vasoactive amines, surface receptors and growth factors that drive their differentiation, as summarized in **Tables 1** and **2** (2, 36–59). This plasticity enables MCs to respond to local specific signals, in normal and pathological conditions.

MCs play key roles in the modulation of diverse physiological processes (60–64). MCs participate in wound healing and bone remodeling, since in their absence both processes are impaired (65–68). MCs store preformed molecules that improve fibroblast and epithelial cell proliferation, leukocyte recruitment and collagen synthesis in damaged tissue, such as tryptase (69–74) and chymase (75, 76). Besides wound healing, angiogenesis and lymphangiogenesis are also influenced by MCs (77–80). They produce several angiogenic mediators, such as histamine, tryptase, matrix metalloproteinase (MMP)-2 and -9, chymase, vascular endothelial growth factor A, platelet-derived growth factor and fibroblast growth factor (77, 81–86). Moreover, MCs are closely residents of nerve endings (87, 88), executing a bidirectional crosstalk with nerve fibers (89–92). MCs also regulate cardiovascular and renal systems (93–96), and participate in cancer control (97, 98).

In addition, a wealth of evidence supports the protective role of MCs during infectious processes, although, under certain circumstances MC response to microbial encounter may lead to harmful conditions in the host. This dual effect of MC activation in the response to pathogens will be revised in detail in the next sections, firstly reviewing the antimicrobial mechanisms that generate protection in the host, i.e. MC beneficial roles, and finally, those conditions in which the response of the cell to the microbial stimulus induces damage in the host, considered as MC detrimental roles.

ANTIMICROBIAL ROLES OF MAST CELLS

Due to their strategic location and the expression of a wide panel of receptors, MCs represent a sentinel system for the detection of invading pathogens with the capacity to generate an immediate response against them (35, 63, 99). Traditionally, MCs have been categorized as starters of the innate response against pathogens, however they can also promote the activation of adaptive

TABLE 1 | Main characteristics of MC types described in rodents.

	MMCs	CTMCs	References
Distribution	Nasal and pulmonary mucosa, intestinal lamina propria	Skin and peritoneum	(2, 36, 37)
Size	7–12 μm (rat)	17-22 μm (rat)	(2, 38, 39)
Granules	Few granules and with variable size (rat)	Many granules and with little size variability (rat)	(40)
Behavior	Migratory (rat)	Nonmigratory (rat)	(41)
Proteases	MCPT-1, MCPT-2 (chymases) (mouse) MCPT-2, MCPT-5 (chymases) (rat)	MCPT-3, MCPT-4 (chymases) MCPT-5 (elastase) MCPT-6 (tryptase) MCPT-7 (tryptase) CPA3 (mouse) MCPT-1 (chymase) CPA3 (rat)	(37, 41, 42)
Amines	Histamine (low amount) Serotonin	Histamine (high amount) Serotonin	(37, 42, 43)
Proteoglycans	Chondroitin sulfate E (mouse) Chondroitin sulfate di-B, A, E (rat)	Heparin (mouse) Heparin Chondroitin sulfate E (rat)	(37, 41)
T-cell dependence in *in vivo* development	Yes	No	(38, 42, 44)
Cytokine needed to *in vitro* proliferation	IL-3	IL-4 in the presence of IL-3 (mouse) SCF in presence of IL-3 (rat)	(43, 45–47)
Sensitive to C48/80	No	Yes	(40, 48–50)
Activated by SP	No	Yes	(49, 51)
Inhibited by sodium cromoglycate	No	Yes	(42, 48, 50, 52)

MC, mast cell; MMCs, Mucosal-type mast cells; CTMCs, Connective tissue–type mast cells; MCPT, mast cell protease; CPA, carboxypeptidase; C48/80, compound 48/80; SP, substance P; SCF, stem cell factor; IL, interleukin.

response by: i) cytokine secretion, such as tumor necrosis factor (TNF)-α, that induces the migration of dendritic cells (DC) to draining lymph nodes or T cell proliferation; ii) exosome secretion containing class II major histocompatibility complex (MHC) and co-stimulatory molecules; iii) the formation of immunological synapses with DC that facilitate the transfer of endosomal content and other molecules between both cells; and iv) presenting antigens and directly activating antigen-experienced T cell (100). This latter action is highly interesting, because it places MCs as important direct participants in the

TABLE 2 | Main characteristics of MC types described in humans.

	MC_T	MC_{TC}	MC_C	References
Distribution	Nasal and small intestinal mucosa, alveoli	Skin, small intestinal submucosa	Submucosa, mucosa of the stomach, submucosa of the small intestine, mucosa of the colon	(41, 53, 54)
Size	9.2 μm	9.9 μm	Not reported	(55)
Proteases	Tryptase	Tryptase, Chymase, CPA3, Catepsine G, Granzyme B	Chymase	(54, 56)
Amines	Histamine	Histamine	Not reported	(37)
Proteoglycans	Chondroitin sulfate A, E and heparin	Chondroitin sulfate A, E and heparin	Not reported	(37, 41, 57)
T-cell dependence	Yes	No	Not reported	(58)
Sensitive to C48/80	No	Yes	Not reported	(42)
Activated by SP	No	Yes	Not reported	(42)
Inhibited by sodium cromoglycate	Yes	No	Not reported	(42, 59)

MC, mast cells; MC_T, mast cell tryptase-type; MC_{TC}, mast cell tryptase and chymase-type; MC_C, mast cell chymase-type; CPA, carboxypeptidase; C48/80, compound 48/80; SP, substance P.

initiation of adaptive immunity. For example, co-culture of T cells with BMMCs caused T cell proliferation by FcεRI-dependent and FcεRI-independent mechanisms, being the latter dependent on the MC secretion of TNF-α (101). In another study, human psoriatic skin biopsies showed an important infiltrate of IL-22$^+$ CD4$^+$ T cells that were found in contact with MCs (102). *In vitro*, human MCs were observed forming immunologic synapses with CD4$^+$ T lymphocytes, inducing the expansion of Th22 and IL-22/interferon (IFN)-γ-producing Th cells (102). Finally, after FcεRI or Toll-like receptor (TLR)4 activation, murine and human MCs upregulated the synthesis of molecules associated with antigen presentation, enabling the autologous memory T cell activation (103, 104).

Participation of MCs in responses against microbes occurs by direct interactions with microorganisms, and by recognition of products from the damaged tissue. MC responses can produce a direct antimicrobial effect and the recruitment and activation of effector cells (35, 63, 99). Direct interactions between MCs and pathogens, as in other immune cells, mainly occur *via* the activation of pattern-recognition-receptors (PRRs), while antibody-mediated interactions occur through Fc receptors. Evidence collected from distinct MC preparations has shown that they express all the main families of PRRs (105). Members of the TLR family (TLR1, 2, 3, 4, 5, 6, 7, 8 and 9) have been detected by real-time PCR in murine MCs (106, 107). Also, nucleotide-binding oligomerization domain (NOD)-like and retinoic acid-inducible gene-I-like receptor families (108), together with the C-type lectin receptors and the Mas-related G protein-coupled receptors have been identified in cultured and/or in freshly isolated MCs from mice and humans (109).

The different roles played by MCs in the elimination of pathogens can be classified as follows: phagocytosis, formation of MC-derived extracellular traps (MCETs), and secretion of preformed and newly synthesized mediators. In the following sections, information about each one of those processes, together with the known signal transduction pathways involved, is presented.

Phagocytosis

Distinct MC preparations have shown the capacity to internalize microbes by canonical cellular processes, although the details of all involved receptors and signaling cascades have not been fully described (110) (**Figure 2A**). In MCs, several phagocytosis-inducing receptors have been described. Some of them activate the process through the direct recognition of pathogen-associated molecular patterns, such as TLR2 or the mannosylated protein CD48, whereas other receptors (like CR3 and FcγRI and FcγRII enable phagocytosis of opsonin-bound pathogens (111–113). Early evidence about phagocytosis in MCs was described in an opsonin-dependent manner in rat peritoneal MCs, where it was shown that sheep erythrocytes covered with IgG and C3b were actively phagocytosed (114). Later works showed that the phagocytosis in MCs also represented a mechanism of pathogen elimination. Human CBMCs engulfed and destroyed Gram-negative (*Citrobacter freundii* clinical isolate (CI)125, *Klebsiella pneumoniae* CI128) and Gram-positive (*Streptococcus faecium* CI126, *Staphylococcus aureus* CI127) bacteria (115). This broad recognizing capacity was proposed to be mediated by specific antibodies and complement proteins. Once bacteria were attached to the surface of the cell, protoplasmic protrusions started to surround them (**Figure 2B**), and then, internalized bacteria could be observed in vacuoles together with a time-dependent decrease in their viability (**Figure 2C**).

MCs can internalize pathogens expressing the mannose-binding FimH from type I fimbriae such as *Escherichia coli*, with subsequent bactericidal activity through the production of reactive oxygen species (ROS), mainly the superoxide anion (**Figure 2D**) (116, 117). The capacity of MCs to phagocytose microbes could be specific for certain MC populations or conditions, since it was also reported that some preparations of MCs, such as mice BMMCs were unable to phagocytose *Salmonella typhimurium* and *Listeria monocytogenes* (118). Recognition of FimH-expressing bacteria by MCs was mediated by the glycosylphosphatidylinositol-anchored molecule CD48 (115). When phagocytosis occurs, it is

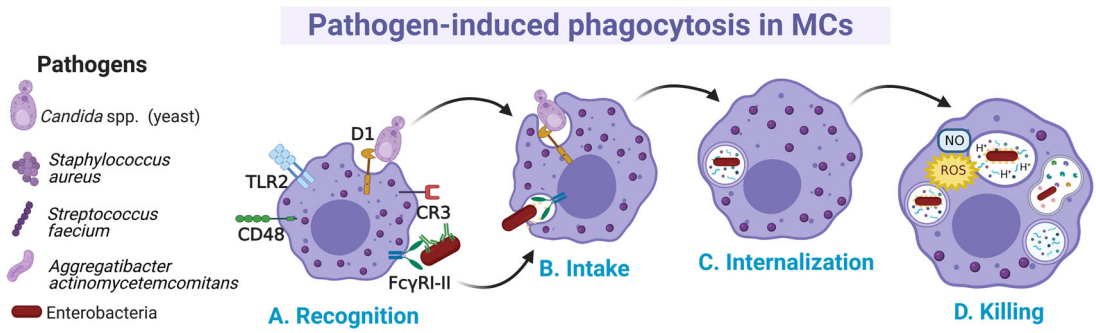

FIGURE 2 | Main events occurring during pathogen phagocytosis in MCs. **(A)** Several membrane receptors bind free or opsonized pathogens. After the recognition phase, **(B)** signal transduction pathways involving cytoskeletal re-arrangements lead to the intake of pathogens and **(C)** internalization into a phagocytic vesicle named phagosome. **(D)** Pathogens are killed by the fusion of phagosome with lysosomes and by the generation of nitric oxide (NO) and reactive oxygen species (ROS) as part of the respiratory burst.

assumed that phagocytic vacuoles are acidified, as the treatment with ammonium chloride reduced the microbicidal activity (116, 117) (**Figure 2D**). On the other hand, human CBMCs were shown to internalize *S. aureus* through a process mediated by CD48 and TLR2 receptors and dependent on alive bacteria and a functional cytoskeleton (119). In this case, *S. aureus* internalization was associated with increased survival of bacteria and the extracellular release of IL-8 and TNF-α. Nevertheless, in serum-free conditions the mechanism of FimH-expressing *E. coli* uptake by BMMCs was mediated through cellular caveolae, since intracellular bacteria were contained in chambers surrounded by caveolin (120). CD48 was co-localized with caveolin in the plasma membrane of the cell. This endocytic route of *E. coli* internalization was distinct from the classical endosome-lysosome pathway, which might allow bacteria to remain in a viable state (121). Similarly, it was reported that internalization of *Aggregatibacter actinomycetemcomitans* by murine BMMCs happens at different rates depending on whether opsonization was present or absent, being higher without opsonization (122). Whether A. *actinomycetemcomitans* is killed once internalized under each condition needs to be further investigated.

MCs also phagocyte and kill yeasts, which indicate that they may have an important role against fungal infections (123). Members of the family *Candida* spp. are common inhabitants of human skin and mucosal cavities, and they behave as opportunistic pathogens in superficial and systemic infections (124). Rat peritoneal MCs had discrete phagocytic activity on heat-killed *Candida albicans*; while yeast opsonization with rat serum increased the percentage of phagocytizing cells. Nevertheless, the percentage of killing of non-opsonized yeast was notably higher than those opsonized, which might suggest that extracellular killing capacity is more important than the one achieved intracellularly (113). The phagocytosis rate of *C. albicans* diminished when TLR2-deficient BMMCs were employed or an antagonistic antibody against Dectin-1 was used. Moreover, the killing capacity of murine BMMCs against *C. albicans* was found dependent on intracellular nitric oxide (NO) production (125).

A few studies have shown that once MCs have phagocytosed microbes, they can process microbial antigens for presentation to T cells. Using an assay in which a well-characterized T cell epitope was expressed within bacteria as a fusion protein, it was demonstrated that MCs are capable of processing bacterial antigens for presentation through class I MHC molecules to T cell hybridomas (126). Recently, MCs have been shown to take up and process both soluble and particulate antigens in an IgG opsonization- and IFN-γ-independent manner, however, while OVA or particulate antigens can be internalized through different pathways, viral antigen capture by MCs was mainly mediated through clathrin and caveolin-dependent endocytosis but not through phagocytosis or micropinocytosis (104). MC secretory granules were used for antigen processing, although the specific proteases involved were not described and require further research. When MCs were stimulated with IFN-γ, they expressed HLA-DR, HLA-DM as well as co-stimulatory molecules, which enable them to activate an antigen-specific recall response of $CD4^+$ Th1 cells (104).

Extracellular Traps

Since 2003, a few studies proposed direct and phagocytosis-independent antimicrobial activity of MCs against bacteria, although the precise mechanism was unclear. The cathelicidin LL-37, a broad-spectrum antimicrobial peptide (AMP) stored in MC granules, was implicated in the antimicrobial mechanism of the cell against group A *Streptococcus* (GAS), proposing that its activity could be due to intracellular (after phagocytosis) or extracellular mechanisms (127). Furthermore, supernatants from cultured MCs were able to kill *Citrobacter rodentium*, indicating a possible extracellular antibacterial effect consistent with the cell capacity to produce AMPs (128). In 2008, four years after the description of extracellular trap (ET) formation by neutrophils (NETs) (129), it was demonstrated that MCs produced extracellular structures like NETs (named as MCETs) with antimicrobial activity (130). Those studies showed that the extracellular death of *Streptococcus pyogenes* (M23 serotype GAS) by MCs depended on the formation of MCETs, which consisted of a chromatin-DNA backbone decorated with histones, and specific granule proteins, such as tryptase and LL-37, that ensnared and killed bacteria. MCET formation was dependent on the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and occurred 15 minutes after exposure of MCs to the bacteria. The inhibition of *S. pyogenes* growth was unaffected by treatment with the phagocytosis inhibitor cytochalasin D, ruling out the possibility that antimicrobial activity was mediated through the phagocytic uptake of *S. pyogenes* by the cells; although a closeness between both elements, the bacteria and the MC, was required. For the first time, MCET formation was described in HMC-1 cells and murine BMMCs as an antimicrobial mechanism in which DNA backbone embedded with granule components and histones forms a physical trap that catches pathogens into a microenvironment highly rich in antimicrobial molecules (**Figure 3**).

ET formation by MCs was later described in response to other GAS strain (131), or to other extracellular bacteria. For example, by HMC-1 in contact with *Pseudomonas aeruginosa* (130), HMC-1 or BMMCs co-cultured with *S. aureus* (132), or BMMCs infected with *Enterococcus faecalis* (133). Bacteria entrapped in MCETs were killed (132, 133). Although the cathelicidin LL-37 has been designated as an important weapon in the antimicrobial activity of MCs against *E. faecalis* (133), its direct activity as part of MCET structure still needs to be investigated. In good correlation, M1 protein of GAS was an important contributor to the MCET response in HMC-1 cell infection, but at the same time it conferred resistance to MCET-dependent killing of the bacteria, at least in part by binding/sequestration of the cathelicidin LL-37 (134). Concerning intracellular bacteria, the cell line HMC-1 stimulated with *L. monocytogenes* also released MCETs that contain histone, tryptase and β-hexosaminidase (135). ET formation in response to *L. monocytogenes* was also a NADPH- and ROS-

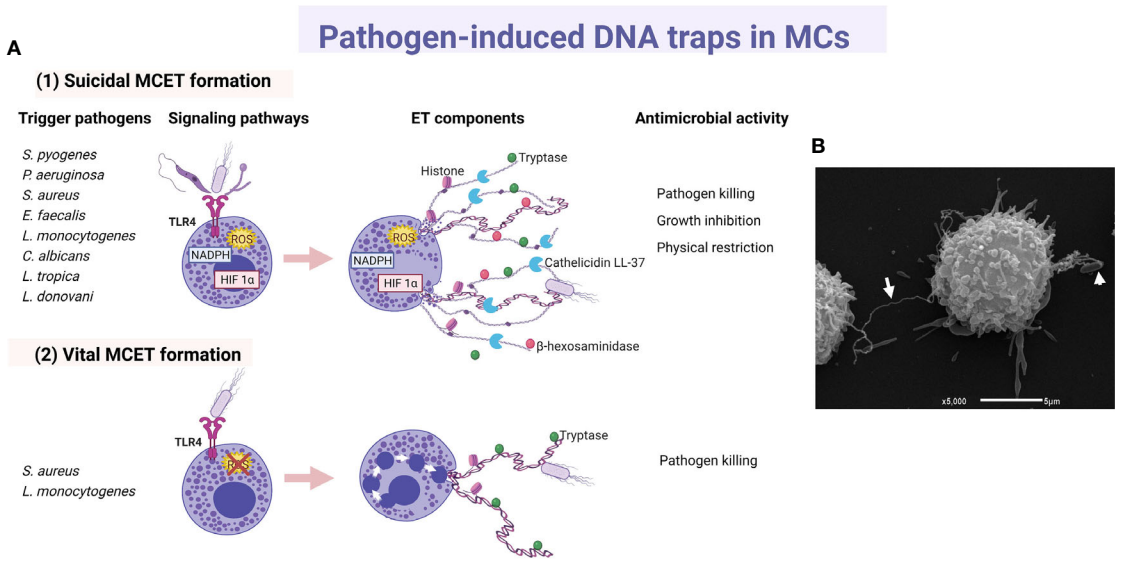

FIGURE 3 | Main characteristics of pathogen-induced MC extracellular traps. **(A)** Principal triggers, activated signaling cascades and components of (1) suicidal and (2) vital MC extracellular traps (MCET) leading to distinct antimicrobial activity. **(B)** Scan electron micrograph of MCET (white arrow) emerging from a bone marrow-derived mast cell in the presence of *E. coli* (white arrowhead). ET, extracellular traps; HIF, hypoxia-inducible factor.

dependent process and, interestingly, the inhibition of the bacterial growth was partly due to β-hexosaminidase. The role of β-hexosaminidase in MCETs still requires to be elucidated.

As aforementioned, most studies in mouse MCs or human MC cell lines about MCET formation describe a ROS-dependent process, that resembles neutrophil cell death involving ETs (suicidal ET formation), a phenomenon that occurs through chromatin decondensation and disruption of the nuclear membrane (see **Figure 3A**1) (136). Interestingly, cathelicidin LL-37 can reach the nucleus and disrupt the nuclear membrane during NET generation in human and murine neutrophils (137). In this context, cultured human LAD2 cells treated with a high concentration of exogenous LL-37 released nucleic acids extracellularly, suggesting that LL-37 is permeabilizing both nuclear and plasma membranes; nevertheless, no ET-like structures were released (138). As LL-37 can disrupt membranes both in bacterial and normal eukaryotic cells (139, 140), the role of LL-37 in the formation of MCETs through the alteration of cellular membranes remains to be elucidated. Recently, using a flow cytometry assay, it was described that *L. monocytogenes*, and to a lesser extent *S. aureus*, induced DNA externalization without intracellular ROS production in human primary MCs (141). Induction of DNA release by *L. monocytogenes* occurred in live human MCs, and the process was associated with a low level of cell death and the presence of tryptase in extracellular DNA (see **Figure 3A**2). A similar type of vital ET release had been described in neutrophils in response to *S. aureus*, in which the release of DNA occurred by fusion of DNA-containing vesicles with the plasma membrane (142). Although more research is needed, the rapid and vital release of MCETs more adequately matches the long-living nature of these tissue-resident mature cells.

MCs express different PRRs and produce inflammatory mediators traditionally involved in the antiviral, antifungal and antiparasitic response in other cells (62, 105, 143). Nevertheless, few studies have investigated the participation of MCETs in host protective response against these non-bacterial pathogens. Concerning fungi, human CBMCs and HMC-1 cells released MCETs decorated with tryptase upon *C. albicans* stimulation (144). Although ET formation increased over the time of fungal infection, it affected only a very low percentage of cells. *C. albicans* was ensnared in DNA backbone, but in contrast to results reported in bacteria, fungal viability was not affected by MCETs as shown by DNase treatment assays. In accordance, MCETs might be contributing to the physical restriction of the fungal pathogen. On the other hand, promastigotes of *Leishmania tropica* (causing cutaneous Leishmaniasis) and *Leishmania donovani* (causing visceral Leishmaniasis) triggered ET release from mouse peritoneal MCs and RBL-2H3 cell line, the greatest effect being in response to the last parasite (145). These MCETs were composed of DNA, histones and tryptase, and apart from killing the promastigotes they might physically restrict the parasite dissemination (145). As tryptase has been involved in the killing of other parasites, such as *Toxoplasma* tachyzoites (146), it would be interesting to investigate its role in *Leishmania* promastigotes death induced by MCETs.

Many questions are still unanswered regarding the formation of MCETs and its role on MC responses to pathogens; among them, whether MCETs might restrict the inflammatory response by breaking down cytokines and chemokines, as described in NETs (147). In this context, *in vitro* assays showed that MC tryptase and chymase could cleave a lower number of cytokines and chemokines than neutrophil proteases (148–150). Interestingly, when combining both MC proteases, three of the

most potent Th2 cytokines (thymic stromal lymphopoietin, IL-18 and IL-33) were cleaved (149), indicating that *in vivo* they might exert a potent negative feedback loop or a regulatory role on anti-parasitic immunity.

Activation of MCs: Release of Pre-Formed and Newly Synthesized Mediators

MCs release immunoregulatory compounds in a specific and intensity-dependent fashion (82, 151). The best-characterized ones are the pre-formed mediators stored in secretory lysosomes (granules), such as histamine, proteases, TNF-α, serotonin and heparin, among others. Secretion of those mediators can occur in a massive event known as anaphylactic degranulation, which is highly dependent on intracellular Ca^{2+} increase and cytoskeletal re-arrangements (152). Degranulation involves the fusion of granule membrane to plasmatic membrane and the extrusion of almost all granule content in few minutes (152). On the other hand, pre-synthesized mediators can also be secreted by a process named piecemeal degranulation, that implies the gradual emptiness of granule content without apparent fusion of granule membrane with the plasma membrane, by a yet poorly described mechanism [Reviewed in (152)]. Also, the triggering of different receptors leads to *de novo* synthesis and secretion of lipid mediators by enzymes localized in plasma membrane, and the activation of transcription factors that induce the synthesis of mRNAs encoding cytokines, chemokines, angiogenic and growth factors. *De novo* synthesized cytokines and chemokines seem to be secreted by budding vesicles from the Golgi apparatus utilizing elements of the constitutive secretory pathway (63, 152), and, recently, secretion of exosomes containing regulatory molecules has also been described in MCs (reviewed in 153). Anaphylactic degranulation occurs through compound exocytosis within 15 to 90 seconds upon cell activation when triggered with a high intensity stimulus (such as the crosslinking of FcεRI receptor), while piecemeal degranulation can take up to 30 minutes after stimulation of TLR4 receptor (99, 154, 155). On the other hand, the production of *de novo*-synthesized mediators can take from few minutes (arachidonic acid derivatives) to several hours (cytokines or growth factors). The best described mechanism of activation of MC is that triggered by the high intensity activation of the FcεRI receptor. Antigen-dependent crosslinking of the IgE molecules bound to FcεRI receptors causes the activation and autophosphorylation of Lyn and Fyn kinases. In turn, those kinases phosphorylate the immunotyrosine-activation-motifs located in the γ and β subunits of the receptor, creating docking sites for the amplifying kinase Syk. This event initiates a complex signaling cascade that leads to degranulation, synthesis of derivatives of arachidonic acid and activation of transcription factors that will give origin to cytokine mRNAs (156, reviewed in 157). Interestingly, a new mechanism of MC degranulation was described in 2015, and was named antibody-dependent degranulatory synapse (ADDS). This process was mediated by crosslinking of FcεRI or FcγRIIA receptors by cell-bound IgE or IgG and it resulted in a polarized and sustained release or exposure of the granule content at the contact surface between both cells. The signalling pathways activated in ADDS involved tyrosine and the phosphorylation of the adapter protein LAT (linker for activation of T cells), together with the clearance of cortical actin (146).

In this section, we will review the preformed and *de novo*-synthetized mediators released by MCs in response to bacteria, viruses, parasites, and fungi, making emphasis on their antimicrobial activity.

Bacteria

In 1996, it was demonstrated the crucial role of MCs against acute bacterial infections. Echtenacher et al. showed that MC-deficient mice were significantly more sensitive to experimental acute bacterial peritonitis induced by cecal ligation and puncture (CLP) than normal mice of the same strain (158). Intraperitoneal reconstitution of MC-deficient mice with matured and differentiated BMMCs before peritonitis induction protected animals from its harmful effects. Nevertheless, the administration of anti-TNF-α antibodies immediately after CLP suppressed these protective effects. Simultaneously, it was reported an increased number of alive *K. pneumoniae* in MC-deficient mice after their intraperitoneal or intranasal inoculation, compared to that found in wild-type animals (159). These results revealed the role of MCs in the elimination of the bacteria, which is dependent on their activation by FimH, the production of TNF-α and the subsequent neutrophil chemoattraction. MCs were mainly related to an early (15 min) peak of TNF-α production after antigen administration (160). Using MC protease (MCPT)4-deficient mice with CLP of moderate severity, it was demonstrated that MCPT-4 enhanced survival of animals, at least in part by degrading peritoneal TNF-α at the initial stage of the infection that subsequently avoided an excessive recruitment of neutrophils to peritoneal cavity (161). The protective role of MCs in acute bacterial infection was further demonstrated using another model of genetically modified MC mouse, such as C57BL/6 *tg/tg*, that shows a diminution in the number of MCs in the skin and the peritoneal cavity due to a mutation that affects the expression of the microphthalmia-associated transcription factor (162). MCs also played important roles in the elimination of bacteria in other tissues, such as during the early stage of otitis media caused by *Haemophilus influenzae* (163), as well as in pneumonia caused by *Mycoplasma pulmonis* (164), decreasing the seriousness of the pathology.

Bacterial activation of MCs is accomplished by a variety of stimuli (**Figure 4**). Gram-positive bacteria such as *Streptococcus equi* (165), or peptidoglycan from *S. aureus* (166) directly activated the cell through TLR2 receptor, although the participation of heterodimers composed by TLR2 and other members of the TRL family of receptors was not evaluated in the mentioned studies. Moreover, Gram-negative bacteria, such as *E. coli*, seemed to trigger TLR4 by its interaction with lipopolysaccharide (LPS) (166), or through CD48 *via* FimH protein (167). Mycobacteria, such as *Mycobacterium tuberculosis*, caused CD48 aggregation and histamine secretion (168). On the other hand, complement proteins were essential in MCs activation during bacterial infections (169), mainly through the CD21/CD35 (CR2/CR1) receptors (170). In addition, *P.*

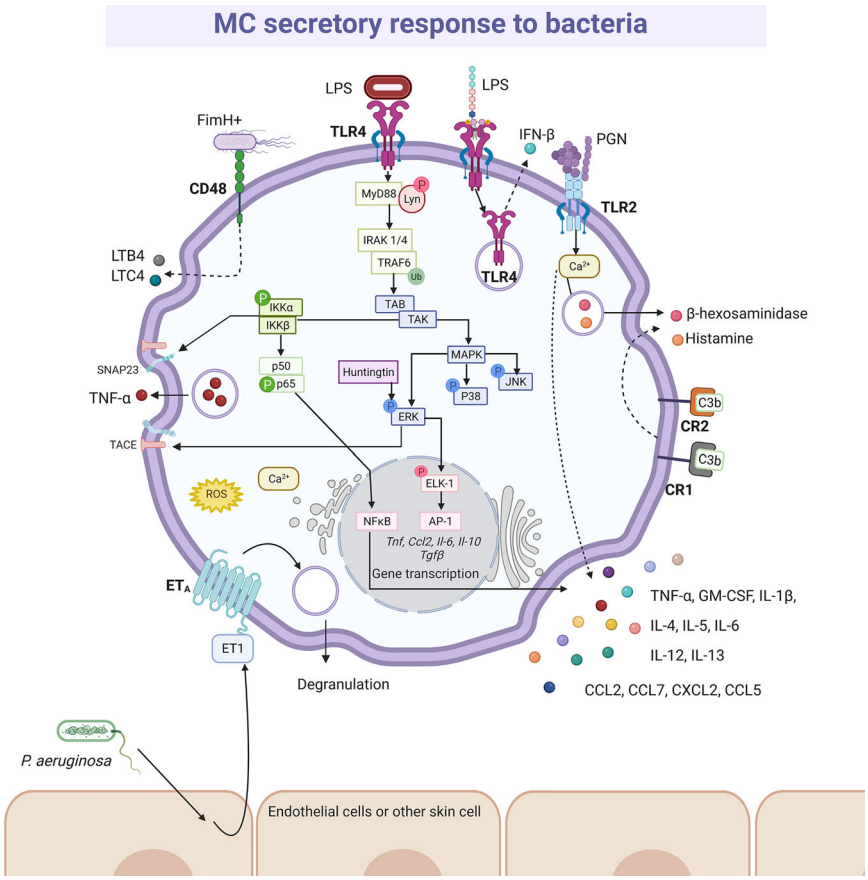

FIGURE 4 | MC-released mediators and signaling pathways elicited by bacteria. After recognition by specific pattern recognition receptors (i.e. TLR4 or TLR2) or specialized receptors (i.e. CR1, CR2 and CD48), distinct signaling cascades are activated in MCs causing the synthesis and secretion of numerous pro-inflammatory mediators, such as leukotriene B4 (LTB4), leukotriene C4 (LTC4), tumor necrosis factor (TNF)-α, interleukins (IL)-4, IL-5, IL-6, IL-12, IL-13, granulocyte and monocyte colony stimulating factor (GM-CSF), and preformed mediators, such as β-hexosaminidase and histamine. The better-known signaling pathway activated by bacteria is the MyD88-dependent cascade leading to cytokine gene transcription after NFκB and AP-1 activation, that requires classical IKK and MAPK (ERK1/2, P38 and JNK) phosphorylation, together with the recruitment of Huntingtin. IKK also contributes to TNF secretion through the phosphorylation of SNAP23 and the ERK1/2-dependent TACE activation. Still controversial, TLR4 is internalized upon LPS recognition and translocated to acidic endo-lysosomes, inducing IFN-β releasing. A particular mechanism has been described for *Pseudomonas aeruginosa*, that promotes endothelin (ET)-1 release from surrounding cells, triggering ETA receptor in MCs. In this scheme solid-lines indicate known pathways and dashed-lines show reported effects of receptor triggering in cases where signaling cascades have not been described. LPS, lipopolysaccharide; PGN, peptidoglycan.

aeruginosa mediated indirectly skin MC activation by the cutaneous production of endothelin-1, a protein that induces MC degranulation through ET_A receptors (171, 172). Nevertheless, it is important to highlight that after cell activation the mediators released are not always the same. Thus, BMMCs co-cultured with alive *S. equi* secreted high levels of chemokines such as CCL2/monocyte chemotactic protein (MCP)-1, CCL7/MCP-3, CXCL2/macrophage inflammatory protein (MIP)-2α, CCL5/RANTES (regulated upon activation normal T-cell expressed and secreted), IL-4, IL-6, IL-12, IL-13 and TNF-α. The release of these mediators was activated by stimulation of TLR2 receptor and was dependent on cell-to-cell contact. Under those conditions, although cytokine release was significant, cells showed a reduced degranulation with a low release of histamine (165). Nevertheless, activation of BMMCs through TLR2 receptor by peptidoglycans from *S. aureus* led to calcium mobilization and cell degranulation as well as *de novo* synthesis of cytokines such as TNF-α, IL-4, IL-5, IL-6, and IL-13, but not IL-1β (166). On the other hand, activation of BMMCs through TLR4 by LPS from *E. coli* did not induce degranulation or significant calcium release, although it triggered the *de novo* synthesis of cytokines such as TNF-α, IL-1β, IL-6 and IL-13 after activation of kappa-light-chain-enhancer of activated B cells transcription factor (also known as nuclear factor κB, NFκB) (166). Since heterodimerization of TLR1 or TLR6 with TLR2 has been demonstrated in other cells with distinct consequences on signaling pathway activation (173, 174), further investigation is needed to gain insight into the detailed activation mechanisms of MCs by bacterial products through TLR receptors.

Evidence have shown that *in vitro* exposure of MCs to FimH-expressing *E. coli* generated a high release of LTB4 and LTC4

(175). Thus, the administration of a potent pharmacological LT-synthesis inhibitor reduced the differences in neutrophil influx and bacterial survival induced by intraperitoneal injection of *E. coli* between MC-deficient and MC-proficient (wild-type and MC-deficient but reconstituted) mice. Moreover, MCPT-6(-/-) mice, that lack the protease homologous to human tryptase β-1, lost their ability to eliminate *K. pneumoniae* from the peritoneal cavity; highlighting the role of this protease in the innate immune response against bacteria. That phenomenon was associated with early extravasation of neutrophils to the peritoneal cavity (176). Supporting these results, mouse MCPT-6 triggered the release of CXCL-2/MIP-2 from endothelial cells, a cytokine equivalent to human IL-8 that enhances the release of TNF-α from MCs (177, 178). Additionally, complement activation was essential in MC activation in response to bacterial infection. Particularly, C3 was associated with MC degranulation, TNF-α production, neutrophil infiltration, and bacterial elimination in the CLP model in C3-deficient mice (169). The anaphylatoxin C3a is a potent activator of connective tissue-type MCs, although C3a and related peptides are also shown to inhibit FcεRI activation in mucosal-type MCs (179). Besides, C3b and C3bi mediate opsonin-dependent phagocytosis in MCs (111, 115), and C3d can activate MCs through CD21/CD35 (170). As human skin MCs can produce C3, process that can be up-regulated by various cytokines (180), and both tryptase and chymase can cleave C3 (181, 182), the participation of locally produced C3 in MC response to bacterial infection requires deeper investigation. Other MC-mediators have been implicated in antibacterial response. BMMCs co-cultured with macrophages inhibited the uptake and growth within macrophages of the Gram-negative bacteria *Francisella tularensis*. Both MC-deficient mice and IL-4R(-/-) mice showed greater susceptibility to infection with *F. tularensis* compared to normal animals, which point out their beneficial roles; although results showed that IL-4 is not mainly produced by MCs in pulmonary infection by *F. tularensis* (183). On the other hand, MC-derived IL-6 improved mice survival following *K. pneumoniae* lung infection and sepsis (184). In line with these results, it was demonstrated the important role of MCs in the healing of skin wounds infected with *P. aeruginosa*; specifically, MCs protected mice from skin infection by secreting IL-6 that induced anti-bacterial effects on keratinocytes by up-regulating the production of AMPs (185). Moreover, it was demonstrated *in vitro* that *M. tuberculosis* activated cultured MCs, triggering the release of preformed mediators such as histamine and β-hexosaminidase, and newly synthesized cytokines such as IL-6 and TNF-α (168). Concerning proteases, the mouse MCPT-4 was associated with the protective role of MCs during urinary tract infections caused by uropathogenic *E. coli* and during the female lower genital tract infections caused by group B *Streptococcus* (GBS) in mice models (186, 187); in the first infectious condition by directly cleaving and activating caspase-1 that induced the death and shedding of bladder epithelial cells and in the last one by cleaving the host extracellular matrix protein fibronectin that diminished GBS adherence.

More recently, the antibacterial activity of β-hexosaminidase was described. MC-deficient mice reconstituted or not with MCs without β-hexosaminidase (β-hexosaminidase(-/-) MCs) presented greater severity in symptoms and a higher rate of death due to intraperitoneal infection with *Staphylococcus epidermidis*, as compared to wild-type mice and MC-deficient mice reconstituted with β-hexosaminidase(+/+) MCs (188). Nevertheless, β-hexosaminidase absence did not change serum allergen-specific IgE levels neither lung infiltration of inflammatory cells in asthmatic animals (188). On the other hand, *in vitro* bacterial growth was inhibited with the addition of β-hexosaminidase(+/+) MCs lysate, but not with that of β-hexosaminidase(-/-) MCs. The authors suggested that β-hexosaminidase together with lysozyme act by destroying the cell wall of *S. epidermidis via* degradation of peptidoglycans (188). However, the microbicidal effect of MC-derived β-hexosaminidase cannot be extrapolated to other Gram-positive bacteria, as no effect was observed on *S. aureus* (188).

The existence of canonical PRR-triggered signal transduction cascades leading to NFκB and activator protein-1 (AP-1) transcription factors and the production of ROS (observed in macrophages and DC) has been confirmed in MCs and explains *de novo* synthesis of cytokines after challenge with bacterial products; in addition, distinctive pathways coupling PRRs to the secretion of pre-formed mediators seem to be quite specific for MCs (**Figure 4**). For example, triggering of TLR4 receptor led to the engagement of the myeloid differentiation primary response 88 (MyD88)-dependent signaling cascade that includes the activation of downstream molecules such as the TNF receptor associated factor 6 (TRAF6) and the IκB kinase (IKK) together with the nuclear translocation of p65 NFκB (166, 189). However, the TLR4-induced TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent signaling pathway leading to the secretion of IFN-β, whereas broadly observed in macrophages and DC, was reported absent in MCs (190). The absence of this pathway is controversial, since recently, BMMCs showed to release IFN-β after TLR4 induction *via* LPS and the internalization and translocation of the receptor to acidic endo-lysosomal compartments was a prerequisite for cytokine release (191). On the other hand, particular roles of IKK and the mitogen-activated kinase (MAPK) extracellular receptor kinase (ERK)1/2 were found in BMMCs activated through the TLR4 receptor, since those kinases participated in the piecemeal secretion of TNF-α through the phosphorylation of SNAP23 (soluble N-ethylmaleimide sensitive factor attachment protein receptor-23) and the activation of the disintegrin/metalloprotease ADAM-17/TNFα-converting enzyme (TACE), respectively (192, 193). Also, Ca^{2+} mobilization and activation of Lyn and Fyn kinases occurred in BMMCs after LPS-dependent TLR4 triggering (154, 189, 192). Finally, recent evidence indicated that the multifunctional protein Huntingtin was required for the activation of the ERK1/2-AP-1 axis after TLR4 triggering in BMMCs, contributing to the accumulation of TNF-α, IL-6, IL-10 and transforming growth factor (TGF)-β mRNAs and secretion of those cytokines (194).

Regarding NOD-like receptors, although no particular signaling molecules were described in MCs and seems that the formation of inflammasomes and activation of NFκB follows the same pathways that those reported in other immune cells (105, 108), it was shown that those receptors were inducible in response to cathelicidin LL-37 and defensin hBD-2 (108) and were important for MC-microbe interactions leading to exocytosis of mediators. For example, the NOD2-specific agonist muramyl dipeptide promoted TNF-α secretion from MCs and, *in vivo*, a significant increase in NOD2 positive MCs was reported in colonic mucosal biopsies from Crohn´s disease patients compared to those coming from ulcerative colitis or control biopsies (195).

Virus

MCs present a diverse response against viruses (196). Studies on the pathogenesis of viruses in their natural hosts have increased our understanding about what happens in humans. In this regard, we can find many similarities in bovine respiratory syncytial virus (RSV) infection and its human homologous hRSV (197). Although, histopathological findings showed degranulation of MCs during infection by bovine RSV (198, 199), using *in vitro* models it was suggested that degranulation was indirectly induced by hRSV (200). The role of MCs on airway hyperreactivity was studied in the onset of viral infection in guinea pig, since it is a feasible model that resembles the observed signs in humans (201, 202). Parainfluenza virus 3 induced degranulation and histamine release in pulmonary MCs from guinea pig, which may represent a significant mechanism to provoke wheezing and asthma pathogenesis (202). Additionally, viral components can stimulate the synthesis and release of *de novo* mediators alone or in combination with degranulation (**Figure 5**). The extracellular version of protein Nef expressed in the early phase of infection of the human immunodeficiency virus (HIV) triggered the release of CXCL8/IL-8 and CCL3/MIP-1α through the CXCR4 receptor in MCs (203). Besides, the indirect activation of MCs during viral infection was documented. In patients affected by acute and chronic viral hepatitis B, C, A and E, the endogen superantigen Fv is produced in high concentrations by hepatocytes, and it induced the secretion of LTC4 or PGD2, as well histamine or tryptase, presumably by interacting with the variable domain of the IgE heavy-chain (204, 205). Although many of these mediators can contribute under certain circumstances to the physiopathology of viral infections, in this section we will focus on the data that have contributed to position the MCs as crucial elements of defense against viruses.

In vivo and *in vitro* murine models defined that vaccinia virus triggers MC degranulation by activating S1P2 receptor after binding of lipids of the viral membrane, generating the release of cathelicidin that abolished the virus infectivity (206). In this context, MC activation was dependent on the fusion of the virus envelope to cell membrane. In young mice susceptible to atopic dermatitis (AD), MC-derived cathelicidin was a determining factor to avoid eczema vaccinatum in response to vaccinia virus (207). In this regard, as vaccination with vaccinia virus is contraindicated in AD patients, to define the role of MC-derived cathelicidin will allow to establish better strategies to prevent adverse reactions (207). The antiviral activity of AMPs was demonstrated against human influenza A virus (208), hRSV (209), Zika virus (210) and HIV (211). Concerning dengue virus (DENV), it was observed that DENV infection up-regulated the transcription of CCL5/RANTES, CXCL12, CX3CL1/fractalkine, TNF-α and IFN-α in RBL-2H3 cells (212). Besides, human MC cell lines infected with the DENV in the presence of specific antibodies selectively released chemokines such as CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, but not IL-8 or CXCL5 (213). These mediators might be involved in the mobilization of lymphocytes, or other immune cells, which favors the early response against the virus. In a recent study, using a cell line of human mature MCs directly exposed to DENV in an antibody-independent manner, it was evidenced that the virus does not replicate in MCs but triggers its degranulation, the synthesis of tryptase, chymase, PGs and LTs and up-regulates the transcription of genes associated with the antiviral response and the Th1-polarization (214). On the other hand, murine intradermal infection with the herpes simplex virus (HSV)-2 induced the synthesis of IL-33 by keratinocytes, that in turn activated the synthesis of TNF-α and IL-6 by MCs, key cytokines in reducing the severity of the infection (215). The same protective effect was mediated by MCs in HSV-1 infection on the cornea; however, in this immune privileged environment the MCs controlled inflammation and viral replication by reducing the infiltration of polymorphonuclear cells (additional reservoirs of the HSV-1), probably due to changes in levels of chemoattractant (216). Thus, authors described that MC-deficient mice showed a decrease in the PGD2:12-hydroxyeicosatetraenoic acid (12-HETES) ratio, and while PGD2 suppresses neutrophil chemotaxis and endothelial transmigration during acute inflammation, 12-HETES is a potent neutrophil chemoattractant that promotes increased vascular permeability. The increased expression of CXCL2/MIP-2α in the corneas of MC-deficient mice might be also facilitating the neutrophil influx during HSV-1 infection. Recently, it was shown that the human placental MCs and HMC-1 cell line were permissible to *in vivo* and *in vitro* Zika virus infection, respectively; in HMC-1 cells, viral infection triggered degranulation as well as the release of TNF-α, IL-6, IL-10, which might induce an optimal defense against the pathogen; however, the pro-inflammatory environment coupled with the viral replication in placental MCs suggest a role of the cell in vertical transmission (217). Then, many questions remain to be resolve about the role of MCs in defense against Zika virus.

Regarding receptors involved in MCs response to viruses, the cytosolic receptors participate in the increased expression of TNF-α and IL-1β, as well as type I IFNs, such as IFN-β and Mx-2, as shown by BMMCs infected with the vesicular stomatitis virus (VSV) (118). It is important to mention that type I IFNs play critical roles in innate host defense against viral infections (218), since after binding to their receptors they activate the expression of hundreds of genes that promote an "antiviral state"

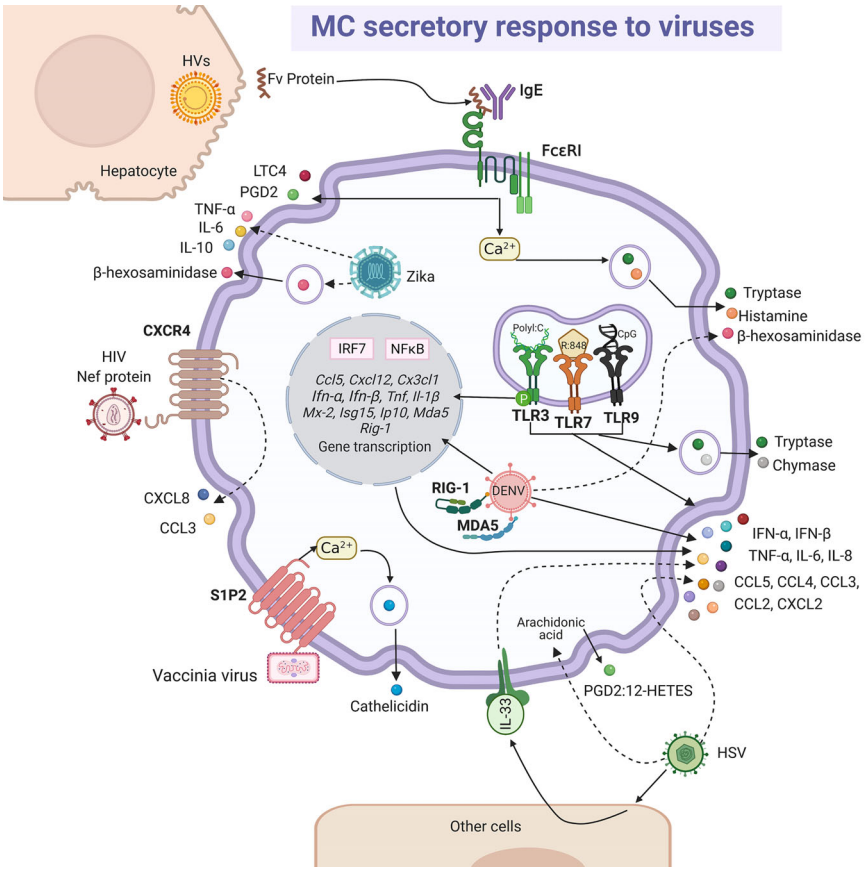

FIGURE 5 | MC-released mediators and signaling pathways in response to viruses. Some viral particles are recognized directly by membrane receptors, i.e. vaccinia virus binds sphingosine-1-phosphate 2 (S1P2) receptor and human immunodeficiency virus (HIV) to CXCR4, triggering signaling pathways leading to cathelicidin or CXCL8 and CCL3 chemokines release, respectively. Intracellular dengue virus (DENV) is probably recognized by RIG-1 and MDA5 and herpes simplex virus (HSV) directly or through the release of alarmin IL-33 by other cells lead to the secretion of cytokines and chemokines, together with the arachidonic acid derivatives prostaglandin 2 (PGD2) and 12-hydroxyeicosatetranoic acid (12-HETES). Fv endogen superantigen from hepatocytes infected by hepatitis viruses (HVs) promotes MC degranulation and the release of leukotriene C4 (LTC4) and prostaglandin D2 (PGD2) by a mechanism that seems to depend on the activation of FcεRI receptor and calcium mobilization. Zika virus infection promotes MC degranulation and cytokine secretion. Finally, classical responses to viral compounds *via* TLR3, TLR7 and TLR9 receptors have been observed in MCs, that lead to the synthesis of interferon (IFN)-α and IFN-β through the activation of interferon regulatory factor (IRF)-7 and NFκB, and also to the release of tryptase and chymase. Solid-lines indicate known pathways and dashed-lines show reported effects of receptor triggering or MC-virus interactions, although specific signaling cascades remain to be described.

in cells (219). Transcripts for MDA5 and retinoic acid-inducible gene-1 were found up-regulated after the infection of MCs with DENV (212, 220) and with VSV, leading to the synthesis of IL-6, IFN-β and IFN-α during VSV infection (221). The activation of the cell by viruses was also dependent on the TLR pathways (222). Activation of TLR3, TLR7 and TLR9 by their respective ligands, polyI:C (double-stranded (ds)RNA analog, TLR3 agonist), R:848 (synthetic TLR7 agonist), and CpG oligodeoxynucleotide (unmethylated consensus DNA sequences, TLR9 agonist), respectively, did not trigger degranulation, but induced the production of TNF-α, IL-6, CCL5/RANTES, CCL3/MIP-1α and CXCL2/MIP-2 by murine fetal skin-derived MCs but not by murine BMMCs (223). Besides, a recent study showed that the stimulation of cultured human peripheral blood-derived MCs (PBMCs) with polyI:C or R848 induced MC activation and the release of chymase, tryptase, IL-8, CCL3/MIP-1α and CCL4/MIP-1β (224), highlighting the diverse functionality of MCs depending on their location and origin. In this context, cultured human PBMCs produced IFN-α through TLR3 in response to RSV, reovirus type 1 and polyI:C, but not TNF, IL-1β, IL-5 or granulocyte-macrophage colony stimulating factor (GM-CSF) (225). The phosphorylation of TLR3 was demonstrated in murine MCs in response to Newcastle disease virus, causing antiviral response mediated by interferon stimulated gene 15 (ISG15), IFN-β, CXCL10/IP-10 and CCL5/RANTES, which was a MC-degranulation independent process (226).

Parasites

Mucosal and connective tissue MCs play important roles in defense against intestinal parasitosis, as it has been reported in infections with *Trichinella spiralis* (227, 228), *Strongyloides ratti*

(229, 230) and *Toxocara canis* (231), among others (232, 233). In addition, the MCs seem to play a crucial role in the decrease in the fertility rate of *Heligmosomoides polygyrus* (234). One of the most important MC activation mechanisms in the immune response to parasites is mediated *via* FcεRI and Fcγ receptors and anti-parasite-specific IgE and IgG antibodies. This fact was demonstrated to *H. polygyrus*, *Nippostrogylus brasiliensis*, *Strongyloides venezuelensis* and *T. spiralis* infection using IgE (-/-), IL-4(-/-) null mice or MC-deficient mice infected with the parasite in the presence or absence of parasite immune sera-derived IgE or IgG (235, 236). In addition, different models of MC-deficient mice showed that MCs play a more important role during the early phase of primary immune response than in the late phase or the secondary response against helminths (230, 237–239). Protection against *Fasciola hepatica* was associated with infiltration of eosinophils, IgE positive cells and MCs (240). The importance of parasite-specific IgE in the protective response to helminths was evidenced by the impaired protective activity in animals with high levels of non-specific IgE that compete for Fc receptors in MCs (241). In this context, degranulation, and histamine production, together with the release of distinct lipid mediators and cytokines was shown in studies where the interaction of MCs and parasites was addressed and the participation of IgE antibodies was identified (reviewed in 242). The pattern of secreted mediators and changes in MC morphology (i.e. degranulation) indicates that the full signaling cascade of FcεRI, which has been characterized in response to allergens, is activated by parasites (235) (**Figure 6**). On the other hand, tachyzoites of *Toxoplasma gondii* opsonized with IgG specific to the SAG-1 surface antigen and co-cultured with MCs induced a polarized degranulation mediated by ADDS that resulted in tryptase-dependent parasite death. In addition, MCs were activated to produce CCL2/MCP-1, CCL4/MIP-1β, CXCL8/IL-8, GM-CSF, IL-1β and TNF-α (146).

IgE-independent MC activation mechanisms are not underestimated in parasitic diseases. Direct contact with alive *Leishmania* promastigotes induced degranulation of BMMCs, with the release of β-hexosaminidase and TNF-α as well as *de novo* synthesis of the latter (243). *Giardia intestinalis* trophozoites and their total soluble extract increased tryptase expression and IL-6 and TNF-α production by a hybrid rat MC line, and the histamine secretion by peritoneal MCs (244); while the total soluble extract activates the release of IL-6 and tryptase, but not degranulation by BMMCs (245). In addition, it was identified that arginine deiminase from *G. intestinalis*, maybe directly or through its metabolic product citrulline, triggered the release of IL-6 and TNF-α (246). Arginine deiminase is an immunodominant antigen that has been identified *in vivo* and *in vitro* after infection by the parasite (247–249). *Giardia intestinalis* infection induced mRNA expression of MC-derived proteases in intestinal tissue of mice. Besides, MMP-7 was one of the most up-regulated genes and together with NO played a key role in the decline of *Giardia* trophozoites. As MMP-7 is responsible for the production of α-defensins in mice, the protective effect of MCs might be mediated by this AMP (250). Whether the cellular source of MMP-7 was MC or another cell it needs to be elucidated. Interestingly, mature adult mice with deletion in chymase MCPT-4 gene (MCPT-4-/-) showed a significant weight reduction due to *G. intestinalis* infection, a characteristic clinical sign of the symptomatic giardiasis, as compared to MCPT-4+/+ mice; the weight loss was not observed in MCPT-4-/- or MCPT-4+/+ young mice (251). However, one of the proteases that becomes more important in defense against helminths is MCPT-1, since in its absence the intestinal permeability was blocked, affecting the expulsion mechanisms of *T. spiralis* (252). Additionally, experiments in MC-deficient mice suggested that the expulsion of the parasite was dependent on MC-derived IL-4 and TNF-α (253). Moreover, MC proteases were responsible for degrading the collagen-like proteins in the *Necator americanus* cuticle (254). However, as aforementioned, the diversity of parasites and the complex nature of their antigens generate a broad range of responses in the cells. For example, the secretory products of *Entamoeba histolytica* promoted the synthesis of IL-8 by MCs *via* a protease activated receptor-2 independent mechanism (255).

Interestingly, the interaction between parasites and MCs can also lead to the blockage of mediator secretion in this cell. For example, the ES-62 protein, secreted by the parasitic worm *Acanthochilonema viteae*, exhibited immunomodulatory activities lowering MC responsiveness (256). It was found that ES-62 inhibited the signaling from the IL-33/ST2 receptor independently on the phenotype of MCs. Interestingly, ES-62 sequestered MyD88 and then contributed to the downregulation of cytokine expression triggered by TLR4 and FcεRI receptors (257). On the other hand, parasites may also modulate the activity of MCPTs. In this context, excretory-secretory proteins from *Giardia* increased the enzymatic activity of human and mouse tryptase (245).

Fungi

Although it is estimated that 1 billion people worldwide have some type of fungal infection (258), just a little is known about the release of mediators by MCs upon their activation by fungi. Concerning fungal PRRs, the C-type lectin receptor family member Dectin-1 and Mincle (macrophage inducible Ca^{2+}-dependent lectin receptor) are expressed in MCs and their signaling systems seem to induce the secretion of pro-inflammatory mediators (259, 260). Curdlan, a Dectin-1 agonist, led to histamine release and degranulation, but not to the production of CCL2/MCP-1, IL-6 or LTC4 (261). On the other hand, Mincle seems to interact with γ and β subunits of the FcεRI receptor, activating Syk tyrosine kinase and leading to anaphylactic degranulation as observed with IgE/Ag complexes (262).

Dectin-1 (261, 263) and TLR2 (264) are the receptors mainly involved in the MC antifungal response, which becomes relevant considering that MC is the cell type with the higher expression of Dectin-1 in the skin (259). Zymosan possess β-glucans that are recognized by Dectin-1; however, zymosan can also interact with other receptors due to its complex composition, including heterodimers of TLR1 or TRL6 with TLR2 (265). Therefore, to analyze the specific activation of Dectin-1, ligands such as

curdlan are used (**Figure 7**). In RBL-2H3 cells, curdlan triggered MC degranulation (261) and caused the phosphorylation of phospholipase Cγ2 and the expression of IL-3, CCL2/MCP-1, IL-13, IL-4 and TNF-α mRNAs in a Syk dependent manner, as the effect was abrogated when cells were preincubated with the Syk inhibitor R406 (263). Remarkably, curdlan-induced cytokine mRNAs, such as TNF-α and IL-3 were also sensitive to the MAPK/ERK kinase inhibitor PD98059, showing that several downstream proteins, such as ERK1/2, are shared between Dectin-1 and FcεRI in MCs (263). Besides, zymosan induced *de novo* synthesis of LTs, GM-CSF and IL-1β by CBMCs in a dose-dependent manner (264). In human MCs, LTC4 was released in a Syk-dependent mechanism *via* Dectin-1 receptor (266); meanwhile, zymosan induced the generation of

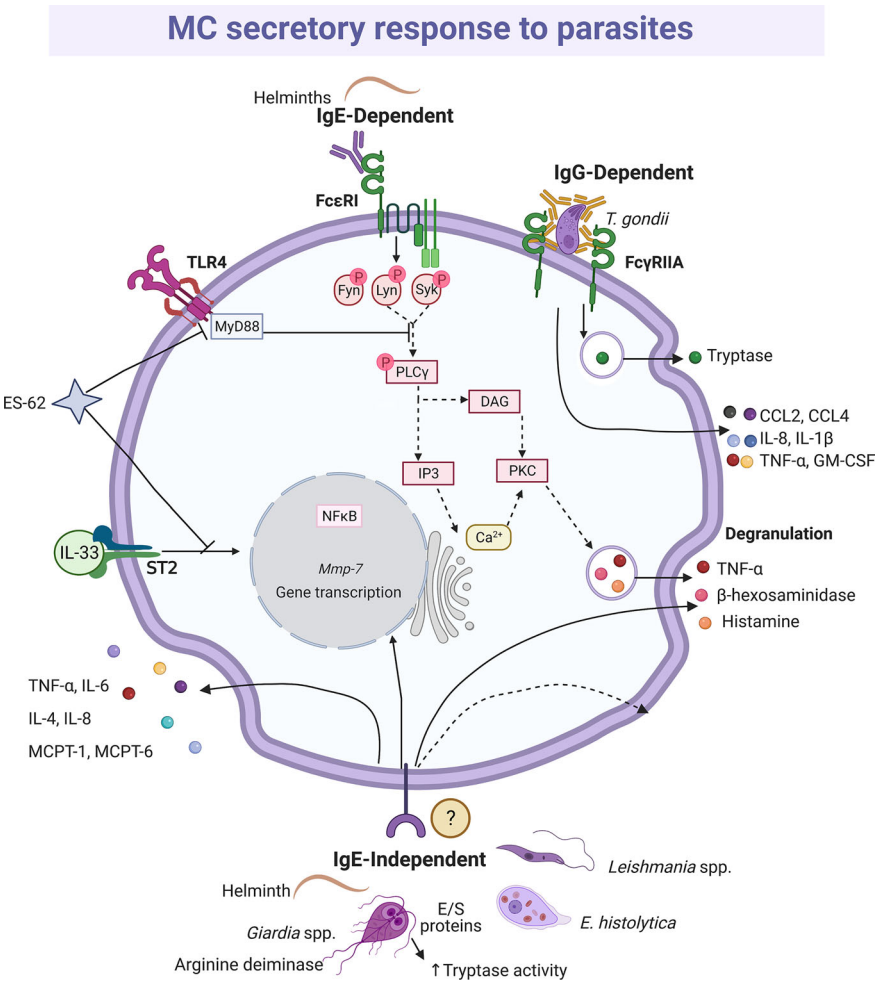

FIGURE 6 | MC-released mediators and signaling pathways elicited by parasites. Distinct parasites promote IgE-dependent and IgE-independent activation patterns. When recognized by IgE, helminths induce full degranulation and cytokine secretion as it has been described for IgE/antigen complexes and the shown intracellular signaling cascade is inferred. Antigen-dependent crosslinking of the IgE molecules bound to FcεRI monomers causes the activation and autophosphorylation of two Src family kinases, named Lyn and Fyn. In turn, those kinases phosphorylate the immunotyrosine-activation-motifs located in the γ and β subunits of the receptor, creating docking sites for the amplifying kinase Syk. Once recruited and activated, Syk phosphorylates membrane adapters that will conform two main protein complexes directing the signaling i) to the main events leading to calcium mobilization and degranulation, and ii) to secondary processes that contribute to sustain degranulation and induce migration and cytokine production. In order to trigger degranulation, the activated phospholipase C (PLC)γ hydrolyses phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol (DAG) and inositol 3-phosphate (IP3). Those messengers activate several isoforms of protein kinase C (PKC) and the IP3 receptor located in endoplasmic reticulum intracellular Ca^{2+} storages. The main final consequences of this signaling branch are the release of Ca^{2+} to the cytoplasm and the phosphorylation of distinct proteins involved in the fusion of granules to the plasma membrane. Crosslinking of FcγRIIA receptors by bound-cell IgGs results in a polarized and sustained release of the granule content at the contact surface between both cells, named antibody-dependent degranulatory synapse (ADDS). ES-62 protein inhibits interleukin (IL)-33-dependent ST2 receptor activation and targets MyD88, which causes downregulation of cytokine synthesis triggered by TLR4 and FcεRI receptors, while excretion/secretion (E/S) proteins from *Giardia* increase tryptase activity. IgE-independent activation is mediated by not well-defined receptors and causes histamine and cytokine secretion. In this figure, solid-lines indicate reported effects of receptor triggering or MC-parasite interactions, whereas dashed-lines show suggested activated pathways, assuming the activation of the high affinity IgE receptor (FcεRI) in this cell type.

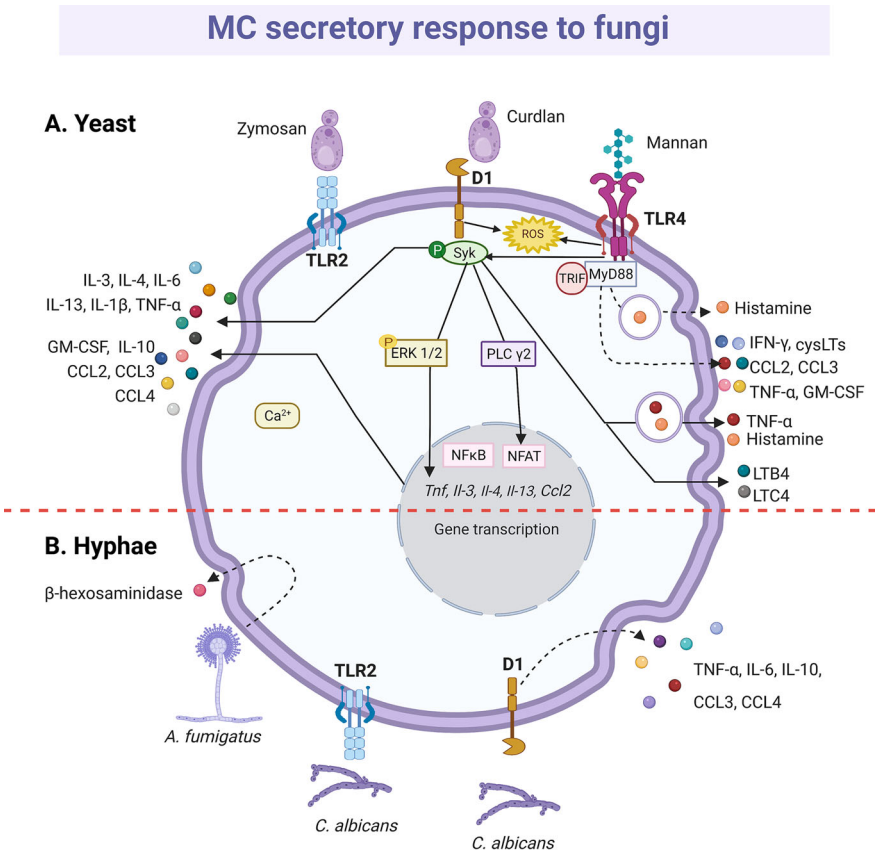

FIGURE 7 | MC-released mediators and signaling pathways in response to fungi. **(A)** Toll-like receptor (TLR)-2 and Dectin-1 (D1) receptors recognize yeasts. Triggering of D1 receptor leads to Syk kinase activation and the release of histamine and cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-3, IL-4, IL-6, IL-10, IL-13 chemokines such as CCL2, CCL3 and CCL4 and granulocyte and monocyte colony stimulating factor (GM-CSF). Secretion of leukotriene (LT) B4 and LTC4 also has been described, together with the reactive oxygen species (ROS)-dependent activation of NFκB. Mannan recognition through TLR4 receptor induces histamine release and ROS, cysLTs, cytokine and chemokine production in a MyD88-, TRIF- and Syk-dependent manner. **(B)** Hyphae also seem to be recognized by TLR2 and D1 receptors that leads to cytokine secretion. Finally, *Aspergilius fumigatus* induces the release of β-hexosaminidase. Dashed-lines show unknown pathways activated in MC response to yeast and hyphae. Solid-lines show fragments of signaling pathways that have been experimentally demonstrated and dashed-lines show reported effects of receptor triggering or MC-fungi interactions. Central red dashed-line separate what is known about the interactions with the yeasts or hyphae of fungi.

intracellular ROS through Dectin-1, and to a lesser extent *via* TLR2, in murine BMMCs (267). BMMCs also released IFN-β in response to zymosan *via* TLR2; where the internalization of the receptor and the endosome maturation were needed (191). Recently, the antifungal response of MCs through TLR4 receptor was demonstrated. Rat peritoneal MCs stimulated with mannan released histamine and produced cysLTs, ROS and pro-inflammatory cytokines and chemokines, such as IFN-γ, GM-CSF, TNF-α, CCL2/MCP-1 and CCL3, *via* TLR4 and dependent on MyD88, TRIF and Syk (268). Mannan also increased the gene expression of different immunoregulatory and pro-inflammatory cytokines and the chemoattraction of MCs. Interestingly, cell response to mannan was enhanced in IgE-sensitized MCs (268), which is important to be considered in the context of IgE-mediated allergic conditions, as ongoing fungal infection in humans could exacerbate and worsen the course of the allergic disease.

The release of mediators by MCs in response to dimorphic fungal pathogens can be different depending on their morphotype (yeast or mycelia) and state of maturation. Degranulation of RBL-2H3 cells was induced by *Paracoccidioides brasiliensis* yeasts and by mature *Aspergillus fumigatus* hyphae, but not by their immature hyphae or conidia (269, 270). Furthermore, a recombinant version of the PbPga1 protein from the yeast surface of *P. brasiliensis*, activated the release of IL-6 *via* NFκB (269). *C. albicans* also induced degranulation and *de novo* synthesis of various cytokines by MCs, although results are still controversial. Nieto-Patlán et al. reported that both yeasts and hyphae induce the production of TNF-α, IL-6, IL-10, CCL3/MIP-1α and CCL4/MIP-1β by BMMCs *via* Dectin-1, without ruling out TLR2 involvement; while IL-1β was only induced by yeast cells (271). Nevertheless, De Zuani et al., using the same MC type, showed that only yeasts triggered the release of TNF-α, IL-6, IL-13, and IL-4 (272). Likely, during the early response, *C. albicans*

extracellular destruction is mediated by products derived from the cell degranulation, such as histamine and TNF-α (113, 144, 271, 273), since the MC fungicidal activity was greater to extracellular than to engulfed yeasts (113, 144).

The MC response to *Sporothrix schenckii*, a dimorphic fungus that causes a chronic subcutaneous mycosis called sporotrichosis that affects both humans and animals, was also studied. Both *S. schenckii* conidia and yeast induced TNF-α and IL-6 secretion by peritoneal MCs without a significant degranulation, and while the former potentiated histamine secretion induced by C48/80, the latter activated MC through ERK1/2 pathway (274, 275). BMMCs also dose-dependent released IL-6, TNF-α, IL-1β and IL-10 in response to *S. schenckii* yeasts (276). Although IL-6 and TNF-α are cytokines that play important roles in the defense against fungi (277–279), it is suggested their participation in the pathogenesis of *S. schenckii* infection, and this injurious side of the MCs will be discussed later.

Modulation of Innate and Adaptive Response to Infection

Through the release of mediators, the MCs establish connections with various cells at the site of infection, supporting the microbicidal activity of macrophages and neutrophils. In addition, MCs participate in the chemoattraction of various pro-inflammatory cells to site of infection. During infection by Gram-negative bacteria, the production of TNF-α, LTs and mouse MCPT-6 participated in neutrophil chemoattraction (159, 160, 175, 176). Additionally, *de novo* production of TNF-α and GM-CSF by MCs was implicated both in the recruitment of neutrophils and the improvement of their phagocytic activity and ROS generation in a model of acute lung inflammation induced by LPS (280). Furthermore, MC-derived GM-CSF decreased neutrophil spontaneous apoptosis (280), and MC-derived IL-6 improved bacterial killing by neutrophils (184). Studies performed in histidine decarboxylase(-/-) mice and infected with *M. tuberculosis* showed that MC-derived histamine mediated the production of TNF-α and IL-6, as well as suppressed the Th1 response, prompting an inflammatory pathology (281). On the other hand, during viral infection, MCs usually produce a series of chemokines that modulate the migration of cells associated to antiviral activity. The chemoattraction of NK and NKT cells in a MC-dependent fashion at the site of DENV infection was associated with MC expression of CCL5/RANTES, CXCL12, CX3CL1/fractalkine, TNF-α and IFN-α (212). While the production of CXCL8/IL-8 by CBMCs after exposure to mammalian reovirus serotype 3 led to the chemoattraction of NK cells (282). In helminth infection, mouse MCPT-6 was associated with eosinophil chemoattraction in an IgE-dependent manner (283). In addition, HMC-1 cells infected with *C. albicans* induced the recruitment of neutrophils, probably due to the increase in IL-8 synthesis (144). Interestingly, a recent study in MC-deficient mice showed that MCs participate in the resolution of zymosan-induced inflammation by promoting the efferocytosis mediated by macrophages, possibly through IL-4 and CXCL1 secretion (191).

In the context of the adaptive immune response, the products secreted by MCs recruit DC precursors, promote the influx of monocyte-derived DCs, activate DCs for antigen presentation and induce their mobilization to draining lymph nodes. In response to peptidoglycans or Gram-positive bacteria, MCs activated skin Langerhans cells, which leads to an increase in the number of these cells at the draining lymph nodes (284). It is known that histamine favors the capture of antigens, the cross-presentation of DCs, the expression of costimulatory molecules by DCs and the induction of Th-differentiation profiles (285, 286). Thus, the histamine secreted during infection by activated MCs might be modulating DC response. In this sense, the histamine receptor (HR)2 expressed by DCs was involved in the attraction of plasmacytoid DCs to draining lymph nodes in response to the pathogen (284). Furthermore, the administration of MC-derived exosomes containing exogenous antigens and heat shock proteins to naive mice enhanced specific humoral responses and induced phenotypic and functional maturation of DC both *in vivo* and *in vitro* (287). Likewise, MC granules exocytosed in response to LPS were captured intact by dermal DCs, promoting the maturation and migration of DC to the lymph nodes and improving the priming of T cells; the TNF embedded in exocytosed MC granule was partially responsible for these effects (288). Besides, TNF released by MCs in mice infected with *E. coli* increased the expression of E-selectin in local blood vessels, facilitating the recruitment of DC to the site of infection (289). Furthermore, activation of murine MCs through TLR3-polyI:C induced CCL5/RANTES, CCL4/MIP-1β and keratinocyte-derived chemokine production, triggering the recruitment of $CD8^+$ T lymphocytes (226). MCs also interact directly with $CD8^+$ T lymphocytes by presenting antigen *via* MHC molecules class I, and induce $CD8^+$ T lymphocytes to produce IL-2, IFN-γ, and CCL3/MIP-1α. At the same time, they regulate the cytotoxic activity of $CD8^+$ T lymphocytes by increasing their degranulation and up-regulating granzyme expression. This effect is enhanced when MCs are activated *via* PRR, by LPS or polyI:C (290). The presentation of antigens to $CD4^+$ T lymphocytes by MCs is not ruled out, since *in vitro* stimulation of murine MCs with LPS and IFN-γ or *in vivo* with LPS or *Leishmania major* induced the expression of MHC class II and costimulatory molecules (103). The just-mentioned *in vitro* experiments showed that MCs *via* MHC II can reactivate antigen-experienced $CD4^+$ T lymphocytes and antigen-specific T regulatory (Treg) cells, over naïve T cells. In this sense, it was demonstrated using longitudinal intravital multiphoton microscopy and DC/MC double reporter mice, that after cell-to-cell contact DCs transferred class II MHC proteins to dermal MCs in the context of skin inflammation (291); although this DC-to-MC communication exacerbated the subsequent T-cell driven skin inflammation and promoted T cell survival, more studies are needed to clarify the physiological impact of this phenomenon. Finally, there is a cross-communication between MCs and Treg lymphocytes. The adoptive transfer of *in vitro*-stimulated $CD4^+$ $CD25^+$ Treg cells to mice with polymicrobial sepsis increased the number of peritoneal MCs and the production of TNF-α, in addition to improving bacterial elimination and animal survival (292). In addition, histamine released by BMMCs activated by FcεRI cross-linking inhibited the suppressive activity of $CD4^+$ $CD25^+$ Treg cells through the HR1 receptor, probably due to the

reduction in the expression level of CD25 and Foxp3 (293). Despite the discoveries made so far concerning MC-Treg intercommunication (294–296) there are still many questions to be resolved in the setting of the antimicrobial response.

DETRIMENTAL ROLES OF MAST CELLS DURING ANTIMICROBIAL RESPONSE

Different studies support that under a high microbial load in the body, the uncontrolled secretory response of MCs can contribute to the development of a pathological conditions. In this sense, while MCs showed a protective role in CLP mice models that caused moderate peritonitis, the MC response was detrimental in severe peritonitis with a high bacterial load, leading to an increase in animal mortality (297). Using MC-deficient mice (W^{sh}/W^{sh}) intraperitoneally engrafted with either wild-type MCs or TNF-deficient MCs, it was shown that MC-derived TNF contributes to the deleterious effects of MCs after severe CLP induction or after intraperitoneal inoculation of *S. typhimurium*. In these experimental conditions, MCs might be susceptible to activation by bacteria carried within the blood stream, and the resulting release of mediators could potentially have lethal effects on the host as they quickly reach the blood vessels due to perivascular location of MCs (298), resulting in severe systemic effects. Accordingly, when animals with CLP were administered with the MC stabilizer sodium cromoglycate clinical manifestations of sepsis were attenuated and there was an improved mice survival by preventing splenocyte apoptosis and the consequent increase in serum levels of the high mobility group box-1 alarmin, suggesting that MCs contribute to systemic inflammation during sepsis (299). The functional importance of MC systemic degranulation during infection was evaluated by compartment-specific MC reconstitution in W^{sh}/W^{sh} mice with CLP-induced septic peritonitis. This study demonstrated that while MC reconstitution only at the peritoneal cavity improved the survival of animals, MC reconstitution both at the peritoneal and systemic levels decreased animal survival (300). In addition, systemically reconstituted animals with IL-6(-/-) BMMCs improved survival compared to those reconstituted with IL-6(+/+) BMMCs, suggesting that degranulation and IL-6 release from MCs located distant to the site of infection play a detrimental role during CLP-induced infection (300). A later study described a potential mechanism of indirect harmful participation of MCs during severe peritonitis, which was mediated by the early release of preformed IL-4, achieving immunosuppressive effects on the ability of macrophages to phagocytose bacteria (301). A similar double-face behavior of MCs has been described in DENV infection. Localized MC response to DENV might protect the host by recruiting key cells involved in virus clearance and by limiting the number of cellular targets to viral infection (212, 302). On the other hand, granule particles released extracellularly by virus-infected skin MCs contained DENV and could disseminate and propagate the infection in mice through lymph (303). This newly proposed mechanism of virus spreading is in accordance with the described interaction between DENV envelope proteins and heparin (304). Concerning dengue pathology, the MC participation in the vascular loss induced during viral infection in severe states of disease was reported. In experimental models of systemic DENV infection using a virus CI, MC mediators able to modulate vascular endothelium, such as the mice chymase MCPT-1, were elevated in serum (305). Chymase levels were also increased in serum of dengue fever and dengue hemorrhagic fever patients as compared to healthy controls (305). Two indicators of vascular leaking, dye leakage into tissues and hematocrit levels, were decreased in MC-deficient mice, and recovered after MC reconstitution. Besides, this study confirmed the involvement of MCs and LTs in dengue-induced vascular permeability using the MC-stabilizing compound cromolyn and ketotifen and the antagonist of LT receptor montelukast (214, 305). Besides chymase and LTs, MC-derived serotonin was also recently implicated in thrombocytopenia in a severe model of dengue-induced disease (306); thus, the potential of MCs as a therapeutic target to limit dengue vasculopathy or thrombocytopenia should be evaluated in clinical trials. According to results in peritonitis and DENV infection models, while local and immediate MC activation during infection seems to be beneficial, sustained, and systemic activation may not be.

In tuberculosis, it is speculated that TNF-α released by MCs might play a role in the formation of the mycobacterial granuloma, which results in latent disease that can be reactivated later in life (115, 307). A correlation between MCs number and granuloma formation has been described. Analyzing lymph nodes from patients with tuberculous lymphadenitis, MC number was positively correlated with the number of granulomas and the number of multinucleated giant cells (308). The data about MCs in leprosy, a chronic dermato-neurological granulomatous disease caused by *Mycobacterium leprae*, are controversial. Most of the studies indicate an increased number of MCs in skin biopsies of lepromatous lesions, in comparison with other leprosy forms (309–311), except for one study in which a higher dermal MC number was found around granulomas in skin biopsies from patients with tuberculoid or mild-borderline leprosy in comparison to lepromatous leprosy biopsies (312). A more recent study showed that there is a greater amount of degranulated versus intact MCs and a predominance of tryptase positive versus chymase positive MCs in the skin of leprosy patients, independently of leprosy form and reactional episodes (313). These data suggest that MC derived mediators can perpetuate inflammation during *M. leprae* infection, and MC tryptase might be exerting detrimental effects on tissue structure and remodeling in leprosy lesions, as it has mitogenic activity on fibroblasts and increases type I collagen production (69). In support of this notion, an association between collagen increase and tryptase-rich MC density in the epineurium of leprous nerves was described (314). Whether MC response contribute to immunity or disease pathogenesis in chronic granulomatous diseases remains to be deeply studied.

Data also suggest that MCs develop harmful roles during antimicrobial response when the infection is associated with a pre-existing inflammatory disorder. Skin colonization with *S. aureus* was associated with worsening of the inflammatory process linked to AD (315). Among *S. aureus* exotoxins, δ-toxin can activate MC degranulation in an IgE- and allergen-

independent manner (316). In experimental models of AD, mice colonized with wild-type *S. aureus* developed higher IgE levels and a more severe inflammatory skin disease than mice inoculated with the bacterium deficient in δ-toxin. Strikingly, in MC-deficient mice (W^{sh}/W^{sh}) inoculated with the wild-type *S. aureus* the level of IgE and the intensity of skin inflammation induced by epicutaneous sensitization was decreased in comparison with wild-type mice, but the severity of the skin disease was restored upon adoptive transfer of MCs into the skin of W^{sh}/W^{sh} mice (316). As different studies show an indispensable role of MCs in the pathogenesis of experimental AD induced by epicutaneous sensitization (317, 318), these results suggest that MC activation by *S. aureus* in the setting of AD exacerbates the pre-existing inflammatory and atopic process. However, more research is needed in this field as it was also suggested protective effects or no participation of MCs in spontaneous AD-like disease or inflammation developed by genetically modified mice (319, 320). *M. sympodialis* infection is also related to the exacerbation of the inflammatory response in AD. MCs responded to *M. sympodialis*, but the response was higher when cells were obtained from patients with AD than those derived from healthy donors (259). *Malassezia* extract induced the production of LTs by sensitized and non-sensitized MCs, the degranulation and production of CCL2/MCP-1 by sensitized cells, as well as improved IgE-dependent degranulation and impaired the synthesis of IL-6 *via* TLR2/MyD88. These changes in the MC response induced by *M. sympodialis* might cause an exacerbated inflammatory response in patients with AD (260). Similarly, MCs are implicated in the pathogenesis of gastritis. An increased MC density was found in mucosa biopsy from subjects with gastritis, and the number was even higher in *Helicobacter pylori*-infected gastric mucosa specimens (321). While MCs in *H. pylori*-infected gastric mucosa showed degranulation, no findings of degranulation were seen in the normal stomach (322). These data suggest that MC response to *H. pylori* infection might be exacerbating the inflammatory response underlying gastritis, as a positive correlation between MC density and intensity of inflammation was described (321). According to all these studies, MC hyperactivation by recurrent infections in the context of an inflammatory disorder can exacerbate pathological tissue damage.

MCs also play crucial roles in the pathogeny associated with some infectious diseases, such as that caused by viruses. It was described that the gp120 glycoprotein of HIV-1, characterized as a superantigen that interacts with the heavy chain of IgE, triggers the release of proinflammatory, angiogenic and lymphangiogenic mediators from human lung MCs (323). As serum IgE levels were elevated in subjects with HIV infection compared to controls (324, 325), this study was the first approach to decipher the possible involvement of MC mediators in chronic lung diseases, that are prevalent among HIV patients (326–328). Besides, human MC progenitors can be HIV infected and retain the virus with their maturation (329). MC participation as a virus reservoir is of great impact on pathology as they are long-lived cells, abundant at viral replication sites and chemoattracted in response to HIV antigens, resistant to the virus cytotoxic effects, and able to contribute to HIV transmission (330–332). In this line, MC precursors cultured *in vitro* from fetal or adult $CD34^+$ progenitors co-expressed CD4, CXCR4, and CCR5 and were susceptible to R5 tropism in viral infection, but only marginally susceptible to X4-HIV infection. When IgE-FcεRI aggregation was induced by HIV gp120 or antigen from *Schistosoma mansoni* eggs, the expression of CXCR4 in MC precursors was up-regulated, increasing their susceptibility to X4 and R5X4 virus infection (333). These data suggest that HIV-positive individuals with pre-existing comorbid conditions associated with elevated levels of IgE, such as atopic diseases or helminth infections, may predispose to a predominant X4 virus phenotype, which has been associated with a more rapid progression to AIDS in infected individuals (334). In the same context of viral infections, it was reported that the activation of brain MCs was causative of worsening infection, morbidity, and mortality in a mice model of Japanese encephalitis virus infection (335). MCs are resident immune cells in the central nervous system that are strategically located near the blood-brain barrier and the neurovascular unit (336). Particularly, MC chymase was identified as the key mediator involved in the increase of permeability in the blood-brain barrier that promotes Japanese encephalitis virus neuroinvasion and neurological dysfunction (335). In addition, MC-deficient mice (W^{sh}/W^{sh}) exhibited resistance to inflammatory disease induced by influenza A virus infection, suggesting that the histamine, LTs, cytokine and chemokine secreted by cultured MCs upon influenza A virus infection might be contributing to the excessive host immune response against the virus (337). Similarly, MC-deficient mice (both W^{sh}/W^{sh} and Sl/Sl^d; the latter harbors deletions in the SCF coding region) showed reduced myocardial inflammation and necrosis, accompanied by an increase in animal survival, compared to normal mice after infection with the encephalomyocarditis virus. Histopathological severity of the myocardial lesions induced by the virus was significantly increased in MC-reconstituted animals, which indicates that MCs are participating in the pathogenesis of viral myocarditis (338). Besides viral diseases, MCs have been also implicated in the development of other infectious pathologies. As previously mentioned, MCs activated by yeast of *S. schenckii* secrete cytokines, mainly TNF-α and IL-6 (275, 276). Nevertheless, when tissue fungal dissemination was evaluated in rats infected with the fungus, the absence of functional MCs in the inoculation site reduced fungal dissemination and the setting of a more severe sporotrichosis (274). The MC contribution to sporotrichosis was recently corroborated using models of MC-depleted mice, and *Sporothrix* virulence was linked to MC cytokine production and the latter to disease activity in patients with sporotrichosis (276).

MCs have been described as potential reservoirs for different pathogens. *S. aureus* promoted its internalization within skin MCs during infection to avoid the extracellular antimicrobial activities (132). *S. aureus* responded to stress imposed by extracellular antimicrobial weapons released by MCs by up-regulating α-hemolysin and other fibronectin-binding proteins. The former was involved in *S. aureus* internalization within MCs (339). Particularly, the interaction between bacterial α-hemolysin and ADAM10 of MCs and the subsequently

activated signaling induced the up-regulation of β1-integrin expression on MCs, which mediated *S. aureus* internalization through a pathway different from the normal phagocytic one. Bacterial α-hemolysin was also involved in bacterial survival within the MCs (339). Through hiding within MCs, staphylococci not only avoid clearance but also establish an infection reservoir that could contribute to a chronic carriage. In the same context, it was shown that *E. coli* was up-taken by mice BMMCs in antibody deficient conditions upon FimH-CD48 interaction through a mechanism mediated by caveolae (120). In macrophages, internalized *E. coli* by FimH employing a similar caveolar endocytic pathway showed an increased intracellular survival as compared to opsonized bacteria internalized *via* antibody (340), which suggests that *E. coli* contained in MC caveolar chambers might be also avoiding intrinsic bactericidal activity bypassing phago-lysosomal fusion. However, bacteria viability inside MC caveolae needs a further demonstration, as an interaction of internalized caveolae with lysosomal compartment was described (341, 342).

The detrimental roles described to MC as a consequence of interaction with microbes are summarized in **Figure 8**; nevertheless, and before closing this section it is worthy to mention that it was reported the first evidence that MC response to an opportunistic pathogen might be associated with allergy onset. Gastrointestinal *Candida* colonization promotes sensitization against food antigens in mice, at least partly due to MC-mediated hyper-permeability in the gastrointestinal mucosa (343). Previous reports had positively associated *H. pylori* infection and the development of food allergy and AD by linking the infectious process with the inhibition of oral tolerance (344–346). Recent works showed that the interaction of *C. albicans* with different MC types, i.e. mucosal or stromal MCs, induced different cytokine microenvironments which contributed respectively to barrier function loss, fungal dissemination, and inflammation or to increase mucosal immune tolerance in gastrointestinal or vulvovaginal candidiasis. The IL-9/MC axis was associated with this dual role of the cell (347, 348).

Finally, few works have suggested the MC participation in the development of both COVID-19 pathology and post-COVID syndrome (349, 350), although more studies are needed to demonstrate the direct implication of the cell in both conditions. An increased MC density was a distinguishing pathological feature in the lungs of COVID-19 patients compared to H1N1-induced pneumonia and control subjects (351), and the levels of chymase, tryptase and carboxypeptidase A3 were higher in serum from SARS-CoV-2 infected patients with generalized inflammation than in uninfected donors (224). Besides, a retrospective cohort study showed that famotidine intake by COVID-19 patients during hospitalization statistically reduced the risk of intubation or death (352). It was suggested that the principal famotidine mechanism of action for COVID-19 was targeting HR2 activity, and that the development of clinical COVID-19 involved dysfunctional MC activation and histamine release (353).

CONCLUSIONS AND PERSPECTIVES

MCs can respond to parasites, bacteria, viruses, and fungi. They perform different antimicrobial mechanisms, such as phagocytosis, ET formation and the release of granular content or *de novo*

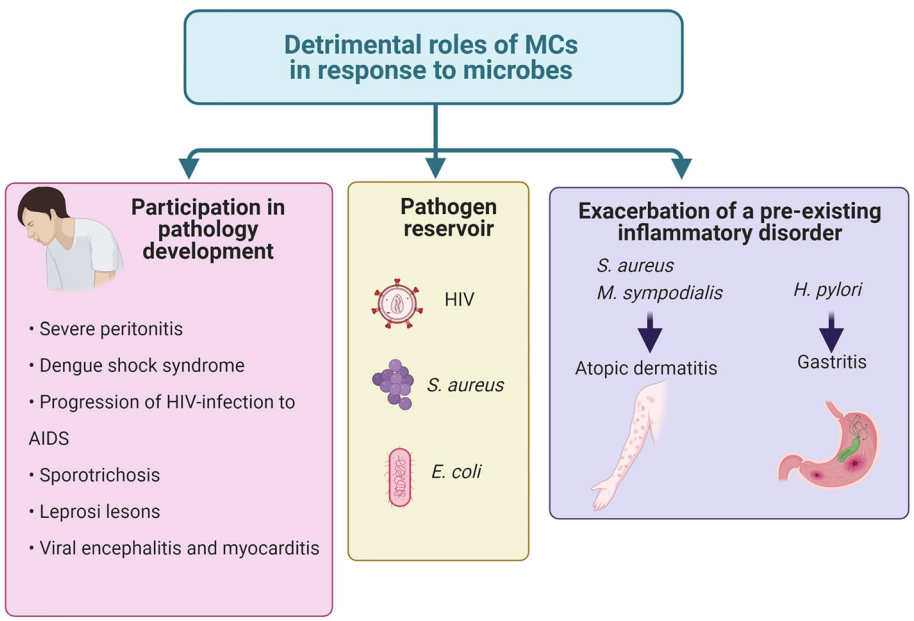

FIGURE 8 | Harmful actions of MCs during infection. MCs have been found to contribute to the worsening of complex pathologies and distinct pre-existing inflammatory conditions. Also, they have been proposed to be reservoirs for distinct virus and bacteria.

synthesized mediators. MC mediators efficiently initiate the recruitment of additional innate effector cells crucial to pathogen clearance, such as neutrophils, monocytes/macrophages, NK cells, NKT cells, or eosinophils. MCs are also associated with the regulation of the adaptive response developed in response to the invading pathogen by directly promoting T-cell activation or by modulating the migration and functionality of DCs.

However, the wide variety of MC mediators allow multifaceted effects, promoting host defense against pathogens on one hand but inducing damage to the host on the other. The outcome of MC response to pathogens seems to depend on the context in which the cell is activated, being able to entail protection or damage. The main factors involved in this dual role are the followings: *i*) the pathogen distribution, load, and location; *ii*) the compartment or organ in which the activated MC is located; *iii*) the previous existence of a pathological condition associated with the infection; *iv*) the potential use of MCs as a reservoir; and *v*) whether it is an acute or chronic infectious process. More research is needed to complete the signaling pathways described in MCs when responding to pathogen encounters and to identify the points of connection or the distinctive molecules among the pathways involved in phagocytosis, ET release and secretion of mediators (summarized in **Figure 9**). Also, future research should consider the possible pathogen-induced epigenetic changes that chronic infections could

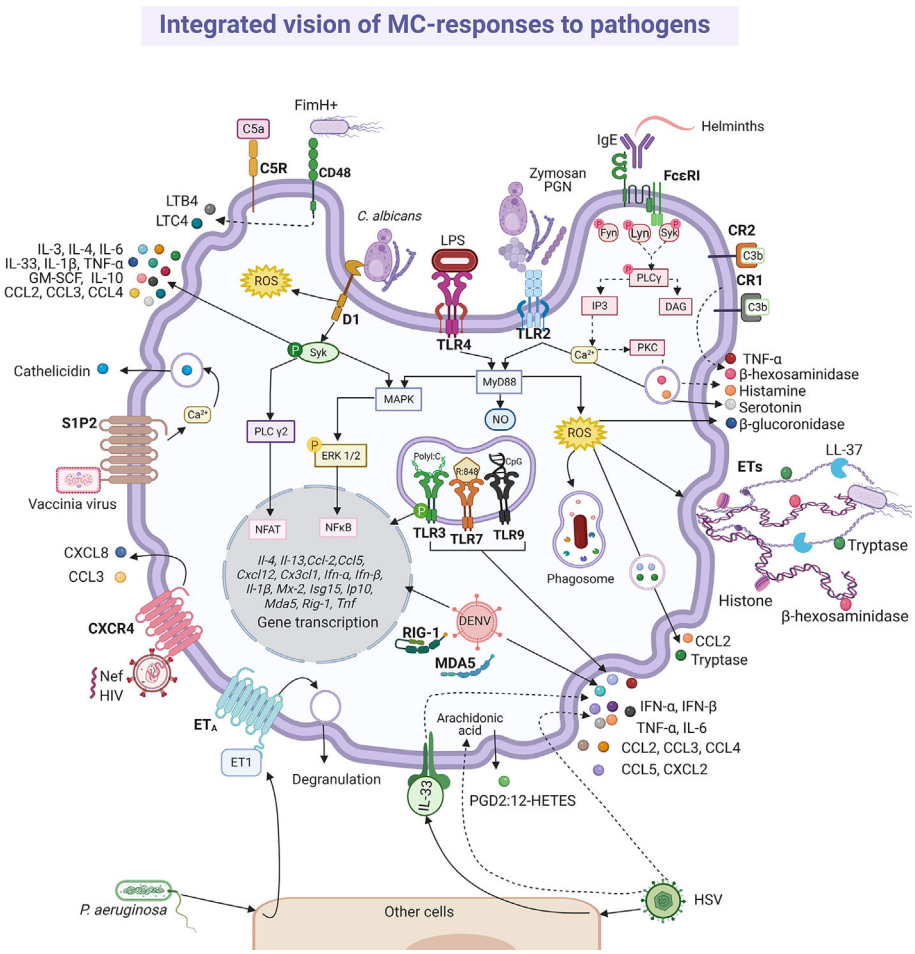

FIGURE 9 | Integrated responses of MCs to distinct pathogens. Distinct pattern recognition receptors (PRRs) expressed in MCs directly recognize pathogens, promoting phagocytosis, extracellular traps (ETs) formation and the release of pre-formed and *de novo* synthesized mediators. Canonical signaling pathways described for PRRs have increased complexity in MCs, where PRR triggering leads not only to the activation of NFκB but also to the secretion of granule content by anaphylactic and piecemeal degranulation. MC activation is observed also as a secondary event after the production of mediators by other cells, which causes the amplification of the initial inflammatory response. On the other hand, IgE-mediated FcεRI signaling cascade by parasites leads to a protective anaphylactic degranulation response that appears to require the activation of well-described signaling pathways participating in the allergic response. Finally, bacterial phagocytosis, ET release and secretion of mediators seem to be connected by mechanisms to be defined yet. Solid-lines indicate known signaling pathways, whereas dashed-lines indicate suggested pathways or reported effects triggered by receptor stimulation or interactions of MCs with pathogens. LPS, lipopolysaccharide; PGN, peptidoglycan.

induce in MCs, inducing long-term alterations in their phenotype that could modify the response from protective one to detrimental. With that information, it will be possible to suggest potential therapeutic intervention strategies directed not only to generate immune protection or resolve inflammation but also to limit or avoid tissue damage in those infectious scenarios in which the MC plays harmful roles.

The high incidence of infections with fatal outcomes in humans and the goal that we are facing of developing new treatments, as many bacteria have generated resistance to antibiotics (354–360), highlight the importance of generating knowledge about MC response to the infection process. Animal models are mostly used to evaluate the immune response to pathogenic agents as they induce immunological responses homologous to humans, although several differences are present. Therefore, mechanisms demonstrated to MCs during antimicrobial scenario in animals need to be proved to occur in humans, to later propose potential therapies aid to modulate MC activity.

AUTHOR CONTRIBUTIONS

MJ and ES conceived the review. MJ, DC-G, LC-D, MP-R, CG-E, and ES wrote de manuscript and designed the figures. MP-R drew the figures. All authors contributed to the article and approved the submitted version.

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GLOSSARY

12-HETES	12-hydroxyeicosatetraenoic acid
AD	atopic dermatitis
ADAM	disintegrin/metalloprotease
AMP	antimicrobial peptide
AP-1	activator protein-1
BM	bone marrow
BMMC	bone marrow progenitor derived mast cell
CBMC	umbilical cord blood derived mast cell
CI	clinical isolate
CLP	cecal ligation and puncture
DC	dendritic cells
DENV	dengue virus
EMP	erythro-myeloid progenitor
ERK	extracellular receptor kinase
ET	extracellular trap
FcεRI	high-affinity receptor to IgE
GAS	group A *Streptococcus*
GM-CSF	granulocyte-macrophage colony stimulating factor
HIV	human immunodeficiency virus
HR	histamine receptor
HSC	hematopoietic stem cell
HSV	herpes simplex virus
IFN	interferon
IKK	IκB kinase
IL	interleukin
IP3	inositol 3-phosphate
LAT	linker of activation of T cells
LPS	lipopolysaccharide
LTs	leukotrienes
MAPK	mitogen-activated kinase
MC	mast cell
MCETs	mast cell-derived extracellular traps
MCP	monocyte chemotactic protein
MCPT	mast cell protease
MHC	major histocompatibility complex
Mincle	macrophage inducible Ca^{2+}-dependent lectin receptor
MIP	macrophage inflammatory protein
MMP	matrix metalloproteinase
MyD88	myeloid differentiation primary response 88
NADPH	nicotinamide adenine dinucleotide phosphate
NETs	neutrophils extracellular traps
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NOD	Nucleotide-binding oligomerization domain
PBMCs	peripheral blood-derived mast cells
PLC	phospholipase C
PGs	prostaglandins
PRRs	pattern-recognition-receptors
RANTES	regulated upon activation normal T-cell expressed and secreted
ROS	reactive oxygen species
RSV	respiratory syncytial virus
SCF	stem cell factor
SNAP23	soluble N-ethylmaleimide sensitive factor attachment protein receptor-23
TACE	disintegrin/metalloprotease ADAM-17/TNFα-converting enzyme
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF6	tumor necrosis factor receptor associated factor 6
Treg	T regulatory
TRIF	TIR-domain-containing adapter-inducing interferon-β
VSV	vesicular stomatitis virus

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ORIGINAL RESEARCH
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Murepavadin, a Small Molecule Host Defense Peptide Mimetic, Activates Mast Cells *via* MRGPRX2 and MrgprB2

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Pseudomonas aeruginosa is a frequent cause of hospital-acquired wound infection and is difficult to treat because it forms biofilms and displays antibiotic resistance. Previous studies in mice demonstrated that mast cells (MCs) not only contribute to *P. aeruginosa* eradication but also promote wound healing *via* an unknown mechanism. We recently reported that host defense peptides (HDPs) induce human MC degranulation *via* Mas-related G protein-coupled receptor-X2 (MRGPRX2). Small molecule HDP mimetics have distinct advantages over HDPs because they are inexpensive to synthesize and display high stability, bioavailability, and low toxicity. Murepavadin is a lipidated HDP mimetic, (also known as POL7080), which displays antibacterial activity against a broad panel of multi-drug-resistant *P. aeruginosa*. We found that murepavadin induces Ca^{2+} mobilization, degranulation, chemokine IL-8 and CCL3 production in a human MC line (LAD2 cells) endogenously expressing MRGPRX2. Murepavadin also caused degranulation in RBL-2H3 cells expressing MRGPRX2 but this response was significantly reduced in cells expressing missense variants within the receptor's ligand binding (G165E) or G protein coupling (V282M) domains. Compound 48/80 induced β-arrestin recruitment and promoted receptor internalization, which resulted in substantial decrease in the subsequent responsiveness to the MRGPRX2 agonist. By contrast, murepavadin did not cause β-arrestin-mediated MRGPRX2 regulation. Murepavadin induced degranulation in mouse peritoneal MCs *via* MrgprB2 (ortholog of human MRGPRX2) and caused increased vascular permeability in wild-type mice but not in MrgprB2$^{-/-}$ mice. The data presented herein demonstrate that murepavadin activates human MCs *via* MRGPRX2 and murine MCs *via* MrgprB2 and that MRGPRX2 is resistant to β-arrestin-mediated receptor regulation. Thus, besides its direct activity against *P. aeruginosa*, murepavadin may contribute to bacterial clearance and promote wound healing by harnessing MC's immunomodulatory property *via* the activation of MRGPRX2.

Keywords: murepavadin, mast cells, MrgprB2, MRGPRX2, host defense peptides, antimicrobial peptides

INTRODUCTION

The emergence of multidrug-resistant bacterial infections poses a global public health threat that warrants urgent need for alternative therapeutic approaches (1). Host defense peptides (HDPs), previously known as antimicrobial peptides (AMPs), such as the cathelicidin LL-37 and human β-defensins are considered as promising antimicrobial agents (2–4). Recent evidence demonstrated that in addition to their direct antimicrobial activity, HDPs promote the recruitment and activation of various immune cells including mast cells (MCs), neutrophils, monocytes and lymphocytes (5–8). These HDPs also display angiogenic activity and contribute to wound healing (8, 9). However, many HDPs cause the lysis of erythrocytes and display cytotoxicity against a variety of cells (10, 11). In recent years, great strides have been made in optimizing HDPs to minimize their toxicity and to improve their stability, which can also modulate the immune system for therapeutic benefits (12, 13).

Mast cells (MCs) are multifunctional immune cells of hematopoietic origin that are found in vascularized tissues such as the oral mucosa, intestine, airway and the skin. MCs play an important role in host defense and promote wound healing (14–16). In addition to high affinity IgE receptor (FcεRI), a subtype of human MCs (MC_{TC}; contain both tryptase and chymase) expresses a G protein-coupled receptor (GPCR) known as Mas-related GPCR-X2 (MRGPRX2) (17, 18). This receptor is highly expressed in human skin MCs but is also present in lung and gut MCs but at lower levels (17, 19, 20). Mouse connective tissue MCs (CTMC; skin, nasopharynx and peritoneal) express MrgprB2 (ortholog of human MRGPRX2) (16, 21). Both receptors are activated by human and mouse HDPs (22–24). Studies with human MCs expressing MRGPRX2 and $MrgprB2^{-/-}$ mice have strongly implicated these receptors in innate immunity and wound healing (15, 16). The major portal of entry for pathogen is the interface between host and external microenvironment such as the skin, nasopharynx, and peritoneum. MrgprB2-expressing CTMCs are found abundantly at these sites and contribute to host defense against bacterial infection through the release of MC-derived mediators and the subsequent recruitment of neutrophils (16). Furthermore, pharmacological activation of MrgprB2 at these sites results in decreased bacterial count and reduced disease severity *in vivo* (16).

Pseudomonas aeruginosa is a Gram-negative bacterium that often presents a therapeutic challenge due to its ability to form biofilms and to display antibiotic resistance (25, 26). Zimmerman et al. (27), utilized a topical *P. aeruginosa* infection model and demonstrated that MCs contribute to both bacterial elimination and promote wound healing. However, they found that culturing MCs infected with *P. aeruginosa in vitro* is insufficient to eliminate bacteria unless they are co-cultured with keratinocytes. MC mediators released in response to *P. aeruginosa* infection results in the secretion of HDPs such as mouse β-defensin-14 (Defb14, ortholog of human β-defensin-3) from keratinocytes (27). These findings suggest that MC-derived mediators confer protective immunity through the promotion of endogenous HDP secretion, which in turn, directly kill the bacteria and restrain the infection (27).

Protegrin-1 is a HDP that was originally purified from porcine leukocytes (28). We have recently shown that protegrin-1 activates human MCs *via* MRGPRX2 (29). However, small molecule HDP mimetics have a number of advantages over natural HDPs because of their superior stability, bioavailability, and reduced toxicity (30, 31). Moreover, several approaches have been used to increase hydrophobicity and membrane activity of HDP mimetics (32). Murepavadin is a lipidated protegrin-1 mimetic, (also known as POL7080), which specifically targets *P. aeruginosa*, including multidrug-resistant clinical isolates (33). Thus, it could be used for the treatment of antibiotic-resistant *P. aeruginosa* skin infection (34, 35). Given that HDPs including protegrin-1 activate human MCs *via* MRGPRX2 (22, 23, 29), raises the interesting possibility that potential therapeutic action of murepavadin for *P. aeruginosa* skin infection likely reflects both MRGPRX2-mediated MC activation and its direct antimicrobial activity. However, the possibility that murepavadin activates MCs has not been tested.

Besides G proteins, most GPCR agonists activate another signaling pathway that requires the recruitment of adapter proteins known as β-arrestins. This β-arrestin-mediated pathway was first identified for its role in receptor desensitization (uncoupling of receptor/G protein interaction) and internalization (36). Agonists that prefer to activate G proteins over β-arrestins are known as G protein-biased agonists, whereas agonists that selectively activate β-arrestins are known as β-arrestin-biased agonists. By contrast, agonists that activate both pathways are designated as balanced agonists. We have recently shown that while compound 48/80 (C48/80) acts as a balanced agonist for MRGPRX2, an angiogenic host defense peptide serves as a G protein-biased agonist (37). The purpose of this study was to test if murepavadin activates human MCs *via* MRGPRX2 and to determine if it serves as a balanced or biased agonist for the receptor. The data presented herein demonstrate that murepavadin activates human and murine MCs *via* MRGPRX2 and MrgprB2, respectively and that it serves as a G protein-biased agonist for MRGPRX2 without the involvement of β-arrestin-mediated receptor regulation. These findings have important implications for the potential utilization of murepavadin in modulating antibiotic-resistant cutaneous infections.

MATERIALS AND METHODS

Materials

All reagents used for cell culture were purchased from Invitrogen (Gaithersburg, MD, USA). Recombinant mouse interleukin-3 (IL-3), mouse stem cell factor (SCF), and recombinant human SCF (rhSCF) were obtained from Peprotech (Rocky Hill, NJ, USA). Compound 48/80 (C48/80) was obtained from AnaSpec (Fremont, CA, USA). Murepavadin (Catalog HY-P1674A) was from MedChem Express. P-nitrophenyl-N-acetyl-β-D-glucosamine (PNAG) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pertussis toxin (PTx) was from List Biological Laboratories

(Campbell, CA, USA). Fura-2 acetoxymethyl ester was from Abcam (Cambridge, MA, USA). Bright-Glo Luciferase was from Promega (Madison, WI, USA). Phycoerythrin (PE)-conjugated anti-human MRGPRX2 antibody was from BioLegend (San Diego, CA, USA). Amaxa Nucleofector Kit V was from Lonza (Gaithersburg, MD, USA). DuoSet ELISA kits were from R&D Systems (Minneapolis, MN, USA). Hemagglutinin (HA)-tagged MRGPRX2 plasmid in pReceiver-MO6 vector was obtained from GeneCopoeia (Rockville, MD, USA). MRGPRX2-Tango plasmid (Addgene no. 66440) was a gift from Dr. Bryan Roth.

Mice

C57BL/6 (wild-type; WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed in pathogen-free cages. WT mice with the deletion of *MrgprB2* transcript (MrgprB2$^{-/-}$ mice) were generated as previously described (38). Eight-to-twelve-week-old male and female mice were used. All animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Cell Culture

The human MC line (LAD2 cell) was kindly provided by Drs. Kirshenbaum and Metcalfe (Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), USA). LAD2 cells were cultured in complete StemPro-34 medium supplemented with l-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL), and rhSCF (100 ng/mL), and the medium was hemi-depleted weekly (39).

Rat basophilic leukemia (RBL-2H3) cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/mL) and streptomycin (100 µg/mL) (40). RBL cells stably expressing human MRGPRX2 were maintained similarly in the presence of 1 mg/mL G418. HTLA cells (HEK-293T cells stably expressing a tTA-dependent luciferase reporter and a β-arrestin2-TEV protease fusion gene) were cultured in DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL), hygromycin (200 µg/mL), puromycin (5 µg/mL), and G418 (500 µg/mL) (41). All cell cultures were kept at 37°C incubator with 5% CO_2.

Peritoneal mast cells (PMCs) were established from peritoneal lavages of WT and MrgprB2$^{-/-}$ mice and were cultured for 4–8 weeks in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% FCS, and recombinant mouse IL-3 (10 ng/mL) and SCF (30 ng/mL). Cells were then determined for MC receptor expression and function, and were used within 4–8 weeks (38, 42).

Degranulation

Human LAD2 (1 x 10^4 cells/well), RBL-2H3, RBL-MRGPRX2 (5×10^4 cells/well), or murine peritoneal MCs (5×10^3 cells/well) were washed and plated in a total volume of 50 µL HEPES buffer in 96-well plates. Cells were then stimulated with murepavadin for 30 min at 37°C. Total level of β-hexosaminidase release were assessed by lysing the cells with 0.1% Triton X-100, whereas cells without any stimulation were designated as controls. Aliquots (20 µL) of supernatants were incubated with 1 mM PNAG (20 µL) for 1 h at 37°C. The reaction was then stopped by adding stop buffer (250 µL; 0.1 M Na_2CO_3/0.1 M $NaHCO_3$). Quantification of β-hexosaminidase level was determined by measuring the absorbance at 405 nm using a Versamax microplate spectrophotometer (Molecular Devices, San Jose, CA, USA) (40).

In some experiments, cells were pretreated with PTx (100 ng/mL, 16 h) prior to any stimulation to assess the inhibitory effect of PTx on MC degranulation.

Calcium Mobilization

Human LAD2 cells (3×10^5), RBL-2H3 or RBL-MRGPRX2 (2×10^6) were loaded with Fura-2 acetoxymethyl ester (1 µM for 30 min at 37°C) in 1.5 mL of HEPES buffer containing 0.1% BSA. Cells were then washed and allowed complete de-esterification for 15 min at room temperature. Cells were then resuspended in buffer and stimulated with Murepavadin or C48/80. Calcium mobilization was determined by the ratio between dual excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm using a Hitachi F-2700 Fluorescence Spectrophotometer.

Cytokine and Chemokine Production and Measurement

LAD2 cells (3×10^5 cells/mL) were washed with medium, resuspended in fresh medium, and stimulated with indicated concentrations of Murepavadin for 24 h at 37°C with 5% CO_2. Cell-free supernatants were collected and kept at -80°C until further analyses. Similarly, RBL-2H3 or RBL-MRGPRX2 cells (2×10^5 cells/mL) were seeded in a 24-well plate and cultured overnight in a 37°C incubator with 5% CO_2. The next day, the medium was aspirated, fresh medium was added to the cells, and the cells were stimulated with indicated concentrations of Murepavadin for 24 h at 37°C with 5% CO_2. Cell-free supernatants were collected and kept at -80°C until further analyses. The cytokine and chemokine production were measured using human CCL3/MIP-1 alpha, human IL-8/CXCL8, rat JE/MCP-1/CCL2 and rat TNF-alpha DuoSet ELISA kits (R&D Systems) following the manufacturer's protocols.

To determine the inhibitory effect of PTx on cytokine and chemokine production, pretreatment with PTx (100 ng/mL, 16 h) was performed prior to any stimulation.

Generation of Cells Transiently Expressing MRGPRX2 and Its Variants

Transient transfections in RBL-2H3 cells expressing WT-MRGPRX2 and its naturally occurring missense variants within MRGPRX2's ligand binding cradle (G165E) or G protein-coupling region (V282M) were performed as described previously (43, 44). RBL-2H3 cells (2×10^6) were transfected with 2 µg of HA-tagged plasmid using the Amaxa Nucleofector Device and Amaxa Kit V and were used within 16 – 20 h post-transfection.

Flow cytometry was used to determine cell surface expression of transiently transfectants. Cells (5×10^5) were incubated with PE-

anti-MRGPRX2 antibody for 30 min, washed in ice-cold FACS buffer (PBS containing 2% FCS and 0.09% NaN_3), followed by fixation with 1.5% paraformaldehyde. Expression level of MRGPRX2 and its variants were analyzed using a BD LSR II flow cytometer (San Jose, CA, USA) with WinList software, version 8.

Transcriptional Activation Following Arrestin Translocation (Tango) Assay

HTLA-MRGPRX2 cells (5×10^4 cells/well, 96-well plate) were cultured overnight in a 37°C incubator. The next day, the medium was removed and cells were exposed to Murepavadin in an antibiotic-free medium (160 µL) for 16 h at 37°C. The medium was then replaced with 100 µL of Bright-Glo solution and relative luminescence was analyzed using a Thermo Luminoskan Ascent 392 Microplate Luminometer (41).

Receptor Internalization

RBL-MRGPRX2 or HTLA-MRGPRX2 cells (5×10^5) were treated with C48/80 or Murepavadin for 30 min, 3, 6, and 16 h to induce cell surface receptor internalization. After indicated time, cell surface expression was determined by flow cytometry as described above.

Evan's Blue Dye Extravasation

Mice (WT and $MrgprB2^{-/-}$) were intravenously injected with 1% Evan's blue followed by intradermal injection of 20 µL Murepavadin (30 µM) in the right paw and PBS (vehicle) in the left paw. After 30 min, the mice were euthanized and the paws were removed, weighed, dissolved in 500 µL formamide and incubated at 56°C overnight. The Evan's blue dye extravasation was measured by collecting the supernatants and the absorbance was measured at 650 nm using microplate spectrophotometer.

Statistical Analysis

Data shown represent mean ± SEM value derived from at least three independent experiments. Statistical significance was calculated using *t*-test and one-way or two-way ANOVA analyzed by GraphPad Prism version 9.0.1. Significant differences were set at $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

RESULTS

Murepavadin Induces Intracellular Cas^{2+} Mobilization, Degranulation and Chemokine Generation in Human MCs

We previously demonstrated that LL-37, human β-defensins and protegrin-1 activate human MCs *via* MRGPRX2 at concentrations ranging from 1 - 10 µM (22, 23, 29). To determine if murepavadin activates MCs, we initially utilized a human MC line, LAD2 cells and tested the ability of 10 µM murepavadin to induce Ca^{2+} mobilization. As shown in **Figure 1A**, murepavadin at this concentration induced a robust and sustained Ca^{2+} response. Next, we asked if murepavadin induces MC degranulation. We found that murepavadin induced a dose-dependent degranulation as measured by β-hexosaminidase release, reaching ~80% at a concentration of 10 µM with an EC_{50} value of ~3 µM (**Figure 1B**). While MC degranulation promotes increased vascular permeability *in vivo*, its innate and adaptive immune function require the generation of chemokines. We therefore investigated the ability of murepavadin to stimulate chemokine IL-8 and CCL3 production in LAD2 cells. At a concentration of 10 µM, murepavadin induced substantial IL-8 (**Figure 1C**) and CCL3 (**Figure 1D**).

To explore the underlying mechanism *via* which murepavadin activates MCs, we utilized RBL-2H3 cells that were transfected to stably express human MRGPRX2 (RBL-MRGPRX2). We found that murepavadin induced Ca^{2+} mobilization, β-hexosaminidase as well as TNF-α and CCL2 production from RBL-MRGPRX2, but not from untransfected cells (**Figures 2A–E**). Moreover, we found that Pertussis toxin (PTx), an inhibitor of Gαi/o family of G proteins, caused significant inhibition of murepavadin-induced degranulation, TNF-α and CCL2 production (**Figures 2C–E**). Taken together, these findings demonstrate that murepavadin causes the activation of human MCs specifically *via* MRGPRX2 and that these responses are G protein-dependent.

Naturally Occurring MRGPRX2 Missense Variants Are Hypo-Responsive to Murepavadin for MC Degranulation

We have previously identified naturally occurring loss-of-function MRGPRX2 missense variants within the receptor's ligand binding (G165E) and G protein coupling (V282M) domains (**Figures 3A, B**) (43, 44). To further validate

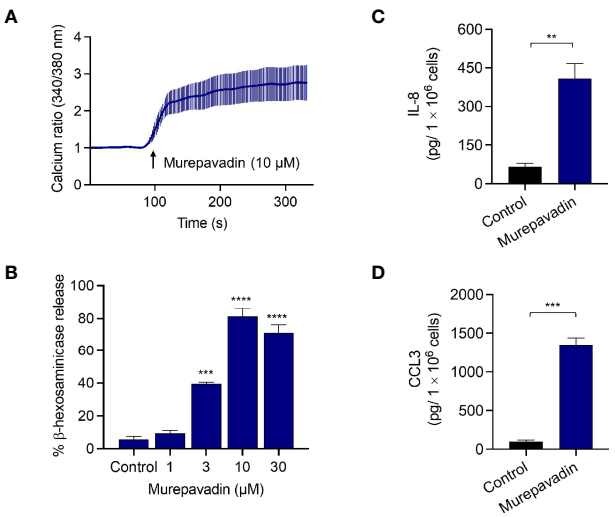


FIGURE 1 | Murepavadin induces intracellular Ca^{2+} mobilization, degranulation and causes chemokine production in human MCs. **(A)** Ca^{2+} mobilization measurement following murepavadin (10 µM) stimulation of Fura-2-loaded LAD2 cells. **(B)** LAD2 cells were exposed to indicated concentrations of murepavadin (30 min) and degranulation was assayed by measuring the release of β-hexosaminidase. **(C, D)** LAD2 cells were exposed to 10 µM of murepavadin for 24 h and the production of IL-8 and CCL3 were determined by ELISA. Data presented are the mean ± SEM of at least three experiments. Statistical significance was determined by *t*-test or one-way ANOVA with Dunnett's multiple comparisons at a value $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

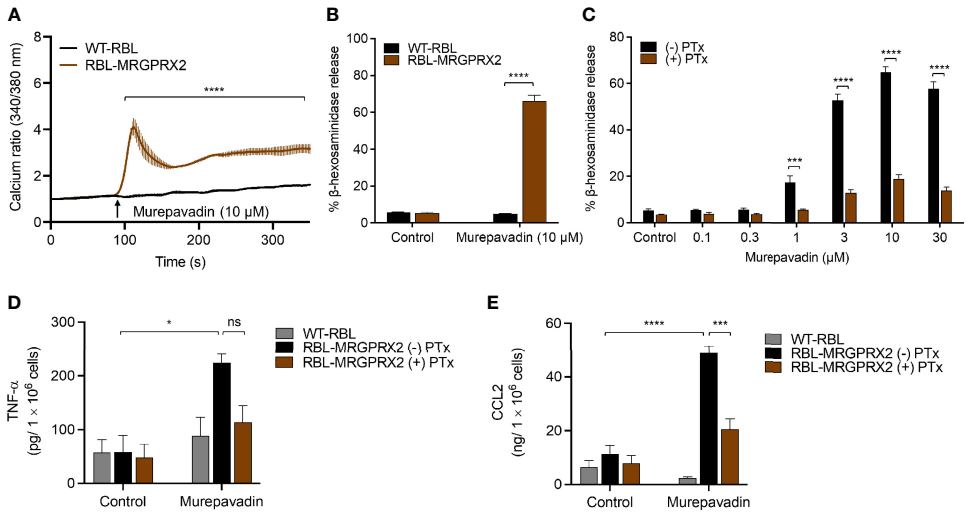


FIGURE 2 | Murepavadin induces intracellular Ca^{2+} mobilization, degranulation and chemokine production in MCs through MRGPRX2. **(A)** Calcium mobilization measurement following murepavadin (10 μM) stimulation of Fura-2 loaded WT-RBL and RBL-MRGPRX2 cells. **(B)** WT-RBL and RBL-MRGPRX2 cells were exposed to 10 μM murepavadin (30 min), and degranulation was assayed by measuring the release of β-hexosaminidase. **(C)** RBL-MRGPRX2 cells were incubated in the presence or absence of pertussis toxin (PTx), at a concentration of 100 ng/mL for 16 h and exposed to indicated concentrations of murepavadin (30 min) and degranulation was assayed by measuring the release of β-hexosaminidase. **(D, E)** WT-RBL and RBL-MRGPRX2 cells were incubated in the presence or absence of pertussis toxin (PTx) at a concentration of 100 ng/mL for 16 h and exposed to 10 μM of murepavadin (24 h) and the production of cytokine TNF-α and chemokine CCL2 were quantified by ELISA. Data presented are the mean ± SEM of at least three experiments. Statistical significance was determined by two-way ANOVA with Šídák's or Tukey's multiple comparisons at a value $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$, and ns denotes "not significant".

murepavadin's specificity for MRGPRX2, we transiently transfected RBL-2H3 cells with cDNAs encoding each of these variants. Flow cytometry analyses confirmed cell surface expression of MRGPRX2 (WT) and its missense variants (G165E and V282M) (**Figure 3C**). While murepavadin (10 μM) induced β-hexosaminidase release in cells expressing WT-MRGPRX2, this response was significantly inhibited in cells transiently expressing the G165E or V282M variant (**Figure 3D**). These findings substantiate the notion that murepavadin utilizes MRGPRX2 to activate MCs.

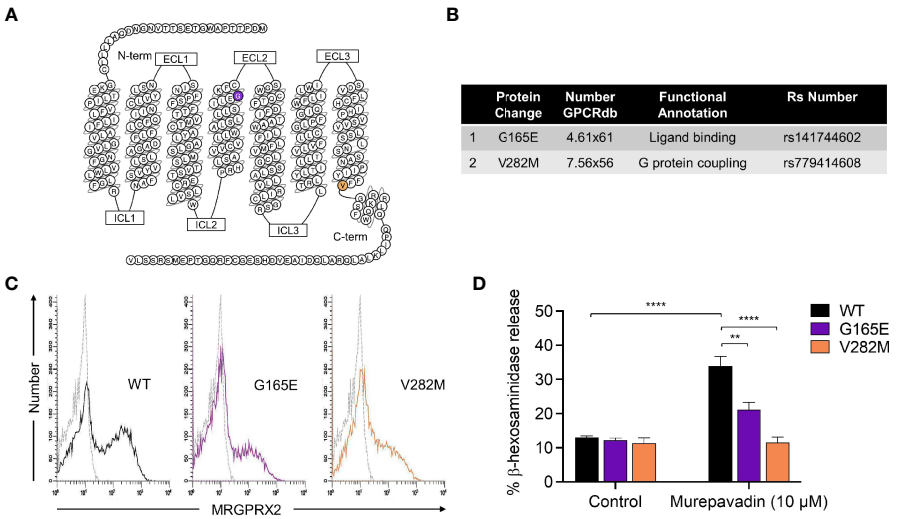
Murepavadin Does Not Promote β-Arrestin Recruitment Following MRGPRX2 Activation

To determine if murepavadin promotes β-arrestin recruitment in addition to G proteins, we utilized HTLA (HEK-293T cells that were transfected with a tTA-dependent luciferase reporter and a β-arrestin2-TEV protease fusion gene) cells stably expressing MRGPRX2 (HTLA-MRGPRX2). We have previously shown that C48/80 strongly promotes β-arrestin recruitment in HTLA-MRGPRX2 cells as determined by a transcriptional activation following arrestin translocation (Tango) assay (37). We therefore utilized C48/80 as a positive control and tested the ability of murepavadin to promote β-arrestin recruitment. As shown in **Figures 4A, B**, while C48/80 (3 μg/mL) induced ~30-fold increase in β-arrestin-mediated gene expression when compared to buffer control, murepavadin was without effect.

Murepavadin Does Not Induce MRGPRX2 Internalization or Desensitization

We have previously shown that C48/80 not only induces β-arrestin recruitment but also causes MRGPRX2 internalization (37). We found that incubation of HTLA-MRGPRX2 cells with C48/80 (3 μg/mL; 0.5 h -16 h) induced substantial receptor internalization but murepavadin had no effect (**Figures 4C, D**). The data presented in **Figures 4E, F** show representative histograms of cell surface MRGPRX2 following incubation of HTLA-MRGPRX2 cells with C48/80 (3 μg/mL) and murepavadin (10 μM) for 16 h. These data clearly demonstrate that while C48/80 promotes β-arrestin recruitment and caused MRGPRX2 internalization, murepavadin does not induce these responses.

To confirm the biological relevance of the findings described above, we performed receptor internalization studies in RBL-MRGPRX2 cells by flow cytometry. Similar to the situation in HTLA-MRGPRX2 cells, C48/80 induced substantial receptor internalization in RBL-MRGPRX2 cells but murepavadin had no effect (**Figures 5A, B**). As for β-arrestin recruitment (**Figure 4A**) and receptor internalization (**Figures 5A, B**), incubation of cells with C48/80 (3 μg/mL, 16 h) resulted in substantial inhibition of Ca^{2+} mobilization response to the stimulation by the same agonist (**Figure 5C**). Conversely, cells preincubated with murepavadin (10 μM, 16 h) had little to no effect on Ca^{2+} response to stimulation by the same agonist (**Figure 5D**).



	Protein Change	Number GPCRdb	Functional Annotation	Rs Number
1	G165E	4.61x61	Ligand binding	rs141744602
2	V282M	7.56x56	G protein coupling	rs779414608

FIGURE 3 | Naturally occurring MRGPRX2 missense variants are hypo-responsive to murepavadin for MC degranulation. **(A)** Snake diagram of MRGPRX2 indicating the two amino acid residues to be investigated. **(B)** Single amino acid substitution for each of the MRGPRX2 variant is shown in the table. **(C)** Cell surface receptor expression of the wild-type MRGPRX2 (WT) and its missense variants (G165E and V282M) was confirmed using flow cytometry. **(D)** RBL cells expressing MRGPRX2 (WT) and its variants (G165E and V282M) were exposed to 10 μM murepavadin (30 min) and degranulation was assayed by measuring the release of β-hexosaminidase. Statistical significance was determined by two-way ANOVA with Tukey's multiple comparisons at a value $^{**}p < 0.01$ and $^{****}p < 0.0001$.

Murepavadin Activates Murine MCs *In Vitro* and *In Vivo via* MrgprB2

It has been previously shown that C48/80 causes substantial degranulation in mouse peritoneal MCs (PMCs) *via* MrgprB2 (21). To test if murepavadin induces degranulation in murine PMCs and to determine the role of MrgprB2 in this response, we cultured PMCs from peritoneal lavage of wild-type (WT) and MrgprB2$^{-/-}$ mice (38, 42). We found that murepavadin induced degranulation in PMCs cultured from WT mice but this response was not observed in cells cultured from MrgprB2$^{-/-}$ mice

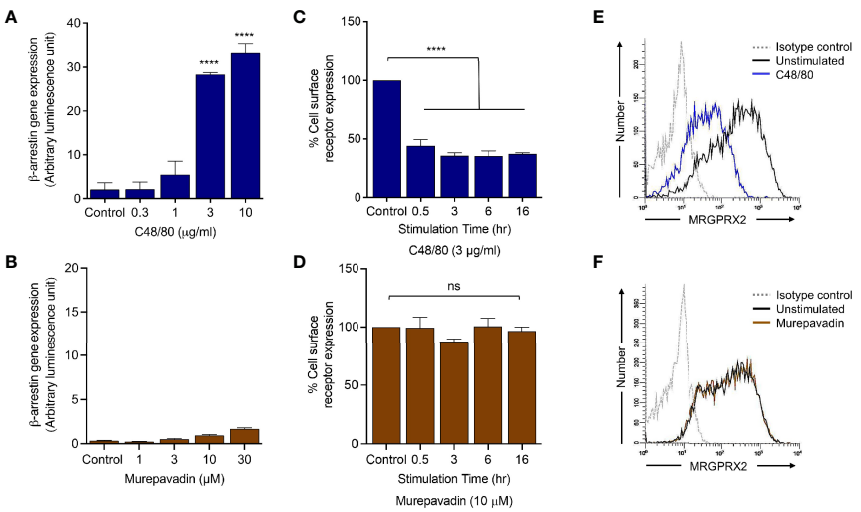


FIGURE 4 | Murepavadin does not promote β-arrestin recruitment following MRGPRX2 activation. **(A, B)** HTLA-MRGPRX2 cells were exposed to indicated concentrations of C48/80 or murepavadin for 16 h. Medium was removed and Bright-Glo solution (100 μL) was added into each well (96-well plate) and β-arrestin gene expression (in relative luminescence unit) was measured. **(C, D)** HTLA-MRGPRX2 cells were exposed to MRGPRX2 ligand (C48/80 or murepavadin, 16 h) and cell surface receptor expression of MRGPRX2 was confirmed using flow cytometry and quantified using a mean fluorescent intensity (MFI) in comparison to the untreated control. **(E, F)** Representative histograms of MRGPRX2 cell surface receptor expression of HTLA-MRGPRX2 cells. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparisons at a value $^{****}p < 0.0001$ and ns denotes "not significant".

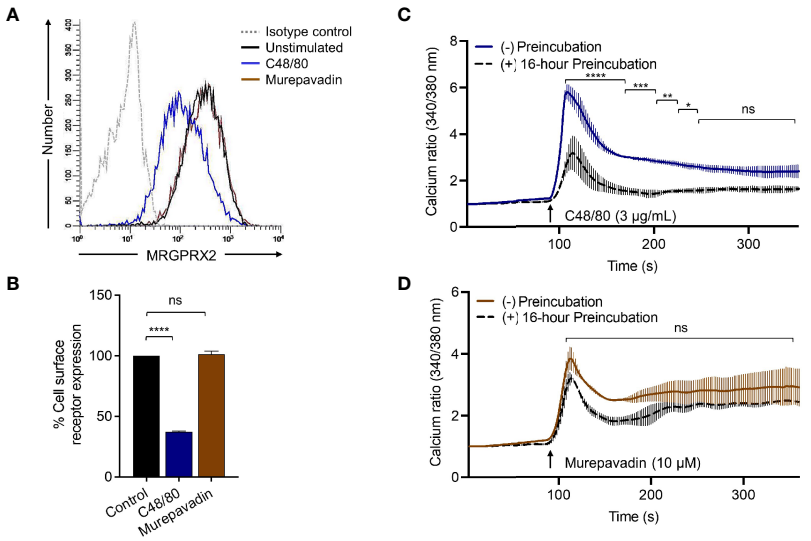


FIGURE 5 | Murepavadin does not induce MRGPRX2 internalization or desensitization. **(A)** RBL-MRGPRX2 cells were exposed to MRGPRX2 ligand (C48/80 3 μg/mL or murepavadin 10 μM, 16 h) and cell surface receptor expression of MRGPRX2 was confirmed using flow cytometry. A representative histogram of MRGPRX2 cell surface receptor expression is shown. **(B)** Quantitative analysis of cell surface receptor expression was calculated using a mean fluorescent intensity (MFI) in comparison to the untreated control. **(C, D)** Calcium mobilization measurements of RBL-MRGPRX2 cells exposed to MRGPRX2 ligand (C48/80 3 μg/mL or murepavadin 10 μM, 16 h) following C48/80 (3 μg/mL) or murepavadin (10 μM) stimulation, respectively. Statistical significance was determined by one-way ANOVA with Dunnett's multiple comparisons and two-way ANOVA with Šídák's multiple comparisons at a value $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, and ns denotes "not significant".

(**Figure 6A**). To determine if murepavadin induces degranulation of cutaneous MC *in vivo*, we performed intradermal injection of murepavadin (30 μM, 20 μL) or PBS into the paw after intravenous injection of Evan's blue dye. Consistent with peritoneal MC degranulation *in vitro*, murepavadin caused a significant increase in vascular permeability when compared to PBS. However, this vascular permeability response was abolished in MrgprB2$^{-/-}$ mice (**Figures 6B, C**). Together, these data demonstrate that murepavadin induces degranulation in murine MCs to cause increased vascular permeability *via* the activation of MrgprB2.

DISCUSSION

Murepavadin is a synthetic cyclic β-hairpin HDP mimetic that targets an outer membrane protein transporter LptD of the Gram-

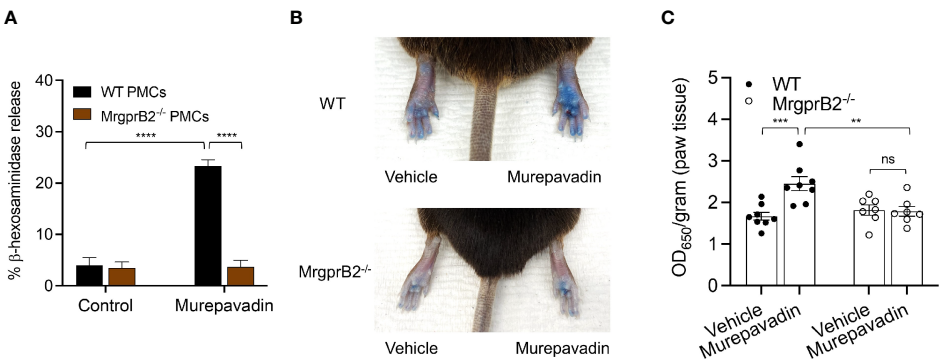


FIGURE 6 | Murepavadin activates murine MCs *in vitro* and *in vivo via* MrgprB2. **(A)** WT and MrgprB2$^{-/-}$ PMCs were exposed to 10 μM murepavadin (30 min) and degranulation was assayed by measuring the release of β-hexosaminidase. **(B)** Representative images of Evan's blue dye extravasation in WT and MrgprB2$^{-/-}$ mice *in vivo*. Mice were intradermally injected with 20 μL of murepavadin (30 μM) or PBS, and extravasation of Evan's blue dye was determined after 30 min. **(C)** Quantification of extravasation of Evan's blue dye in WT and MrgprB2$^{-/-}$ mice (n = 7-8). Statistical significance was determined by two-way ANOVA with Tukey's multiple comparisons at a value $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, and ns denotes "not significant".

negative bacterium *P. aeruginosa*, which makes it highly specific to this pathogen (45, 46). In *in vitro* studies, murepavadin is very effective against a wide-ranging species of multi-drug resistant *Pseudomonas* bacteria and demonstrates exceptional efficacy in sepsis, lung, and thigh infection models *in vivo* (47). However, intravenous administration of murepavadin for treating nosocomial pneumonia has been temporarily halted due to reports of kidney injury. Despite this, an inhaled formulation of murepavadin is under investigation for its potential effectiveness in treating *Pseudomonas* infection in patients with cystic fibrosis (33, 48). We made the novel observation that murepavadin is able to induce human MC activation through MRGPRX2 and murine MC activation through MrgprB2. Because human skin MCs express MRGPRX2 at high levels (17, 19, 49), these findings suggest that murepavadin can be utilized for treating *P. aeruginosa* skin infection through harnessing MC's host defense and wound healing properties.

P. aeruginosa skin infection is associated with high morbidity and mortality rates mainly because of its ability to form biofilms and to resist multiple antibiotics (25, 26). Therefore, novel treatment approach together with rapid wound closure are critical to control this type of infection. Weller et al. (50), showed that skin wounding in mice results in MC degranulation. This, in turn, causes increased vascular permeability and neutrophil recruitment. Using the same wound model but superimposed with *P. aeruginosa*, Zimmerman et al. (27), showed that MCs are essential in controlling bacterial infection and promoting healing. It was proposed that IL-1 and IL-33 generated by keratinocytes in response to infection activate MCs to produce IL-6, which in turn generate HDPs from keratinocytes resulting in the direct bacterial killing. However, recent studies demonstrated that HDPs can also induce MC degranulation in human (*via* MRGPRX2) and mouse (*via* MrgprB2) (22–24). Based on these findings, we suspect that the activation of MCs *via* MRGPRX2/B2 confer protective immunity and contribute to host defense. The minimum concentration of murepavadin required to inhibit 90% growth of *P. aeruginosa* (MIC_{90}) was reported to be in the range of 2 - 32 mg/L (33, 48). We found that murepavadin (1 μM; 1.667 mg/L) induced significant MC degranulation *via* MRGPRX2 and that maximal response was obtained at a concentration of 10 μM (16.67 mg/L). Murepavadin at 10 μM, which is relevant for direct inhibition of *P. aeruginosa* growth, induced TNF-α, IL-8 and CCL3 production from MCs *via* MRGPRX2. A topical application of murepavadin is currently under investigation for treating *P. aeruginosa* cutaneous infection mainly because of its direct and specific activity against the pathogen (34, 35). Based on the data presented herein, we propose that potential effectiveness of murepavadin also reflects its ability to harness MC's immunomodulatory property *via* the activation of MRGPRX2 (**Figure 7**).

It is well documented that *Staphylococcus aureus* is responsible for the majority of bacterial skin infections (51, 52). In a murine model of *S. aureus* skin infection, topical application of mastoparan, an amphipathic peptide found in *Hymenoptera* venom that causes MC activation *via* MrgprB2, results in the recruitment of immune cells particularly neutrophils, This contributes to subsequent bacterial clearance (15). However, a mastoparan derivative that does not induce MC degranulation is unable to control *S. aureus* infection despite the fact that it demonstrates direct antibacterial activity. By contrast, another mastoparan derivative that induces MC degranulation *via* MrgprB2 but has no direct antibacterial activity effectively controls infection. These findings suggest that the potential therapeutic effectiveness of mastoparan could reflect its action on MCs *via* MRGPRX2 rather than its direct antibacterial activity.

Apart from degranulation, mastoparan also triggers the generation of chemokines CCL2, CCL3 and CCL4 from MCs, which promote the mobilization of dendritic cells (DCs) including $CD301b^+$ dermal dendritic cells (DDCs). DDCs have been implicated in promoting re-epithelialization of sterile wounds and accelerating wound closure. Depletion of MrgprB2-expressing MCs results in significantly decreased number of DDCs, suggesting that MCs contribute to the restoration of DDCs population to homeostatic levels, thus contributing to regenerative healing (15). Moreover, mastoparan also contributes to the production of *S. aureus*-specific IgG following initial *S. aureus* skin infection, which is associated with reduced lesion size during subsequent bacterial reinfection (15). Thus, it is highly likely that activation of murine MCs by mastoparan through MrgprB2 confers both innate and adaptive immunity to provide protection against bacterial infection and reinfection. Given that murepavadin activates human MCs *via* MRGPRX2, it should be possible to use this HDP mimetic for the modulation of *S. aureus* skin infection. Because murepavadin appears to have a lower EC_{50} value for MC degranulation than mastoparan (15), our prediction is that lower concentration of the drug will be required to treat *S. aureus* infection. Also, since murepavadin is an HDP mimetic and is less susceptible to degradation than mastoparan, its effectiveness is likely to be greater than that of mastoparan, thus requiring fewer treatments to clear infection and to promote healing.

It is generally accepted that most GPCRs activate two parallel but independent signaling pathways; one involving G proteins while second pathway is independent of G proteins but requires the recruitment of β-arrestins (53–56). For certain GPCRs, agonists can activate only G proteins (G protein biased) or only β-arrestin (β-arrestin biased) or both (balanced). Agonists that induce both pathways not only activate G protein-mediated signaling but also result in dissociation of G proteins from the receptor (desensitization) and promote the receptor internalization. We have previously shown that while C48/80 acts as balanced agonist for MRGPRX2, an HDP angiogenic peptide, AG-30/5C acts as a G protein-biased agonist for the receptor. Thus, C48/80 caused substantial β-arrestin recruitment while AG-30/5C had little to no effect. Most importantly, preincubation of cells with C48/80 resulted in a significant reduction of cell surface receptor expression and loss of cell responsiveness to all MRGPRX2 agonists tested. By contrast, AG-30/5C had little to no effect on cell surface receptor expression or MC degranulation to any of the MRGPRX2 agonists tested (37). The data presented herein demonstrate that similar to AG-30/5C, murepavadin acts as a G protein-biased agonist for MRGPRX2. Thus, it is possible that the resistance MRGPRX2 to undergo desensitization and internalization by murepavadin could enhance its therapeutic potential.

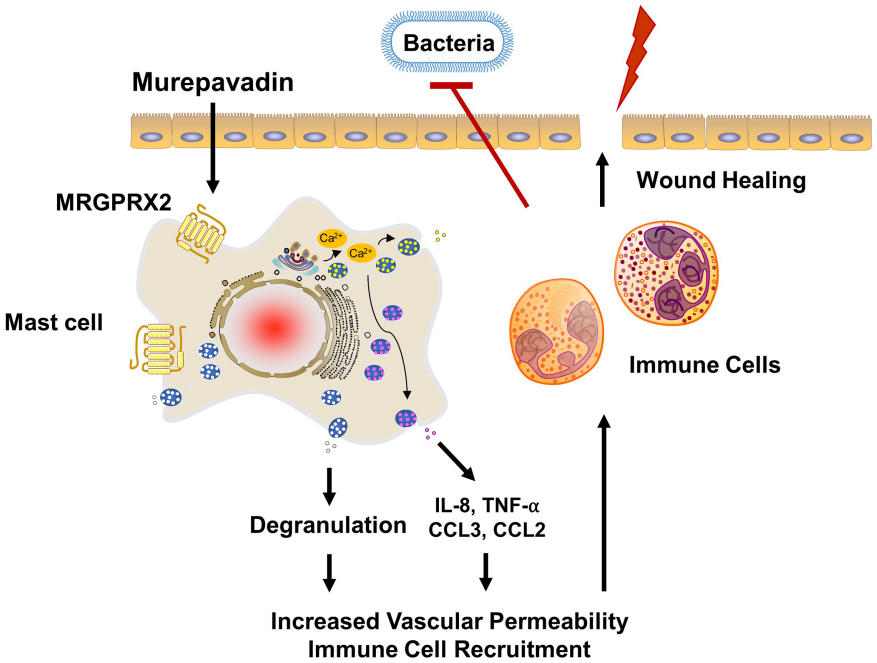


FIGURE 7 | Proposed mechanism of action of murepavadin/MRGPRX2-induced bacterial clearance and wound healing. Murepavadin activates MCs *via* MRGPRX2. An increase in intracellular calcium following murepavadin stimulation triggers MC degranulation and chemokine production (IL-8, CCL3, CCL2, and TNF-α). These MC mediators induce immune cell recruitment and subsequently contribute to host defense and wound healing.

In summary, although HDPs have therapeutic potential for the treatment of multi-drug-resistant bacterial infections, their relative instability and cytotoxicity have limited their usefulness as prospective antimicrobial agents. Small molecule HDP mimetics do not display these limitations and murepavadin was synthesized to specifically target *P. aeruginosa*. However, the possibility that murepavadin could activate MCs has not been suspected. In the present study, we have shown that murepavadin induces degranulation, TNF-α, IL-8 and CCL3 production *via* MRGPRX2, a receptor that is primarily expressed in human skin MCs. Thus, the ability of murepavadin to exploit MC's immunomodulatory functions could form the basis for the treatment of skin infection caused by bacterial species such as *P. aeruginosa* and *S. aureus*. Furthermore, the findings presented herein that MCs expressing missense MRGPRX2 variants G165E (rs141744602) and V282M (rs779414608) were resistant to murepavadin-induced degranulation likely has important clinical implications. It is possible that murepavadin may not effectively clear microbial infection in individuals harboring these MRGPRX2 polymorphisms because of their inability to support MC degranulation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee of the University of Pennsylvania.

AUTHOR CONTRIBUTIONS

HA contributed to conception, supervision and funding acquisition of the study. AA and CC performed the experiments and analyzed the data. AA and HA wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Molecular Mechanisms of Mast Cell Activation by Cholesterol-Dependent Cytolysins

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Mast cells are potent immune sensors of the tissue microenvironment. Within seconds of activation, they release various preformed biologically active products and initiate the process of *de novo* synthesis of cytokines, chemokines, and other inflammatory mediators. This process is regulated at multiple levels. Besides the extensively studied IgE and IgG receptors, toll-like receptors, MRGPR, and other protein receptor signaling pathways, there is a critical activation pathway based on cholesterol-dependent, pore-forming cytolytic exotoxins produced by Gram-positive bacterial pathogens. This pathway is initiated by binding the exotoxins to the cholesterol-rich membrane, followed by their dimerization, multimerization, pre-pore formation, and pore formation. At low sublytic concentrations, the exotoxins induce mast cell activation, including degranulation, intracellular calcium concentration changes, and transcriptional activation, resulting in production of cytokines and other inflammatory mediators. Higher toxin concentrations lead to cell death. Similar activation events are observed when mast cells are exposed to sublytic concentrations of saponins or some other compounds interfering with the membrane integrity. We review the molecular mechanisms of mast cell activation by pore-forming bacterial exotoxins, and other compounds inducing cholesterol-dependent plasma membrane perturbations. We discuss the importance of these signaling pathways in innate and acquired immunity.

Keywords: mast cell, cholesterol-dependent cytolysins, pore-forming toxins, Ca^{2+} signaling, cytokine production, streptolysin O, pneumolysin, listeriolysin O

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INTRODUCTION

Mast cells are critical initial players in innate and acquired immunity and, in this way, ultimately influence the outcome of various diseases. They are dispersed throughout the body tissues, but most abundantly are found at host-environment interfaces such as the skin, respiratory tract, and oral/gastrointestinal mucosa, suggesting their role as sentinels of infection. Mast cells share some features with other immune effector cells, such as basophils, macrophages, and neutrophils. However, they differ by their unique interactions with blood vessels and capacity to rapidly, within seconds and minutes, release an extensive set of inflammatory mediators such as histamine, proteases, lipid mediators, cytokines, and chemokines. Like other cell types of the immune system, mast cells communicate with their environment predominantly *via* surface receptors recognizing various

soluble or membrane-bound ligands. Depending on mast cell location and the overall context, mast cell activation leads to activation of multiple immune effector mechanisms, cell differentiation, chemotaxis, or inhibition of ongoing immune reactions. Various mast cell immune functions have been summarized in several reviews (1–5).

Mast cells can be activated by engagement of various plasma membrane receptors. The most studied receptor on the surface of mast cells is the high-affinity IgE receptor (FcεRI) associated with the mast cells' role in pathological conditions such as allergy, anaphylaxis, and asthma. Besides FcεRI, many other surface receptors recognize a variety of soluble or membrane-bound ligands that tightly upregulate or downregulate mast cell responsiveness. Extensively studied are pattern recognition receptors such as Toll-like receptors (TLRs), which are activated in response to conserved pathogen-associated molecular patterns (6, 7), and the Mas-related G protein-coupled receptor (Mrgpr) family, especially Mrgprb2 in the mouse and its human ortholog MRGPRX2 (8–10). Many other receptors present on the mast cell surface have been covered in numerous reviews (2, 4, 5, 11). Some of the plasma membrane receptors allow the innate system to identify invading bacteria and even viruses by their expression of pathogen-associated molecular patterns and allow mast cells to directly respond to bacteria by degranulation and production of *de novo* synthesized proinflammatory and anti-inflammatory products (3, 12–17). Thus, there is an intricate network of surface receptors and regulatory proteins that can either induce mast cell activation or inhibit mast cell signaling.

There is a widespread notion that mast cells are part of the antimicrobial host defense. This is based on experiments showing that mast cell-deficient mice are more sensitive to bacterial infection than WT mice (13, 18–20). In experiments with *Listeria monocytogenes*, mast cell-deficient mice showed approximately hundred-fold higher bacterial burden and significantly impaired neutrophil mobilization when compared to control mice. Although bacteria bound to mast cells triggered degranulation, their phagocytosis was negligible. Thus, mast cells control bacterial infection not through bacterial uptake, but by activation and rapid degranulation associated with the release of pre-synthesized pro-inflammatory mediators, cytokines and chemokines, which cause influx of other immune cells, mainly neutrophils, to the site of infection. Once recruited, neutrophils not only phagocytose and destroy bacteria, but also become activated and secrete inflammatory mediators, hence amplifying the anti-bacterial inflammatory response (21). Other studies showed that animals lacking mast cells or mast cell signaling molecules respond differentially to bacterial infection when compared with wild type controls. Interestingly, in response to bacteria, mast cells, in contrast to other cell types such as macrophages, elicit only a proinflammatory response but not the type I interferon (IFN-I) response. It has been found that this phenomenon could be attributed to the spatial regulation of proinflammatory and IFN-I responses from different subcellular sites; proinflammatory responses occur from the cell surface, whereas IFN-I responses are induced from endolysosomal compartments (22). This review focuses on the molecular mechanisms of mast cell activation by cholesterol-dependent cytolysins (CDCs).

CDCS

CDCs are a class of pore-forming proteins produced by a wide range of predominantly Gram-positive bacteria. They form the most prominent toxin family, comprising at least 28 bacterial species, that mediate bacterial virulence. The most frequently studied CDCs are those produced by pathogenic *Streptococci*, *Listeria*, and *Clostridia*. The summary of cytolysins discussed in this review, their bacterial producers, and a subset of diseases they cause are presented in **Table 1**. Various strategies have been developed to eliminate the pathogens producing CDCs. One effective way is sensing CDCs produced by the pathogens by mast cells followed by their activation and mobilizing innate and adaptive immunity mechanisms. In terms of their effects on mast cells, extensively studied CDCs were those produced by

TABLE 1 | Summary of cytolysins discussed in this review, their bacterial producers, and a subset of associated diseases.

Toxin	Abbreviation	Bacteria	Diseases
Streptolysin O	SLO	*Streptococcus pyogenes*	Various infectious diseases as pharyngitides, rheumatic fever, scarlet fever, necrotizing soft tissue infection, toxic shock syndrome (23, 24)
Pneumolysin	PLY	*Streptococcus pneumoniae*	Bacterial pneumonia, otitis media, bacterial meningitis (25, 26)
Listeriolysin O	LLO	*Listeria monocytogenes*	Listeriosis (manifestations include abortion, sepsis, meningoencephalitis, febrile gastroenteritis syndrome) (27, 28)
Streptolysin S*	SLS	*Streptococcus equi*	Disease of the upper respiratory tract and associated lymph nodes in equids (29, 30)
Perfringolysin	PFO	*Clostridium perfringens*	Histotoxic infections, pathogenesis of gas gangrene (31, 32)
Vaginolysin	VLY	*Gardnerella vaginalis*	Bacterial vaginosis (33)
Lectinolysin	LLY	*Streptococcus mitis* *Streptococcus pseudopneumoniae*	Infective endocarditis, bacteremia and septicemia (34)
Suilysin	SLY	*Streptococcus suis*	Meningitis (35)
Intermedilysin	ILY	*Streptococcus intermedius*	Associated with brain and liver abscesses (36)

**SLS was also identified in S. pyogenes and most of other group A Streptococcus isolates (37). However, the hemolytic activity of SLS is not affected by cholesterol (29).*

pathogenic *Streptococci* and *Listeria* (see below). Numerous studies have shown that the CDCs' pore-forming ability requires the presence of cholesterol in the plasma membrane of host cells (38–47).

General Mechanism of Plasma Membrane Pore Formation by CDCs

Formation of the plasma membrane pores by CDCs relies on the self-assembly of monomers bound individually to cholesterol-rich membranes, followed by dimerization and oligomerization. At the protein level, CDCs are conserved across multiple organisms (46, 48). Structurally, various CDCs are organized into four functional domains. **Figure 1** shows crystal structures of three CDCs [streptolysin O (SLO) from *Streptococcus pyogenes*, pneumolysin (PLY) from *S. pneumoniae*, and listeriolysin O (LLO) from *Listeria monocytogenes*] used in mast cell research. Domains 1 and 2 retain the contact with the aqueous environment during the pore formation. Domain 3 is composed of two transmembrane helices that convert to β-strands involved in penetrating the host membrane. Domain 4 is involved in cholesterol sensing and membrane binding. This domain, which shows the highest conservation across CDCs, consists of a β-sandwich linked by structural loops and a tryptophan-rich undecapeptide (TR-UDP). A previous study has shown that the threonine-leucine pair of sequential amino acids binds to the hydroxyl group of cholesterol in cholesterol-rich membrane regions (54), but not free cholesterol (55). Thus, the threonine-leucine pair of CDC recognizes specific features of cholesterol at the plasma membrane to initiate the cholesterol-dependent interaction of the CDC with the cell. Although different CDCs have an identical cholesterol-binding motif, they exhibit different binding parameters depending on the lipid (56) and glycan (57, 58) environment. These interactions anchor the CDC monomer in a perpendicular orientation to the membrane surface. The domain 4-lipid interaction triggers conformational changes in spatially distant domain 3, which exposes a previously hidden interface involved in oligomerization and, hence, formation a pre-pore complex. Then, two sets of short α-helices in domain 3 undergo an α-helix to β-sheet transition, leading to creation of two pore-forming transmembrane β-hairpins (TMHs) per monomer, which are still above the membrane surface. Through this process, plasma membrane-bound monomers oligomerize into pre-pores consisting of 35 - 50 monomers sitting on the cell surface (**Figure 2**). Next, the pre-pore components undergo further restructuring, including shape changes, bringing domain 3 and its TMH regions to the membrane proximity. This leads to refolding the transmembrane helices into β-strands and forming a β-barrel pore in the plasma membrane with a diameter of about 30 nm (43, 45, 46, 48, 59–62).

Recent data (57, 58) showed that of eight CDCs studied [SLO, PLY, LLO, perfringolysin (PFO) from *Clostridium perfringens*; vaginolysin (VLY) from *Gardnerella vaginalis*; lectinolysin (LLY) from *Streptococcus mitis* or *S. pseudopneumoniae*; suilysin (SLY) from *S. suis*, and intermedilysin (ILY) from *S. intermedius*], all had high-affinity lectin activity that identified glycans as candidate cellular receptors. Some of the CDCs, including SLO, VLY, and PFO, bound multiple glycans, while PLY, LLY, LLO recognized a single glycan class. All of the glycans functioning as CDC receptors are found as glycolipids, transmembrane glycoproteins, or GPI-qanchored (CD-59) glycoproteins that are frequently associated with the periphery of cholesterol-

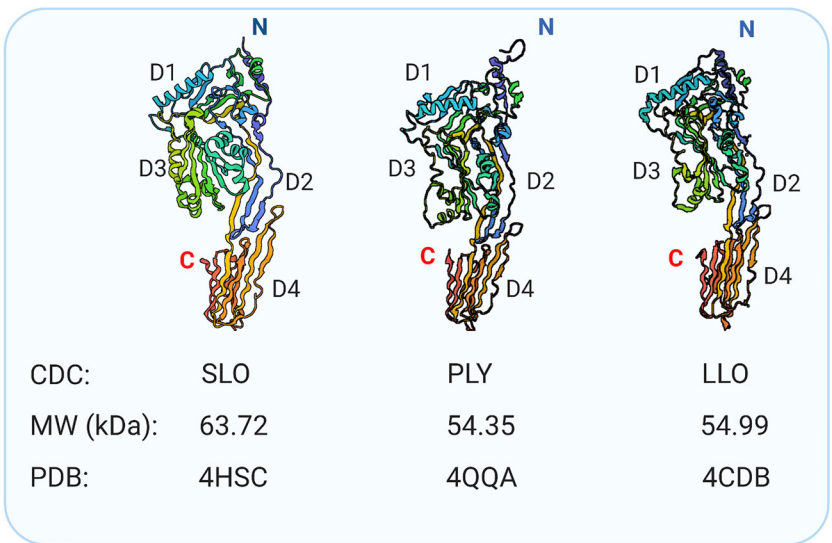

FIGURE 1 | Bacterial pore-forming cholesterol-dependent cytolysins used in mast cell research. Crystal structure of SLO (42), PLY (49), and listeriolysin (LLO) (50). Indicated are N-terminus, C-terminus, and four domains rich in β-sheets: Domain 1 (D1), Domain 2 (D2), Domain 3 (D3) with the transmembrane spanning region, and Domain 4 (D4) involved in the initial direct interaction with cholesterol and glycans (51–53). The molecular weights and protein data bank (PDB) codes are also indicated.

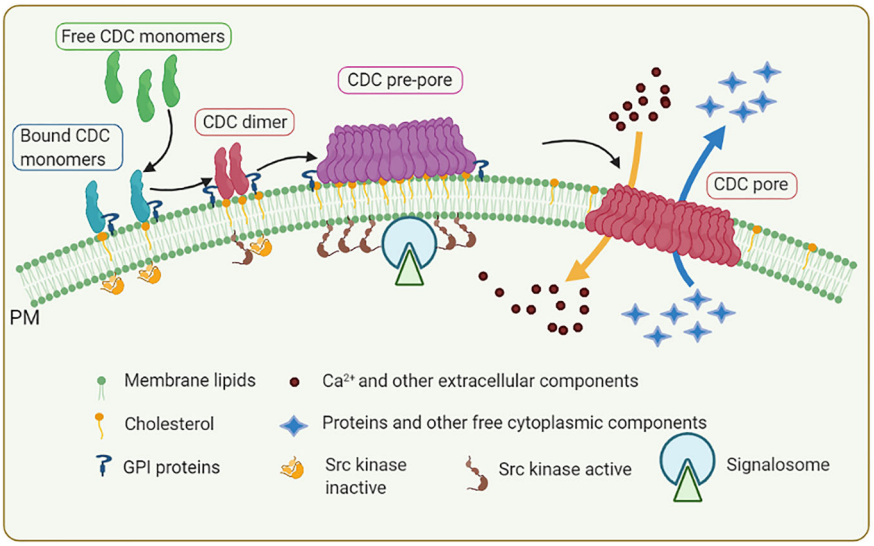

FIGURE 2 | Formation of membrane pores by CDCs. CDC monomers released from bacteria bind through their D4 domain to plasma membrane (PM) microdomains called lipid rafts enriched in cholesterol, GPI-anchored proteins, and Src family kinases. PM-bound monomers form dimers that polymerize into pre-pore structures containing 30 to 50 CDC monomers. In this process, CDCs induce aggregation of lipid raft components leading to formation of signaling assemblies called signalosome. The signalosomes are capable to initiate cell activation events. Individual CDCs in the pre-pore structure undergo conformational rearrangement and formation of membrane-spanning β-strands, leading to concerted membrane insertion and formation of the pore with approximately 25 nm in diameter. Formation of the transmembrane pore results in influx of Ca^{2+} into the cytoplasm and efflux of K^+, other small molecules (e.g., ATP), and proteins through the plasma membrane. These processes trigger various cell responses involved in repairing the plasma membrane and activating innate and acquired immunity.

enriched lipid rafts (63). Further investigation showed no competition between cholesterol and glycan receptor binding, indicating that cholesterol and glycans bind to CDCs independently. Significantly, addition of an exogenous carbohydrate receptor for each CDC inhibited the toxin activity. Thus, binding to both the glycan receptor and cholesterol-rich membrane seems to be essential to the toxic effect of the CDCs. The combined data indicated that glycoprotein and/or glycolipid receptors present on the cellular membrane contribute to the CDCs' cell and tissue tropism.

Cell Response to CDC in General

The cell response to CDC depends on the cell type examined, type of CDC, and its concentration. Sensitivity to the toxic effect of CDC reflects, in part, the cell ability to repair membrane disruptions. This probably explains why erythrocytes are more sensitive to SLO than nucleated mammalian cell lines (64, 65). When used at sublytic concentrations, as is often the case *in vivo*, CDCs trigger several cellular processes, including membrane repair and resealing of the membrane pores by various mechanisms. The cell ability to reseal a limited number of pores generated by CDCs is generally dependent on Ca^{2+} levels (66). Changes in the concentration of free cytoplasmic Ca^{2+} could lead to activation of proinflammatory transcriptional regulators, including nuclear factor (NF)-κB, c-Jun N-terminal kinase (JNK), and NFAT (**Figures 3** and **4**). Various transcriptional regulators could be activated depending on the calcium signal amplitude and duration (67). Initial studies suggested that the CDCs' Ca^{2+} signaling is mainly due to Ca^{2+} influx from the extracellular milieu (68, 69). However, later studies showed that CDCs could also induce Ca^{2+} release from intracellular stores *via* at least two independent mechanisms. The first one induces activation of intracellular Ca^{2+} channels and involves phospholipase (PLC)-inositol triphosphate receptor (IP3R)-operated Ca^{2+} channels activated *via* G-proteins and protein tyrosine kinases. The second one is Ca^{2+} channel independent, involving injury of intracellular Ca^{2+} stores such as endoplasmic reticulum (ER) (70).

Membrane repair after exposure to sublytic concentrations of CDCs is usually executed through microvesicular shedding and endocytosis; this could lead to removing the toxin from the membrane (71). Using SLO mutants with engineered defects in pore formation or oligomerization, the authors found that oligomerization, in the absence of pore formation, was necessary and sufficient for membrane shedding, suggesting that the calcium influx and patch formation were not required for shedding (71). However, the authors did not exclude the possibility that oligomerization and pre-pore formation induced changes in the plasma membrane leading to activation of calcium uptake and other signaling events.

CDC-mediated formation of pores in the plasma membrane leads to many other cell signaling events, which involve apoptosis (72), DNA damage and cell cycle arrest (73), unfolded protein response (74), induction of ubiquitination (75), and transcriptional activation (76, 77).

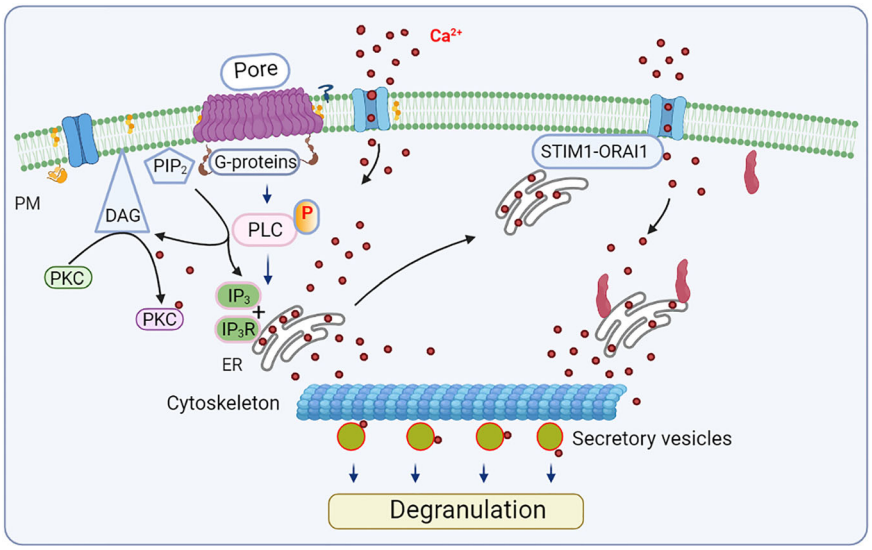

FIGURE 3 | Mast cell activation by CDCs - calcium response and degranulation. Exposure of mast cells to sublytic concentrations of CDCs leads to aggregation of lipid rafts and transmembrane insertion of CDC complexes, resulting in phosphorylation of signal transduction molecules, including phospholipase C (PLC). PLC hydrolyses PM-localized phosphatidylinositol 4,5-bisphosphate (PIP2), producing inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 binds to IP3 receptor (IP3R) on the endoplasmic reticulum (ER), where it stimulates the release of calcium into the cytoplasm. Free cytoplasmic calcium together with DAG activates protein kinase C (PKC). The reduced Ca^{2+} level in ER is sensed by stromal interaction molecule 1 (STIM 1), which then binds to and activates the store-operated ORAI1 calcium ion channel in the PM. Calcium could also be released due to the injury of intracellular Ca^{2+} stores by CDCs. Increased levels of free cytoplasmic Ca^{2+} and other activation events lead to the release of secretory vesicles (degranulation) in which the cytoskeleton plays an important role.

It should also be mentioned that some CDCs can induce cell activation in the absence of pore formation. The toxins bind to plasma membrane microdomains enriched in cholesterol, gangliosides, GPI-anchored proteins, and Src family kinase called lipid rafts, which are involved in signal transduction (78–80). Using LLO and the cholesterol-inactivated form of LLO (CL-LLO), Gekara et al. demonstrated that both forms of the toxin bind to and induce clustering of lipid rafts (81). Consistent with the role of lipid rafts in cell signaling, the authors found that CL-LLO-induced raft aggregation resulted in activation of tyrosine kinases in a pore-independent manner. The aggregation of rafts could have critical physiological consequences in listeriosis. Under *in vivo* conditions, secreted LLO is inactivated by cholesterol present in body fluids. Although cholesterol-inactivated LLO loses its pore-forming capacity, CL-LLO can activate target cells by aggregating lipid rafts and, in this way, influences the course of *Listeria monocytogenes* infection. In this process, the lectin activity of CDCs, discussed above, could play a key role. Interestingly, LLO was found to bind to carbohydrate structures present on gangliosides (58), which are found in lipid rafts (82).

CDC INTERACTIONS WITH MAST CELLS

Most of the studies focused on the molecular mechanisms of CDC interactions with mast cells were performed using rat, mouse, or human mast cell lines, bone marrow-derived mouse mast cells (BMMCs), or human mast cells isolated from the lungs or intestine. While mechanisms described in previous section are common to various cell types, including mast cells, several mast cell-specific responses are directed towards CDCs. This reflects the unique properties of mast cells, based on transcriptional profiles, dramatically different from other cell types of the immune system (83). This could be in part related to the findings that mast cells are evolutionarily ancient, dating back to at least as far as urochordates (84–86), and that mast cells have unique functions as first-line sentinels of the immune system for host defense against pathogens (87).

Molecular Mechanism of Mast Cell Activation With CDCs

The canonical way of mast cell signaling through FcεRI involves, as a first step, ligand (IgE-antigen complexes, lectin, anti-FcεRI antibody)-mediated aggregation of FcεRI. The aggregation of the FcεRI receptors leads to Src family protein tyrosine kinase (PTK) Lyn-mediated phosphorylation of immunoreceptor tyrosine-based activation motives (ITAMs) in the FcεRI β and γ subunits by incompletely understood mechanism (88). The phosphorylated γ subunits then serve as binding and activation sites for the Syk kinase, which phosphorylates many signaling molecules, including transmembrane adaptor protein LAT1 (linker for activation of T cells) and non T cell activation linker (NTAL or LAT2), reviewed in (89). Phosphorylated

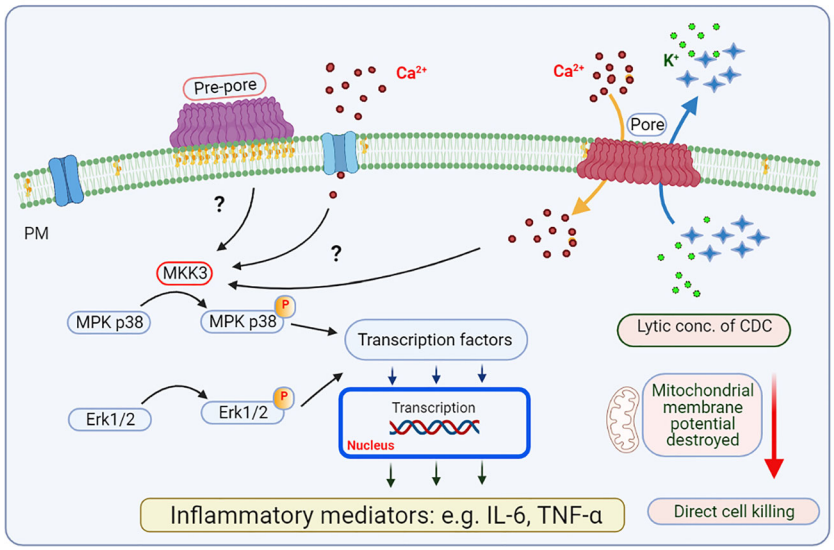

FIGURE 4 | Mast cell activation by CDCs - de novo production of inflammatory mediators. Among proteins phosphorylated and activated by CDC-induced changes is mitogen-activated protein kinase (MPK)3, which is involved in phosphorylation and activation of MPK p38 and Erk1/2. These enzymes are involved in activation of transcription factors regulating transcription of selected genes for inflammatory cytokines and chemokines (e.g., IL-6 and TNF-α). It is not clear whether CDCs at the pre-pore stage have any role in these signaling events. Higher concentrations of CDCs lead to killing of target cells in the absence of production of inflammatory mediators.

LAT1 recruits molecules containing Src homology (SH)2 domains, such as adaptor protein Grb-2 and PLCγ1. An important intermediate is phosphatidylinositol (PI)-3-OH kinase (PI3K), which catalyzes synthesis of PI-3,4-bisphosphate and PI-3,4,5-trisphosphate (PIP3). These phospholipids contribute to the recruitment of PLCγ1 and PLCγ2 and other molecules containing pleckstrin homology domains to the plasma membrane. PLC cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). IP3 diffuses through the cytosol to bind to IP3R, Ca^{2+} channels in the ER, and thus causes a rapid but transient release of Ca^{2+} from ER stores to the cytoplasm. Decreased levels of Ca^{2+} in ER are sensed by ER protein STIM1, which then oligomerizes and interacts with the plasma membrane (PM) protein Orai1 at ER–PM junctions [reviewed in (90, 91)]. STIM1-Orai1 assembly forms the active channel responsible for store-operated Ca^{2+} (SOC) channels, which activates the entry of external Ca^{2+} into the cytoplasm. Phospholipids and DAG are used as substrates by phospholipase A (PLA)2 and diacylglycerol lipase, respectively, leading to arachidonic acid production. Arachidonic acid is a precursor in production of eicosanoids such as prostaglandins and leukotrienes, which exert a complex control over many bodily systems, mainly in inflammation and immunity (92, 93). The maintenance and amplification of FcεRI-generated signals are regulated by the phosphoinositide 3 kinase (PI3K)/Bruton's tyrosine kinase (Btk) axis (94). This pathway contributes to the cytokine and chemokine production regulation through activation of transcription factors NFAT and NFκB (95). Production of cytokines, chemokines, and other proteins also requires activation of mitogen-activated protein (MAP) kinase pathways and enhanced transcription through the activation of various transcription factors, such as those that bind to promoter regions of the genes encoding the proteins mentioned above. There are three major MAP kinase pathways involving extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK), and p38 MAP kinase activated *via* the Ras-Raf pathway in the FcεRI-activated cells. A key role in this process is played by Ca^{2+} influx, as documented by the possibility to induce degranulation in mast cells by bypassing the early aggregated FcεRI-mediated events by thapsigargin or calcium ionophore A23187 (96, 97). Significantly, the PI3 kinase is involved in mast cell activation induced through both IgE-dependent and antigen-independent pathways (98, 99).

Based on these and data from other systems showing the involvement of calcium in the activation of transcription factors (67, 100), it has been proposed that CDCs induce mast cell activation by producing pores through which Ca^{2+} enters the cell (70). However, as described by Usmani et al. (101), the interaction of SLO with the cells could increase the concentration of free Ca^{2+} through IP3-mediated depletion of intracellular stores and activation of store-operated Ca^{2+} (SOC) entry dependent on the STIM1-Orai1 cross-talk. This mechanism of Ca^{2+} entry was independent of the toxin's ability to form Ca^{2+}-conducting pores, allowing the cell to respond to much lower toxin concentrations. Thus, SLO may induce IP3 release by a mechanism comparable to the one used by regular surface receptors. The molecular mechanisms of IP3 release by CDCs, however, remain enigmatic. Below, we will review data on three CDCs (SLO, PLY, and LLO) used in the

studies on the molecular mechanisms of mast cell activation. In several studies, bacteria producing CDCs and their non-producing forms have been used. Summary data on the effects of CDCs on degranulation, cytokine and chemokine production, tyrosine phosphorylation, and Ca^{2+} responses are presented in **Table 2**. **Table 3** provides summary data on the effect of CDCs on cytokines, chemokines, and other mediators produced in mouse and human mast cells.

Streptolysin O (SLO)

SLO is a multifunctional protein with pore-dependent and pore-independent functions (112, 113) produced by *Streptococcus pyogenes*. This pathogen is responsible for various infectious diseases, including pharyngitides, rheumatic fever, scarlet fever, and life-threatening conditions such as necrotizing soft tissue infection (necrotizing fasciitis) and streptococcal toxic shock syndrome (23, 24). In initial studies, peritoneal mast cells were permeabilized by treatment with SLO at concentrations that generated membrane lesions. The permeabilized mast cells released histamine and β-N-acetylglucosaminidase, dependent on the presence of nucleoside triphosphate and micromolar concentrations of Ca^{2+}. SLO-permeabilized mast cells have been used as a simplified system for studies to understand the molecular mechanisms of exocytosis (114–118) and the role of plasma membrane repair mechanisms (65, 76, 119). In these experiments, relatively high SLO doses were used, allowing formation of numerous pores in the plasma membrane of target cells. While such doses are cytocidal, low doses are tolerated because a limited number of lesions are formed and can be resealed by repair mechanisms (120).

Further studies showed that sublethal doses of SLO rapidly activated BMMCs to degranulate and to induce production of mRNAs of several cytokines, including tumor necrosis factor (TNF)-α, IL-13, IL-4, IL-6, and GM-CSF (**Figures 3** and **4**). Production of TNF-α was blunted upon pharmacological depletion of protein kinase C by phorbol-12 myristate-13 acetate. Exposure to low, nontoxic concentrations of SLO also resulted in enhanced phosphorylation of stress-activated protein kinases p38 mitogen-activated protein (MAP) kinase and c-jun N-terminal kinase (JNK). Inhibition of p38 MAP kinase markedly reduced production of TNF-α, suggesting that transcriptional activation of mast cells following transient permeabilization might contribute to the host defense against streptococcal infections *via* the beneficial effects of TNF-α. However, mast cell hyperstimulation might also lead to overproduction of TNF-α, promoting development of the toxic streptococcal syndrome (102). The involvement of TNF-α in the host defense against streptococcal infection was described in previous studies (13, 76, 107, 108).

The resistance to CDCs is affected by the levels of surface cholesterol and proteins exposed to the plasma membrane.

TABLE 2 | Effect of various cytolysins at sublytic concentrations on mast cell degranulation, production of cytokines, chemokines, and other mediators, tyrosine phosphorylation of signaling molecules, and Ca^{2+} response.

MC response Activating agents	Degranulation		Level of cytokines, chemokines and other mediators		Tyrosine phosphorylation			Ca^{2+} levels
	Histamine release	β-Hexosaminidase release*	mRNA	Protein	P38 MAPK	JNK	Erk1/2	
Cytolysins								
SLO		↑ (102) ↑ (103, serotonin*)	↑ (102)	↑ (102)	↑ (102)	↑ (102)		
PLY	– (104)			↑ (104)				
LLO		↑ (70)	↑ (70)	↑ (70)				↑ (70)
Cholesterol-inactivated LLO		– (70)	– (70)					– (70)
Cytolysin-producing and non-producing bacteria								
SLS producing *S. equi* live bacteria	↑ (105)		↑/– (106)	↑ (105, 106)	↑ (106)			
SLS non-producing *S. equi* live bacteria			– (106)	– (106)	– (106)		– (106)	
PLY producing *S. Pneumoniae* live bacteria (107) lysed bacteria (102, 108)	– (104)	↑ (26, 109)		– (109) ↑/– (26) ↑ (104)				
PLY-non-producing *S. pneumoniae*	– (104)	– (26)		– (26, 104)				
LLO-producing *Listeria monocytogenes*		↑ (70) ↑ (110, CD107a*)		↑ (70, 110)				↑ (70)
LLO-non-producing *Listeria monocytogenes*		– (70)						– (70)
Others								
Saponin			↑ (106)	↑ (106)	↑ (106)		↑ (106)	

↑*Increased level, – unchanged level.*

**In these studies, serotonin release or CD107a surface expression were used for quantification of degranulation instead of β-hexosaminidase release.*

TABLE 3 | Effect of cytolysins or bacteria producing the corresponding cytolysins on production of selected cytokines, chemokines, and other mediators by various mast cell types.

Mast cell type	Changes in production of cytokines, chemokines, and other mediators in response to cytolysins or bacteria producing the corresponding cytolysins		
	At the mRNA level	At the protein level	Ref.
Mouse			
BMMC	↑: TNF-α, IL-4, IL-6, IL-13, GM-CSF, MCP-1, Nr4a3	↑: TNF-α, IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, CCL2/MCP-1, CCL3, CCL4, CCL5, RANTES, GM-CSF –: TNF-α, IL-1β, IL-4, IL-10, IL-12, IL-17A, IFN-γ, TGF-β	(26, 70, 102, 105, 106, 110)
BMMC (response to SLS-deficient strain)	–: TNF-α, IL-6, Nr4a3	–: TNF-α, IL-6, CCL2/MCP-1	(106)
BMMC (response to PLY-deficient strain)		–: IL-6, -CCL2/MCP-1	(26)
PCMC	↑: IL-6, Nr4a3 –: TNF-α	↑: TNF-α, IL-6, CCL2/MCP-1	(106)
PCMC (response to SLS-deficient strain)	–: TNF-α, IL-6, Nr4a3	–: TNF-α, IL-6, CCL2/MCP-1	(106)
Human			
HLMC		↑: LTC4	(104)
Human intestinal MC	↑: TNF-α, IL-3, -5, -6, CXCL8	↑: TNF-α, CXCL8, LTB4, sLT	(111)
Human intestinal MC (response to Hly-deficient strain)	–: TNF-α, IL-5, CXCL8	–: LTB4, sLT	(111)
HMC-1		↑: LTC4	(104)
HMC-1 (response to PLY-deficient strain)		–: LTC4	(104)
LAD2		↑: LTC4	(104)

↑ increased level.
– unchanged level.
BMMC, Bone marrow-derived mast cells.
PCMC, Peritoneal cell-derived mast cells.
HLMC, Human lung mast cells.
HMC-1, Human mast cell line.
LAD2, Human mast cell line.

Schoenaurer et al. (121) described that toxin-induced plasma membrane perforation caused by SLO in human mast cells (HMC1) is affected by expression levels of the P2X7 receptor. The P2X7 receptor is an ATP-gated trimeric membrane cation channel, which after activation with ATP triggers Ca^{2+} influx and induces blebbing (122). This protective effect of the P2X7 receptor can be increased by activating the P2X7 receptor with ATP and abolished by selective P2X7R antagonists, A-438079 or blebbistatin (121).

Other studies showed that SLO is not the only activator of the mast and other immune cells, but also inhibits immune cell activity. Experiments with neutrophils revealed that SLO sublytic concentrations suppressed the oxidative burst in neutrophils, facilitating bacteria escape from innate immune killing (123). Thus, SLO functions as a virulence factor that is necessary and sufficient for suppressing bactericidal ROS production, thereby subverting neutrophil ROS-dependent killing. Interestingly, S. aureus, producing pore-forming virulence factor α-hemolysin (H1a), did not modulate the oxidative burst of neutrophils, suggesting a specific unique role of SLO, not a general consequence of membrane perturbation and disruption. Further studies showed that LLO and PFO could suppress the oxidative burst in murine macrophages by preventing NADPH oxidase localization with phagosome (124). In addition to oxidative burst suppression, low concentrations of SLO prevented release of IL-8 and elastase and blocked formation of neutrophil extracellular traps (123). It should also be mentioned that Logsdon and colleagues (113) showed that SLO at low levels reduced bacteria internalization by keratinocytes and concluded that SLO interferes with the internalization through local perturbations of the cell membrane and disruption of clathrin-dependent uptake pathways. They suggested that by forming plasma membrane pores in cholesterol-rich membrane domains, SLO may mimic cholesterol depletion and, in this way, inhibit the clustering of lipid rafts, thereby interfering with integrin signaling and bacterial internalization. These results are compatible with a model in which SLO binding (through fibronectin) to integrins of the cell surface acts to cluster integrin-containing membrane domains, thereby enhancing integrin-mediated cell signaling to stimulate the process of bacterial internalization. The results obtained *in vitro* were confirmed by experiments *in vivo* in which injection of SLO into ears of mast cell-deficient mice (KitW/KitW-v) resulted in a weak inflammatory response when compared to KitW/KitW-v mice that had been selectively engrafted with BMMCs (103).

Pneumolysin (PLY)

Most pathogenic isolates of *Streptococcus pneumoniae* produce PLY. These bacteria are the leading source of bacterial

pneumonia and could cause otitis media and bacterial meningitis (25, 26). Experiments *in vitro* showed that exposure of rat RBL-2H3 cells to various strains of *S. pneumoniae* leads to degranulation in a dose- and time-dependent manner. The degranulation was only partially controlled by cytosolic calcium and was not accompanied by production of TNF-α and IL-6 (109). The authors suggested that the induction of mast cell degranulation by pneumococcal factors not accompanied by the production of proinflammatory cytokines may be a specific strategy elaborated by this bacterium to promote its spreading from the respiratory mucosa and reducing neutrophil infiltration. Thus, the PLY amount could be meager, and other bacterial proteins could play a dominant role.

Human lung mast cells (HLMCs) and human mast cell lines HMC-1 and LAD2 were cocultured with pneumococci or stimulated with PLY. HLMCs and cell lines exhibited antimicrobial activity against *S. pneumoniae*. PLY induced release of antimicrobial peptide cathelicidin LL-37. These data suggested that mast cells limit pneumococcal dissemination early in the course of pneumococcal pulmonary disease (104). A recent study extended these findings and showed that mouse BMMCs degranulated and released IL-6, CCL2, CCL3, and CCL4 (but not IL-1β, TNF-α, IFN-γ, and several other cytokines) after exposure to *S. pneumoniae* (104, 125).

Furthermore, the response of BMMCs varied among different pneumococcal serotypes and was dependent on PLY but independent of TLR activation (26). These studies suggested that the absence of mast cells or pharmacologic mast cell stabilizer (cromoglycate) may reduce inflammation and ameliorate the disease severity following intracisternal infection in mice with *S. pneumoniae*. Surprisingly, experiments *in vivo* using mast cell-deficient strains (WBB6F1-KitW/Wv and C57BL/6 KitW-sh/W-sh mice) showed no significant effect on the disease phenotype of experimental pneumococcal meningitis (26). Thus, the results do not support previous *in vivo* data showing that mast cell-deficient KitW-sh/W-sh mice exposed to *S. pneumoniae* exhibited reduced inflammation, lower bacterial outgrowth, and longer survival compared with wild-type (WT) mice (126).

Listeriolysin (LLO)

Pore-forming toxin LLO is the main virulence factor of *Listeria monocytogenes*. LLO is known to induce a broad spectrum of host responses that ultimately influence the outcome of listeriosis. Unlike the other pathogens producing CDCs, *Listeria monocytogenes* is an intracellular pathogen that requires its CDC, LLO, for intracellular survival. LLO is thus the only cytolysin that is secreted by a facultative intracellular pathogen, while all other CDCs are produced by pathogens that are largely extracellular. LLO monomers bind to plasma membrane microdomains in target cell membranes, dimerize and oligomerize to form pre-pore complexes, followed by formation of pores of 50-80 subunits. Formation of pre-pores leads to aggregation of lipid rafts and signal transduction. Formation of pores and reparation processes are other steps of CDC-mediated cell activation in which extracellular components (Ca^{2+}) enter the cytoplasm, and cytoplasmic components (such as K^+ and proteins) are released from the cell. This leads to signals in target host cells resulting in degranulation, cytokine and chemokine production, suppression of phagocytosis, and induction of apoptosis (127–130) (**Figures 3** and **4**).

Using BMMCs and RBL-2H3 cells, Gekara et al. (70) showed that LLO triggers cellular responses such as degranulation and cytokine synthesis in a Ca^{2+}-dependent manner. They also found that LLO-mediated Ca^{2+} signaling is due to Ca^{2+} influx from the extracellular milieu and release of Ca^{2+} from intracellular stores. Ca^{2+} release from intracellular stores occurs *via* activation of intracellular Ca^{2+} channels, which involve tyrosine phosphorylation of several proteins, including PLC-γ1 and IP_3R-operated Ca^{2+} channels activated *via* G-proteins and protein tyrosine kinases. These data and the fact that the Ca^{2+} release could partially be blocked by tyrosine kinase inhibitor genistein and G-protein inhibitor pertussis toxin suggested that LLO activated the IP_3R Ca^{2+} channels *via* tyrosine phosphorylation and G-protein activation of PLC γ and PLC β isoforms, respectively. Another mechanism of Ca^{2+} release from intracellular stores is Ca^{2+} channel independent, which could reflect injury of intracellular stores, such as the ER (70). The data are relevant to previous studies showing that exposure of human embryonic kidney cells to sublytic concentrations of LLO caused long-lasting oscillation of the intracellular Ca^{2+} levels, leading to a pulsed influx of extracellular Ca^{2+} through pores that LLO forms in the plasma membrane. Calcium influx did not require the activity of endogenous Ca2+ channels. These data indicated that Ca^{2+} oscillations modulate cellular signaling and gene expression and could form a basis for the broad spectrum of Ca^{2+}-dependent cellular responses induced during *Listeria* infection (68).

Later studies using macrophage cell line J774 indicated that LLO is a potent aggregator of plasma membrane components, including GPI-anchored proteins CD14, CD16, ganglioside GM1, and protein tyrosine kinase Lyn. Abrogation of the cytolytic activity of LLO by cholesterol pretreatment was found not to interfere with the ability of LLO to aggregate the above-mentioned surface molecules nor to trigger tyrosine phosphorylation of Lyn and Syk kinases in the cells. When the oligomerization of LLO was blocked by monoclonal anti-LLO antibody, aggregation of surface molecules and tyrosine phosphorylation were blocked. The combined data suggested that LLO induces signaling through coaggregation of the plasma membrane receptors, kinases, and adaptors (81). Recently discovered lectin activity of LLO could play a vital role in this process (58). However, cholesterol-inactivated LLO, which binds and aggregates plasma membrane components such as the active form of LLO, could not induce Ca^{2+} release in mast cells. Thus, it is likely that membrane binding or plasma membrane protein aggregation is not sufficient to activate the IP_3R-dependent pathways and that LLO oligomerization and transmembrane insertion leading to pore formation are essential in this process (70).

Contrary to the study of Gekara et al. (70), Jobbings et al. (110) found that in the absence of Ca^{2+}, LLO-mediated

degranulation was enhanced, whereas antigen and PMA/I-mediated degranulation was completely inhibited. This discrepancy could be explained by LLO-mediated pore formation in granules resulting in degranulation and mediator release. Thus LLO is required for mast cell degranulation, independent of extracellular Ca^{2+}. The authors also found that in mast cells, LLO induces transient downregulation of cell surface c-kit receptor (CD117) without any effect of the FcεRI expression. Detailed analysis showed that in response to *L. monocytogenes*, mast cells release in addition to the key inflammatory cytokines (TNF-α and IL-6) a range of other mediators including Osteopontin, IL-2, IL-4, IL-13, granulocyte-macrophage colony-stimulating factor (GM-CSF), and several chemokines (CCL2, CCL3, CCL4, and CCL5). These cytokines are released in a MyD88-dependent manner.

A recent study (51) showed that four D4 subunits of LLO in the membrane-bound state are placed in the bilayer interface in a pre-pore configuration. In contrast, the membrane-inserted state consists of a tetrameric arc-like pore configuration. The binding of LLO leads to induced spatial heterogeneity that occurs in both membrane-bound and membrane-inserted states. This heterogeneity is primarily driven by the local density enhancement of cholesterol in the vicinity of LLO D4 subunits in the membrane-bound state. The induced heterogeneity after plasma membrane binding of LLO could be at least in part responsible for the observed changes in signaling machinery in cells exposed to low concentrations of CDCs. In this process, aggregation of lipid raft components (**Figure 2**) could play an important role.

COMPARABLE RESPONSE OF MAST CELLS TO CDCS AND OTHER PORE-FORMING COMPOUNDS

The results so far discussed indicate that various CDCs at sublytic concentrations induce similar mast cell activation events. This could reflect the structural similarity of CDCs examined (**Figure 1**) or the general effect of compounds leading to plasma membrane perturbation. Several lines of evidence indicate that the mast cell inflammatory response described for CDCs is comparable to other pore-forming and membrane destabilizing compounds.

Extensive studies have been devoted to SLS produced by *Streptococcus equi*, which causes a highly contagious and common disease of the upper respiratory tract and associated lymph nodes in equids (29). SLS was also identified in *Streptococcus pyogenes* and other *Streptococcus* species. Genomic analysis has identified gene clusters that are similar to the SLS-associated cluster in other pathogens such as *Listeria monocytogenes*, *Clostridium botulinum* and *Staphylococcus aureus* (131). SLS belongs to a distinct group of toxins whose hemolytic activity is sensitive to trypan blue, which are resistant to cholesterol and unaffected by oxidation. In these properties it differs from SLO and other CDCs (29, 131, 132). An initial ultrastructural study using BMMCs as a model showed the extensive formation of dilated ER in response to *S. equi* exposure, indicating enhanced protein synthesis (105). Further analysis revealed that exposure of BMMCs to *S. equi* did not show signs of extensive degranulation. However, the coculture of live bacteria with BMMCs resulted in profound secretion of IL-4, IL-6, IL-12, IL-13, TNF-α, CCL2, CCL7, MCP3, CXCL2, CCL5. In contrast, heat-inactivated bacteria caused only minimal cytokine/chemokine response (105).

A recent study showed that BMMCs responded vividly to wild-type *S. equi* by upregulating a panel of proinflammatory genes and secreting proinflammatory IL-6, TNF-α, and monocyte chemoattractant protein (MCP)1 (106). However, this response was abrogated entirely in *S. equi* lacking the sagA gene encoding SLS. Several lines of evidence indicated that mast cell activation is not the result of mast cell lysis and release of components capable of mast cell triggering. Immunoblotting analysis revealed that exposure of mast cells to wild-type *S. equi*, but not to a SLS-deficient mutant, induced phosphorylation of p38 and Erk1/2, which could be inhibited by the corresponding inhibitors. Based on these data, the authors concluded that bacteria-derived SLS at sublytic concentrations is a major stimulus for mast cell activation leading to proinflammatory gene expression and cytokine production. It should be noted, however, that in contrast to CDCs, SLS induced only week degranulation (106).

Kramer et al. (111) examined the effect of α-hemolysin, a protein toxin that assembles on membranes to form a heptameric pore structure (133). They found that *Escherichia coli* strains producing α-hemolysin induced release of histamine, leukotrienes, and proinflammatory cytokines from intestinal human mast cells. Blocking the extracellular Ca^{2+} and calmodulin/calcineurin pathway by cyclosporine A inhibited the response to α-hemolysin. Activation of mast cells by α-hemolysin was also inhibited by blocking MAPKs p38 and ERK. Pharmacological blockade of Ca^{2+}-dependent PKCα, and PKCβ1 or PI3K had an only weak effect on α-hemolysin activation of mast cells, but a robust inhibitory effect on FcεRI-mediated cell activation. The data indicate that mast cell activation by FcεRI and α-hemolysin utilize different signal transduction pathways (111).

During their experiments focused on *S. equi* interaction with mast cells, von Beek et al. (106) used another pore-forming compound, saponin, and a steroid glycoside detergent digitonin to determine whether SLS and saponins trigger BMMCs in a similar way. They found that saponin, which forms tiny pores in the plasma membrane (134, 135) at sublytic concentrations, triggered IL-6 and TNF production similarly to SLS. They concluded that mast cell activation by *S. equi* SLS could be phenocopied by low sublytic concentrations of saponin. When steroidal saponin, digitonin, was used at sublytic concentrations, profound production of IL-6 in mast cells was also observed. Altogether, these data suggest that multiple lytic agents at sublytic concentrations could induce mast cell activation by similar mechanisms.

CONCLUSIONS AND FUTURE DIRECTIONS

Despite impressive progress in understanding the molecular mechanism of CDCs' interaction with plasma membranes and CDC-mediated activation of the mast and other cell types, the picture is far from complete. The involvement of similar signaling pathways triggered by CDCs and saponins suggests a similar cell response towards plasma membrane perturbations. These perturbations involve (I) binding of ligands to plasma membrane structures (GPI-anchored proteins, cholesterol-rich domains, glycoconjugates), (II) aggregation of monomers (oligomerization process) and pre-pore formation, (III) plasma membrane pore formation, and (IV) activation of the signaling machinery leading to cell activation and production of inflammatory mediators. The molecular-level details of individual steps are yet to be determined. Although studies of membranes can now benefit from the large-scale detailed analysis of lipid molecular species, there is currently a paucity of data regarding some of the critical points, such as (I) lipid compositional analysis of plasma membrane domains from cells at various stages of pore formation and (II) complete compositional analysis of proteins associated with lipid pre-pores and pores at various time intervals after exposure to CDCs. Furthermore, a comparison of phosphoproteomes from cells activated *via* IgE-antigen complexes, sublytic concentrations of CDCs, or sublytic concentrations of saponins could be informative in delineating specific signaling pathways involved in cell activation by CDCs.

Most of the experiments described in this review were performed *in vitro*. Models that more closely resemble *in vivo* conditions are needed to unravel the relevance of CDCs and other pore-forming compounds to the pathogenesis of various diseases caused by CDC-producing bacteria. Such studies will be of great value in rational usage of CDCs as anti-cancer therapeutics (136, 137), vaccine adjuvants (138–141), and adjuvants stimulating inflammasome activity (142).

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Study of Antibody-Dependent Reactions of Mast Cells *In Vitro* and in a Model of Severe Influenza Infection in Mice

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We investigated the reaction of mouse peritoneal mast cells (MCs) *in vitro* after IgG-containing immune complex introduction using A/H5N1 and A/H1N1pdm09 influenza viruses as antigens. The sera of immune mice served as a source of IgG antibodies. The concentration of histamine in the supernatants was determined at 4 hours after incubation with antisera and virus. We compared the contribution of MCs to the pathogenesis of post-immunization influenza infection with A/H5N1 and A/H1N1 influenza viruses in mice. The mice were immunized parenterally with inactivated viruses and challenged with lethal doses of drift A/H5N1 and A/H1N1 influenza viruses on the 14th day after immunization. Simultaneously, half of the mice were injected intraperitoneally with a mixture of histamine receptor blockers (chloropyramine and quamatel). In *in vitro* experiments, the immune complex formed by A/H5N1 virus and antiserum caused a significant increase in the histamine release compared to immune serum or the virus alone. With regard to the A/H1N1 virus, such an increase was not significant. A/H1N1 immunization caused detectable HI response in mice at 12th day after immunization, in contrast to the A/H5N1 virus. After challenge of A/H5N1-immunized mice, administration of antihistamines increased the survival rate by up to 90%. When infecting the A/H1N1-immunized mice, 90% of the animals were already protected from lethal infection by day 14; the administration of histamine receptor blockers did not increase survival. Histological examination of the lungs has shown that toluidine blue staining allows to estimate the degree of MC degranulation. The possibility of *in vitro* activation of murine MCs by IgG-containing immune complexes has been shown. In a model of influenza infection, it was shown that the administration of histamine receptor blockers increased survival. When the protection was formed faster due to the earlier production of HI antibodies, the administration of histamine receptor blockers did not significantly affect the course of

the infection. These data allow to propose that even if there are antibody-dependent MC reactions, they can be easily stopped by the administration of histamine receptor blockers.

Keywords: mast cells, influenza infection, vaccination, IgG antibodies, immune complexes

INTRODUCTION

Influenza virus (the genus *Influenzavirus*, the family *Orthomyxoviridae*) infections remain an important medical and social problem with high incidence during annual influenza epidemics. Despite the improvement of vaccines and the development of vaccine prevention, severe cases of the disease are still encountered. The avian influenza A/H5N1 virus has been causing periodic outbreaks of severe infection in Southeast Asia since it was first detected in Hong Kong in 1997 (1). According to WHO, for the period from 2003 to 2019, the mortality rate from laboratory-confirmed influenza A/H5N1 was 30–66% [https://www.who.int/influenza/human_animal_interface/2020_MAY_tableH5N1.pdf?ua=1]. Avian influenza viruses of the H5 subtype not only circulate among waterfowl, but also continue to be periodically transmitted to humans (2). In this regard, the preparation of the corresponding vaccine strains will be relevant.

Influenza vaccination aimed mainly at the production of serum antibodies may have reduced protection against drift variants of viruses. In addition, infection can occur in a time period before a full-fledged immune antibody response is elicited. The effectiveness of influenza vaccination can go down when the vaccine strain does not match the epidemic virus (3). In some cases, low-affinity antibodies to the influenza virus that do not have virus-neutralizing properties may be involved in intensifying a secondary infection (4). During the 2009 influenza pandemic, the use of seasonal vaccines and the presence of antibodies against influenza A/H1N1pdm09, which do not have neutralizing properties, correlated with an increased risk of more severe influenza-like illness in infected people (5). The role of mast cells in the onset of antibody-dependent enhancement is poorly understood and underestimated.

Mast cells are specialized innate immune cells derived from the bone marrow and have been identified in all vertebrates. They have long been recognized as effector cells in allergic disorders and certain immune responses to parasites. Mast cells (MCs) are truly universal cells involved in complex immunological and non-immunological functions, providing direct effects and indirect regulation of other cells and their functioning in various biological processes (6). MCs synthesize and store cytokines in granules. Mouse MCs have been shown to produce cytokines IL-1β, IL-6 and TNF-α, a potent preformed pro-inflammatory cytokine that is also found in human MCs (7). Some of the granule components are preformed before MCs are activated (proteases, biogenic amines, proteoglycans, TNFα and IL-4 cytokines, as well as many growth factors). Another part of molecules secreted by MCs molecules is synthesized after activation of MCs (interleukins, TNFα, TGFβ, chemokines, eotaxin, lipid inflammatory mediators) (8).

Human IgG antibodies in rheumatoid arthritis and systemic lupus erythematosus, as well as diabetes and allergies, cause pathology through interaction with MCs (9). Monovalent IgG4 antigenic complexes are unable to bind and cross link Fcγ receptors (FcγR) and cannot stimulate antigen-presenting cells activation, but can bind FcγIIb receptors and cause suppression of MCs, monocytes and macrophages (10). MCs, the main effector cells in allergies, release various vasoactive substances, including histamine, SSRA (slow-reacting anaphylaxis factor), and serotonin (11). Leukotrienes, prostaglandins and platelet activating factors are synthesized by MCs activated by arachidonic acid. Cytokines, chemokines, and growth factors are synthesized *de novo* and released shortly after activation (12).

Allergic immunotherapy reduces the production of proinflammatory mediators with reduced migration of MCs in target organs (13). Different classes of FcγR are expressed on many immune effector cells and mediate various cellular responses such as macrophage phagocytosis, antibody-dependent NK- and T-cell cytotoxicity, and MC degranulation. High affinity mouse FcγRI is able to bind with high affinity only IgG2a isotype, while low affinity FcγRIII binds polymeric forms of all IgG subclasses (IgG1, IgG2a and IgG2b), except IgG3. Not so long ago, a third activating receptor FcRIV was discovered in mice, which binds immune complexes containing IgG2a and IgG2b with intermediate affinity (14).

MCs mainly express low-affinity and, under certain conditions, high-affinity IgG receptors. Mast cell survival and cytokine secretion depend on the ITAM (Immunoreceptor Tyrosine-based Activation Motif) presence in cytoplasmic tails of FcRγ-receptors, which provides for the signal transmission and cell activation (15).

Antibody-dependent enhancement (ADE) has been described for some viral infections in people with repeated illnesses or in previously vaccinated people, which is manifested by a severe course of infection, often fatal. ADE is believed to develop through the mechanism of facilitated penetration of the virus in complex with IgG antibodies and/or complement factors into cells with Fcγ and C3 receptors, which increases its infectivity and contributes to the development of a severe, life-threatening course of viral infection (16). ADE syndrome is characteristic of Dengue fever, in which it attracted attention and was first described (17).

Abbreviations: ADE, Antibody-dependent enhancement; AH, antihistamines; CE, chicken embryos; EID_{50}, fifty percent embryonic infectious doses; ELISA, enzyme-linked immunosorbent assay; FcγR, Fcγ receptors; HA, hemagglutinin; HAU, hemagglutinating units; HI - hemagglutination inhibition reaction; HRP, horseradish peroxidase; IM, intramuscularly; ITAM, Immunoreceptor Tyrosine-based Activation Motif; LD_{50}, 50% mouse lethal dose; MCs, mast cells; NA, neuraminidase; PBS, phosphate-buffered saline; VIP, vasoactive intestinal peptide.

Seasonal influenza vaccination successfully prevents the disease when the antigenic structure of vaccine strains and circulating viruses coincides. However, the positive effect of such vaccination is reduced in case of infection with the shift variants of the influenza virus, to which people have no immunity, resulting in increased severity of the infectious process and mortality when infected with the shift variants of the influenza virus, to which people have no immunity (18). In this regard, studies aimed at increasing the cross-protection of existing influenza vaccines, as well as at deciphering the mechanisms of aggravating viral infections during re-infection, are of particular relevance.

In connection with the above, the aim of the work was to test the hypothesis that MCs make a tangible negative contribution to the pathogenesis of viral infection due to the histamine secreted by them, which makes the course of the infectious process more severe. To study the role of MCs in the development of severe viral infection in immunized animals we used a model of infection with a drift variant of the influenza virus.

MATERIALS AND METHODS

Viruses

In this study we used the following influenza viruses: A/Vietnam/1194/2004(H5N1) NIBRG-14 (National Institute for Biological Standards and Control, UK) [A/Vietnam(H5N1)] and A/Indonesia/5/2005(H5N1) IDCDC-RG2 (Centers for Disease Control and Prevention, USA) [A/Indonesia(H5N1)]. These strains were produced by reverse genetics by the National Institute of Biological Standards and Control (NIBSC, United Kingdom) and the Center for Disease Control and Prevention (USA) using Vero-certified vaccine-producing cells and laboratory protocols that take into account that the end use of the vaccine is administration to humans. The WHO Influenza Center recommends these strains for the preparation of inactivated influenza vaccines against avian influenza. Viruses contain modified H5 subtype hemagglutinin (HA) and are safe for humans. Also, we used the A/South Africa/3626/2013 (H1N1)pdm09 [A/South Africa(H1N1)pdm09] and A/New York/61/2015 (H1N1)pdm09 [A/New York(H1N1)pdm09] influenza viruses obtained from the National Institute for Biological Standards and Control (NIBSC, UK) repository. The A/California/07/2009(H1N1)pdm09 was obtained from the Institute of Experimental Medicine collection of viruses. All viruses were propagated in 10-day old chicken embryos (CE) and stored at -70°C.

Determination of Histamine Production *In Vitro* by Mast Cells of Mouse Peritoneal Exudate

As a material containing MCs, we used cells from mouse peritoneal exudate. 5 ml of 0.85% phosphate-buffered saline (PBS) was injected into the abdominal cavity of the mouse, then MCs containing exudate were collected. The resulting cells were used at a concentration of 1 million cells/450 μl, among which MC content averaged at an estimated 7-10%. Influenza viruses A/Vietnam(H5N1) and A/New York(H1N1)pdm09 purified by ultracentrifugation in a 30/60 stepwise sucrose gradient were used as antigens. The source of IgG was the sera of CBA mice immunized intramuscularly (IM) with the same viruses at a dose of 20,000 hemagglutating units (HAU)/1ml in a volume of 0.1 ml. The sera were collected at day 21 after immunization and the content of antibodies to the A/Vietnam (H5N1) or A/New York(H1N1)pdm09 influenza virus in sera was confirmed by ELISA as described previously (19). Fcγ receptors of MC were loaded with IgG antibodies (incubation of peritoneal cells with hyperimmune mouse serum at a dilution of 1: 300 and 1: 900 (contact for 1 h, 4°C). Then the cells were washed from unbound antibodies and additionally loaded with antigens (influenza viruses) with a content of 5-50 HAU/1 ml (contact 1 h, 4°) C, followed by incubation for 40 min at 37° for degranulation and histamine release. The setup of the experiment on murine peritoneal cells is shown in **Figure 1**.

After the end of all incubations, results of the reaction were read with a plate modification of Shore's method (20). The method is based on the processing of the luminescent condensation product of histamine with orthophthalic

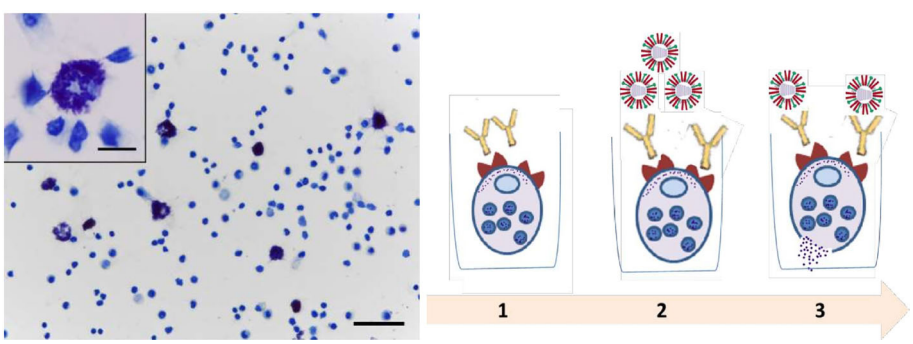


FIGURE 1 | Main steps of peritoneal mast cell experiments. Peritoneal cell smear stained with toluidine blue. Mast cells are metachromatically stained purple with toluidine blue. Obj. 40x, scale bar = 50 μm; insets, obj. 100x, scale bar = 10 μm. (1) Loading of Fcγ-receptors of MC with IgG antibodies (hyperimmune serum of mice). (2) The load of MC that bound Ab using influenza viruses. (3) Degranulation and histamine release.

aldehyde at wavelengths 355/460 nm. The level of histamine was expressed in arbitrary units.

Ethics Statement

The conditions for keeping animals in the vivarium provide them with a normal biological background and fully comply with the requirements of the Sanitary Rules for the Arrangement, Equipment and Maintenance of Experimental Biological Clinics (vivariums) dated 06.04.1973. The diet of animals complies with the order of the Ministry of Health No. 1179 dated 1983. The study was approved by the Local Ethics Committee for Animal Care and Use at the Institute of Experimental Medicine, Saint-Petersburg, Russia (protocol № 3/17 of 30.11.2017). Non-terminal procedures were performed under ether anesthesia inhalation. To control viral load in the lungs, animals were euthanized under ether anesthesia inhalation and cervical dislocation. The health status of the challenged mice was monitored and recorded once a day for 10-14 days post infection.

Animals

The 8-to-10-week-old female CBA mice were provided by the laboratory-breeding nursery of the Russian Academy of Sciences (Rappolovo, Leningrad Region, Russia). Mice were maintained under standard conditions and given ten days to acclimate to the housing facility. Feeding was carried out ad libitum, in the morning with free access to water. The weight of animals at the start of the experiments was 20.0 ± 2.0 grams (mean ± SEM).

Study of Drifted Influenza Infection in A/H5N1 and A/H1N1 Sensitized Mice

Immunization of Mice

Viral antigens for immunization of mice were prepared as earlier described (19). Formaldehyde-inactivated influenza viruses A/Vietnam/1194/2004(H5N1) NIBRG-14 and A/New York/61/2015 (H1N1)pdm09 or A/California/07/2009 (H1N1)pdm09 were used for IM immunization at a dose of 20,000 HAU/1ml in a volume of 0.1 ml. The control group received IM PBS as a placebo in the same volume.

Immunogenicity Study

Blood sera were taken from the mice on day 12 after immunization and the sera were stored at -20°C until serological tests were performed. For the hemagglutination-inhibition (HI) assay the sera were treated with receptor-destroying enzyme (RDE, Denka Seiken, Tokyo, Japan) and tested for HI antibodies against A/Vietnam/1194/2004(H5N1) NIBRG-14, A/Indonesia/5/2005(H5N1) IDCDC-RG2, A/New York/61/2015 (H1N1)pdm09, and A/South Africa/3626/2013 (H1N1)pdm09 influenza viruses as previously described (19). The enzyme-linked immunosorbent assay (ELISA) was conducted to determine serum IgG antibodies against influenza viruses in 96-well microplates (Sarstedt AG & Co, Nümbrecht, Germany) as previously described (19). For absorption we used 20 HAU/0.1 ml of the whole purified A/H5N1 or A/H1N1 influenza viruses. As a conjugate, we used rabbit horseradish peroxidase (HPR) labeled antibodies to the Fc fragment of mouse IgG (Sigma, St. Louis, United States). The end-point ELISA titers were expressed as the highest dilution that yielded an optical density at 450 nm (OD_{450}) greater than the mean OD_{450} plus 3 standard deviations of negative control wells containing conjugate only.

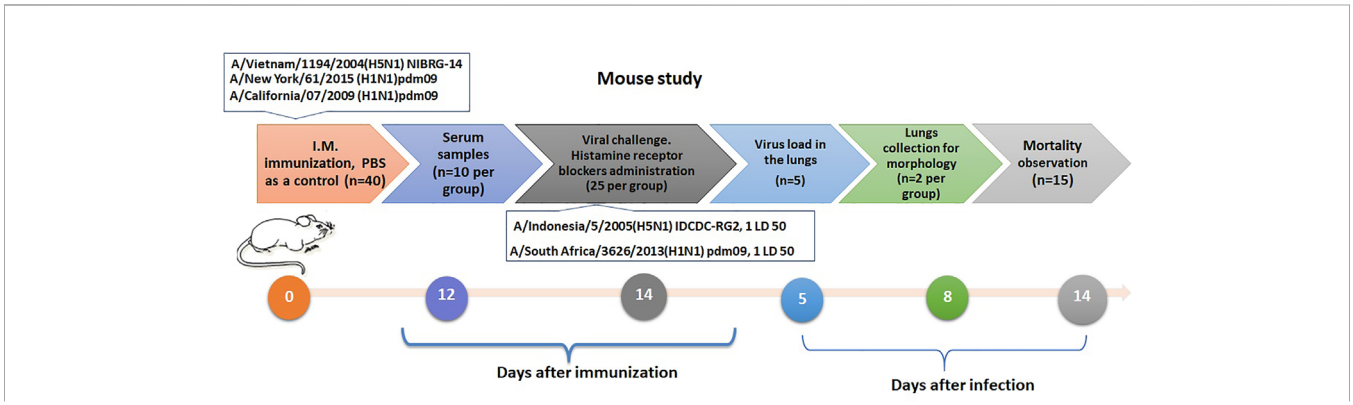
Challenge Study

On day 14 after immunization, the mice were infected intranasally with the influenza viruses at a concentration of one 50% mouse lethal dose (LD_{50}) which was approximately $10^{4.5}$ EID_{50} for A/Indonesia(H5N1) influenza virus and 10^5 EID_{50} for the A/South Africa(H1N1)pdm09 influenza virus. The virus was administered intranasally in a volume of 50 µl, evenly distributed between the nostrils. The LD_{50} was preliminary determined after infection with serial dilutions of virus A/Indonesia and A/South Africa from 10^2 to 10^7 EID_{50}.

Simultaneously with the infection, 50% of the immune mice were administered intraperitoneally with a mixture of histamine receptor blockers H1 and H2, chloropyramine (Egis, Guyancourt, France) and quamatel (Gedeon Richter, Budapest, Hungary), each 6.7 mg/kg body weight, in a volume of 0.1 ml, or PBS in the same volume. The experimental design is shown in **Figure 2**.

Evaluation of the Viral Titers in the Lungs

The lungs were collected from mice at day 5 after viral infection, homogenized using a Retsch MM-400 ball vibratory mill in PBS containing 100 U/ml penicillin, 100 µg/ml streptomycin and



centrifuged for 10 min at 6000 g. Lung homogenates were titrated in developing chick embryos starting with an initial dilution of 1:10. The viral titers were calculated as a log10 of the fifty percent embryonic infectious doses (EID_{50}) using hemagglutination as the endpoint, as described previously (21).

Histamine and Levels Measuring in the Blood Sera

In order to determine histamine levels using ELISA, blood samples were collected on the 5^{th} day after infection. Histamine production was evaluated using the EIA Histamine kit (Beckman Coulter, Brea, United States) according to the manufacturer's instructions. All tested samples were duplicated

Histological Examination of the Lungs

Left- and right-lung samples from CBA mice (n = 2 per group) were used for the histological study. The samples were fixed in formalin and embedded in paraffin as previously described (22). Lung sections were stained with toluidine blue (LabPoint, Russia). Briefly, a dye solution was applied to the previously dewaxed sections and incubated for 10 min at room temperature. The sections were then rinsed, dehydrated, cleared, mounted and cover slipped.

Statistical Processing of Results

Data was processed using Statistica software, version 6.0 (StatSoft, Inc. Tulsa, Oklahoma, USA) and graphics data were generated using Prism 8 (GraphPad software, San Diego, USA). When analyzing the results obtained, the mean values and standard deviation ($M \pm \sigma$) were determined. The statistical significance of the differences was assessed using the Wilcoxon - Mann - Whitney nonparametric tests. The log-rank (Mantel–Cox test) was used to compare the survival distributions. The differences were considered significant at $p < 0.05$.

RESULTS

Estimation of Histamine Production by Mast Cells of Mouse Peritoneal Exudate *In Vitro*

The immune complexes formed on the cell surface by successive incubations with IgG antibodies and an antigen specific to these antibodies (A/Vietnam(H5N1) virus) with a content of 50 HAU) caused a significant increase in histamine release at serum dilutions of 1: 300 and 1: 900 (**Figure 3A**). This release of histamine was significantly higher than after incubation with IgG alone or with A/H5N1 virus alone, although it was inferior to that when exposed to mouse IgE antibodies.

While the concentration of the A/Vietnam (H5N1) virus was 5 HAU, a significant increase in the level of histamine in the culture supernatants was observed only when the A/H5N1-specific antiserum was diluted 1: 900 (**Figure 3B**).

For the A/New York(H1N1)pdm09 influenza virus, a significant increase in histamine was observed only at a virus concentration of 50 HAU and a 1:900 dilution of H1N1-specific serum (**Figure 3C**).

Thus, for both A/H5N1 and A/H1N1 viruses, there was a dose-dependent effect on virus concentration. The release of histamine was more pronounced with a higher dilution of the immune serum.

A Model of Lethal Influenza Infection in Immunized Mice

Immunogenicity

Groups of mice were administered IM with inactivated influenza viruses of A/Vietnam(H5N1) or A/New York(H1N1)pdm09 influenza viruses. Immunogenicity was assessed 12 days after immunization by the production of serum antibodies. The results of the study of virus-specific serum antibodies in the HI-test and ELISA are presented in **Figure 4**.

A **Figure 4A**, shows that on the 12^{th} day after A/H5N1 immunization, the levels of HI antibodies to homologous A/Vietnam(H5N1) and drifted A/Indonesia(H5N1) viruses were lower than 1:40, i.e. less than protective level, which is traditionally associated with at least a 50% reduction in the risk of influenza (23). HI antibodies to A/H1N1 viruses were formed at a protective level against the homologous vaccine virus and, in some cases, against the drift virus (**Figure 4A**). The levels of virus-specific IgG among immunized mice both to A/H1N1 and A/H5N1 influenza viruses significantly exceeded the levels in the control group (**Figure 4B**). Thus, viruses A/H1N1 and A/H5N1 differed in immunogenicity. When the A/New York (H1N1)pdm09 virus was administered to mice, a HI immune response was formed on the 12th day after immunization, in contrast to the A/Vietnam(H5N1) virus. These data correlate with previously obtained data on the reduced immunogenicity of A/H5N1 influenza viruses in comparison with epidemic influenza viruses (24), and may suggest that the antibodies formed as a result of A/H5N1 immunization were mainly of the non-neutralizing type.

Evaluation of Survival After Lethal Infection of Immunized Mice With Drifted Variant of the Same Influenza Virus Subtype

On day 14 after immunization, the mice were infected intranasally with the A/Indonesia(H5N1) or A/South Africa (H1N1)pdm09 influenza virus at a concentration of one 50% mouse lethal dose (LD_{50}). Five independent experiments were carried out for the pair of A/H5N1 influenza viruses, and three independent experiments for the A/H1N1 pair. **Figure 5** shows data on mortality, weight loss, and lung virus titers.

Figure 5A shows that after infection of immune mice with the A/Indonesia(H5N1) virus of immune mice, when part of the mice was injected with a mixture of histamine receptor blockers, the survival rate of mice increased from 70% to 80%. The differences between vaccinated and unvaccinated animals became statistically significant. After infection with the A/South Africa(H1N1)pdm09 virus, 90% of A/New York(H1N1)pdm09 immunized mice were protected from lethal infection and the administration of antihistamines did not increase the survival rate of the mice (**Figure 5B**). Interestingly, in this case,

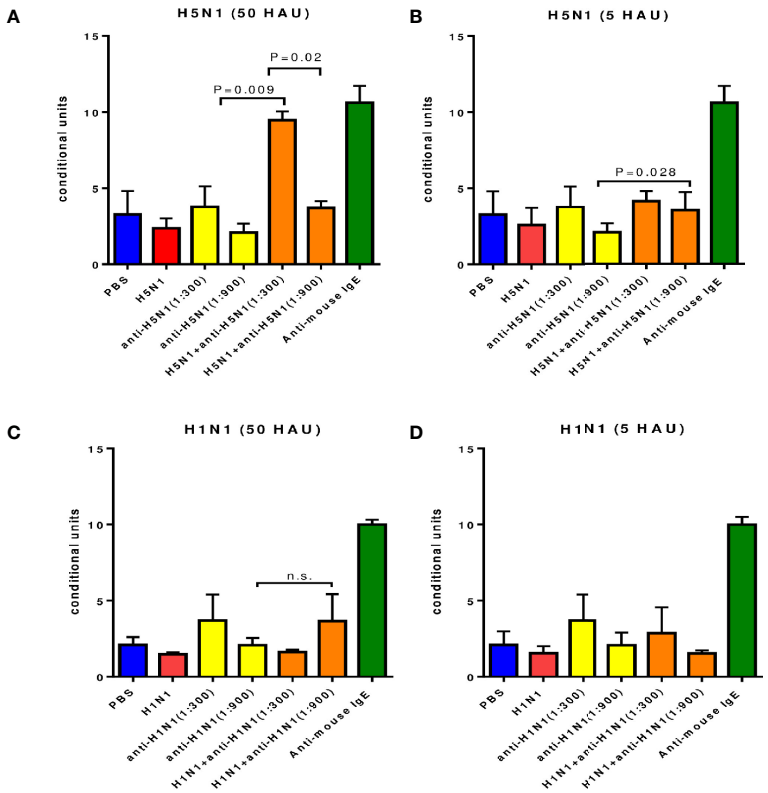


FIGURE 3 | Degranulation of mast cells contained in peritoneal exudate of intact CBA mice under the influence of the immune complex. Average data from three independent experiments are presented. **(A, B)** - antibodies and influenza virus A/Vietnam/1194/2004(H5N1) NIBRG-14). **(C, D)** - antibodies a and A/New York/61/2015 (H1N1)pdm09 influenza virus); n.s., no significance.

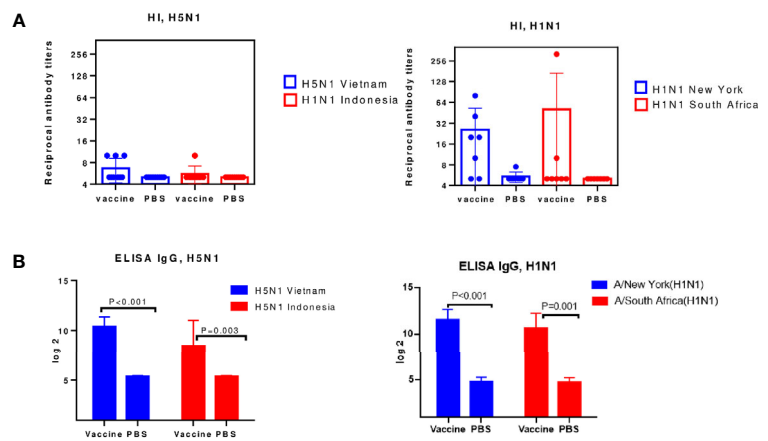


FIGURE 4 | Serum antibodies on day 12 after immunization (n=8-10). Each chart shows data from one of three experiments. **(A)** The HI antibody levels against the A/H5N1 and A/H1N1 subtype. **(B)** ELISA serum IgG antibodies.

the administration of antihistamines to non-immunized mice increased the survival rate of the mice by up to 100%, although the increase was not statistically significant. Administration of antihistamines for both A/Indonesia(H5N1) and A/South Africa (H1N1)pdm09 lethal infections did not significantly affect weight loss. **Figure 5C** shows that the isolation of infectious A/Indonesia (H5N1) viruses from the lungs of A/Vietnam(H5N1) immunized mice did not differ from those among the mice in PBS group.

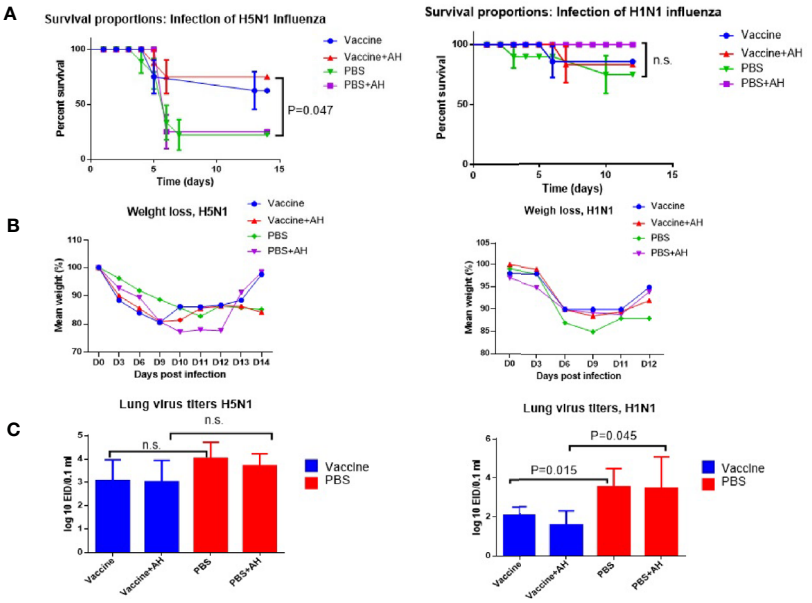


FIGURE 5 | Lethal influenza infection of immunized mice. A/Vietnam/1194/2004(H5N1) immunized mice were infected with A/Indonesia/5/2005(H5N1) influenza virus. A/New York/61/2015 (H1N1)pdm09 immunized mice were infected using A/South Africa/3626/2013(H1N1)pdm09 influenza virus. AH - antihistamines. Data from one of each series of experiments are presented. **(A)** Survivals (n=20). Log-rank (Mantel-Cox) test, P – compared to PBS; n.s., no significance. **(B)** Weight loss (n=12). **(C)** Virus isolation from the lungs (n=5); n.s., no significance.

And in the case of the A/South Africa(H1N1)pdm09 infecting virus, the levels of the virus in the lungs differed from those among non-immunized mice, both with and without the use of histamine receptor blockers. These data show that immunization with inactivated A/H1N1 virus may cause a higher level of protection against infection with drifted variant of virus on day 14 after immunization compared to the A/H5N1 virus. This could be due to a higher level of HI antibodies to the A/H1N1 virus which had already managed to form by the 12th day after IM immunization, although 3-4 weeks are considered the optimal time for the formation of a full-fledged humoral immune response to influenza vaccination (25).

Sublethal A/South Africa(H1N1)pdm09 Influenza Infection in Immunized Mice

The above pair of viruses did not differ in the structure of hemagglutinin so significantly, since the challenge virus was isolated in 2013, and the vaccine virus was isolated in 2015. So, we tried to find out what will happen if immunization and infection with even more distant variants of the influenza A/H1N1 pandemic strain are carried out. The pandemic virus A/California (H1N1) pdm09 was first isolated in 2009 and has been included in the strains recommended for the preparation of influenza vaccines for almost 10 years. For immunization, we used the A/California (H1N1) pdm09 virus, inactivated as indicated above. Infection was carried out with virus A/South Africa at a sublethal dose, which was approximately equal to 10^4 EID_{50}. The results are shown in **Figure 6**.

Thus, it has been shown that both A/California/07/2009 (H1N1)pdm09 and A/New York/61/2015 (H1N1)pdm09 influenza viruses have a higher immunogenicity in mice than A/Vietnam/1194/2004(H5N1) NIBRG-14 influenza virus as estimated using HI assay. It was shown in models of lethal and sublethal infection of immunized mice that in A/H5N1-immized mice, mortality and weight loss were reduced after A/Indonesia/5/2005(H5N1) IDCDC-RG2 infection by histamine blockers administration but introduction of antihistamines did not affect the survival or weight loss of A/H1N1-immunized mice upon infection with the A/South Africa/3626/2013(H1N1) pdm09 virus. The use of antihistamines slightly reduced mortality among control (non-immune) animals after A/South Africa/3626/2013(H1N1)pdm09 lethal infection. Unlike A/H1N1 immunization, A/H5N1 immunization did not reduce pulmonary infection according to lung virus titers data.

Determination of Serum Histamine Levels After Infection of Immunized Mice

Figure 7 shows that histamine levels in the sera of immunized mice were not affected by administration of antihistamines during infection with the A/Indonesia/5/2005(H5N1) IDCDC-RG2 influenza virus, and slightly decreased in the case of infection with this virus in non-immune mice, although the differences were not statistically significant. Administration of antihistamines led to a decrease in serum histamine levels only among non-immune mice following infection with the A/South Africa/3626/2013(H1N1)pdm09 virus (P=0.02).

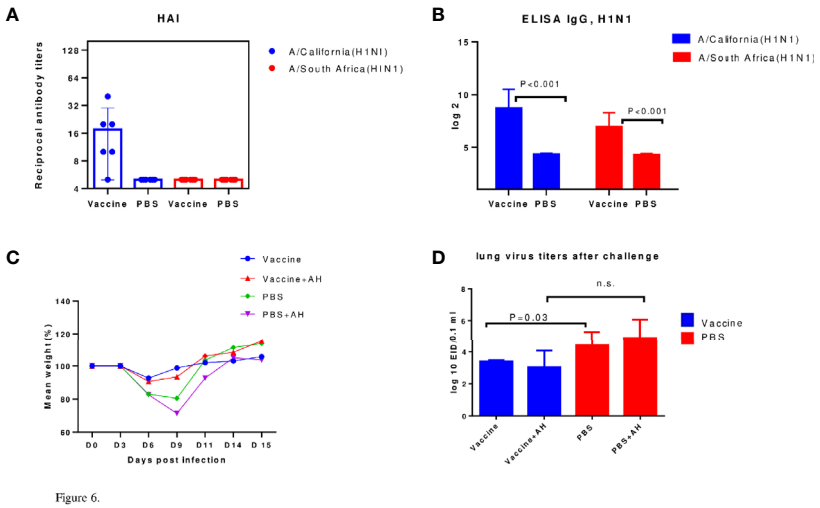


Figure 6.

FIGURE 6 | Protection against sublethal infection with drift A/H1N1pdm09 influenza virus. A/California/07/2009 (H1N1)pdm09 – immunized mice were infected using A/South Africa/3626/2013(H1N1)pdm09 influenza virus. AH - antihistamines. **(A)** The HI data (n=6). **(B)** ELISA IgG (n=6). **(C)** Weight loss dynamics (n=10). **(D)** Virus isolation from the lungs (n=5); n.s., no significance.

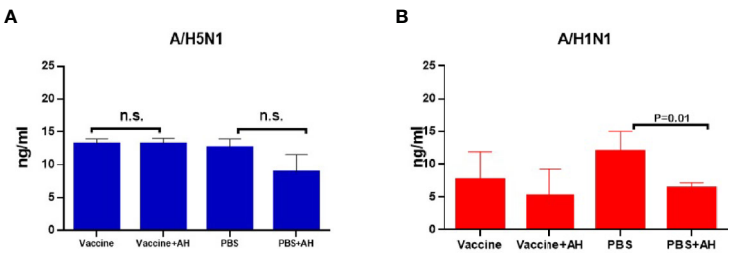


FIGURE 7 | Serum histamine levels (n=5, in duplicates). **(A)** The A/Vietnam/1194/2004(H5N1) NIBRG-14 immunization followed by A/Indonesia/5/2005(H5N1) IDCDC-RG2 infection. The chart shows data from one of three independent experiments; n.s., no significance. **(B)** The A/California/07/2009 (H1N1)pdm09 immunization followed by A/South Africa/3626/2013(H1N1)pdm09 infection.

Histological Examination of Lung Tissue

A/Indonesia/5/2005(H5N1) Influenza Virus Infection of A/Vietnam/1194/2004(H5N1)- Immunized Mice

MCs were identified in lung tissue on day 8 after immunization (**Figure 8**). When using toluidine blue, MCs in the mouse lung are stained metachromatically to a rich purple color. Due to the intense staining, MCs can already be identified at a low microscope magnification (x10). This greatly facilitates the quantitative analysis of MCs. The MC cytoplasm contains a large number of densely packed metachromatically stained granules, often shielding the nucleus. Several of lung MCs showed signs of degranulation such as extracellular granules. In this case, the granule boundaries are visible. This makes it possible to identify the different stages of MC degranulation (from weak degranulation with the secretion of single granules into the extracellular space to strong degranulation with rupture of the plasma membrane and secretion of all granules) and to assess the MC degranulation degree.

On day 8 after A/H5N1 infection of non-immune mice, rare non-activated (intact) mast cells were found in the walls of the large bronchi (**Figure 8A**) and in the alveolar septa (**Figure 8B**).

After A/H5N1 infection of the immunized mice, the enrichment of lung MCs was observed (**Figures 8C, D**, arrowhead). MCs were typically found in close proximity to both inflamed bronchi and blood vessels (**Figures 8E, F**, arrowhead). Intact MCs, as well as MCs with mild/moderate degree of degranulation were identified.

Infection of A/California/07/2009 (H1N1)pdm09-Immunized Mice With the A/South Africa/3626/2013 (H1N1)pdm09 Influenza Virus

The lungs of intact mice (non-immunized and non-infected) had a normal histological structure (**Figure 9A**). In some airway walls, the bronchus-associated lymphoid tissue was found (**Figure 9B**).

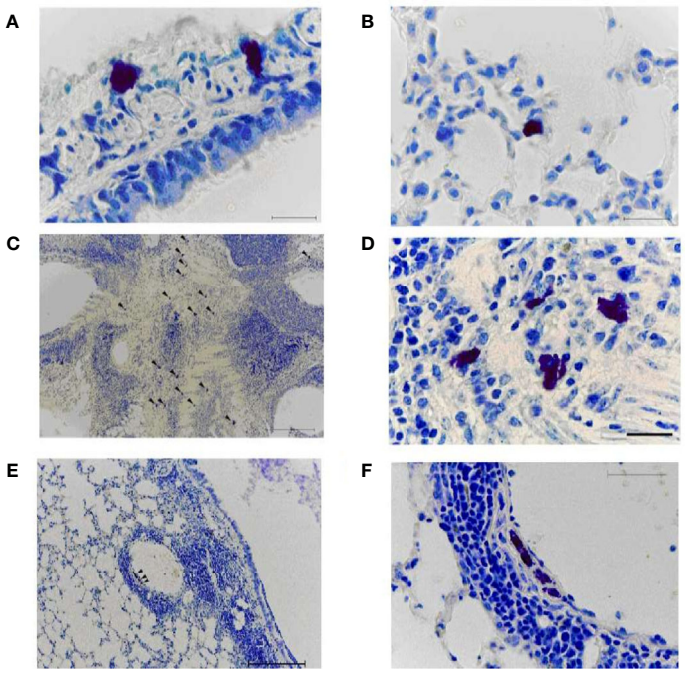


FIGURE 8 | Mast cells in the lungs of vaccinated mice on day 8 after infection with A/Indonesia/5/2005(H5N1) IDCDC-RG2 influenza virus. A, **(B)** The lungs from mock-immunized mice on day 8 after infection with A/Indonesia/5/2005(H5N1) IDCDC-RG2 influenza virus. **C–F**. The lungs from A/Vietnam/1194/2004(H5N1) NIBRG-14¬-immunized mice. The arrowhead indicates a mast cell. Obj. 10x **(C, E)**, 40x **(F)**, and 100x **(A, B, D)**; scale bar = 200 μm **(C, E)**, 50 μm **(F)**, and 20 μm **(A, B, D)**.

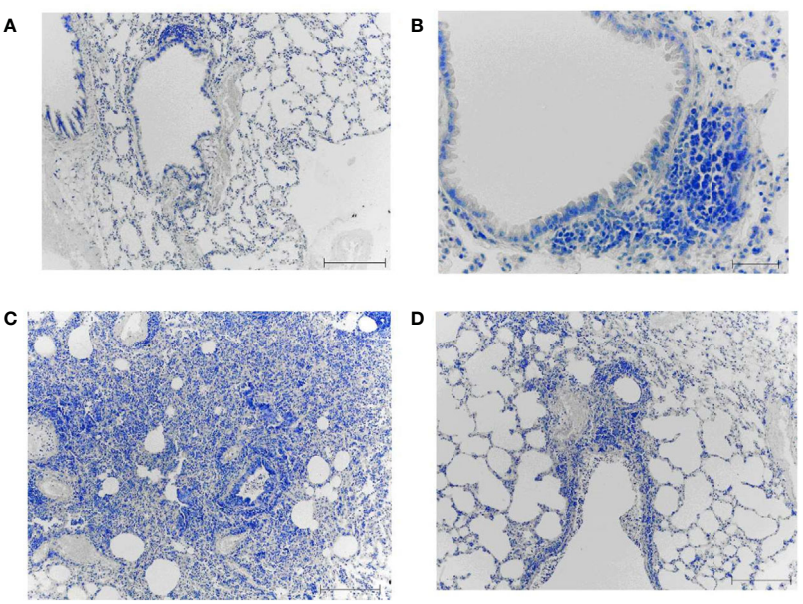


FIGURE 9 | The lungs from non-immunized mice on day 8 after infection with A/South Africa/3626/2013(H1N1)pdm09 influenza virus. A, **(B)** Intact non-immunized non-infected mice. C, **(D)** The PBS-immunized mice infected with A/South Africa/3626/2013(H1N1)pdm09 influenza virus. Obj. 10x **(A, C, D)** and 40x **(B)**; scale bar = 200 μm **(A, C, D)** and 50 μm **(B)**.

In the PBS-immunized A/South Africa/(H1N1)pdm09–infected group, a focal pneumonia was detected (**Figure 9C**). Large areas of atelectasis and emphysema, extensive mononuclear infiltrates in the walls of the airways and blood vessels, interstitial infiltration with thickening of the interalveolar septa were detected (**Figure 9D**).

After A/H1N1–immunized animals were infected with A/South Africa/3626/2013(H1N1)pdm09 infection of AH1N1–immunized animals, extensive mononuclear infiltrates were present in the airway walls (in particular, along their entire length) and around the blood vessels (**Figures 10A, B**). Dystrophic changes in the ciliated epithelium and desquamation of epithelial cells in the bronchi were revealed. Some signs of diffuse alveolar damage including interstitial infiltration with thickening of the interalveolar septa and emphysema were observed. At the same time, when antihistamines were administered simultaneously with infection to the A/H1N1–immunized animals (**Figures 10C, D**), the lung tissue looks the same as in the intact control. Extensive mononuclear infiltrates, dystrophic changes and desquamation of the epithelium were not revealed. No signs of alveolar damage were found. In some airway walls, the presence of bronchus-associated lymphoid tissue was noted (**Figure 10C**). Intact MCs were also identified (**Figure 10D**, arrow).

DISCUSSION

Previously, it has been noted in mouse macrophage cell lines that antibodies against influenza virus hemagglutinin (HA) or neuraminidase (NA), the two main structural components of the viral envelope, were able to induce ADE (18, 26). The enhancing activity was strain-dependent and FcR-mediated (27–29).

Our *in vitro* experiments were carried out not with a purified population of MCs, but with a pool of peritoneal cells, in which the proportion of MCs is about 10% relative to other cell types (lymphocytes, macrophages, neutrophils, eosinophils). MCs are the main cells with an immediate release of histamine. This suggests that the response to the immune complex in our experiments in the form of a histamine release reaction was predominantly in response to the action of MCs.

The A/Vietnam(H5N1) and A/New York(H1N1)pdm09 strains were used as antigens for the formation of immune complexes on antibody-loaded MCs from peritoneal exudate of mice, and for both viruses, the dose of 50 HAU was more effective in stimulating the production of histamine compared to 5 HAU (**Figure 3**). The opposite trend was observed with respect to IgG containing sera when a higher dilution of immune sera caused a higher production of histamine. Thus, in our *in vitro* study of histamine production under the influence of IgG-containing immune complexes, a dose-dependent effect on the concentration of the virus was demonstrated.

Interestingly, in previous studies of antibody-dependent reactions in SARS-Cov-1 *in vitro* it was shown that the more diluted anti-S mouse sera exhibited significantly greater SARS CoV ADE effects on HL-CZ cells compared to less-diluted mouse sera (30).

As is generally known, MCs are the main source of histamine in the body. To block the action of histamine in experiments on mice, we used a mixture of H1 and H2 blockers (although the main type of receptors on cells responsible for the anaphylactogenic effects of histamine are H1 receptors). Our previous data indicate that the introduction of histamine

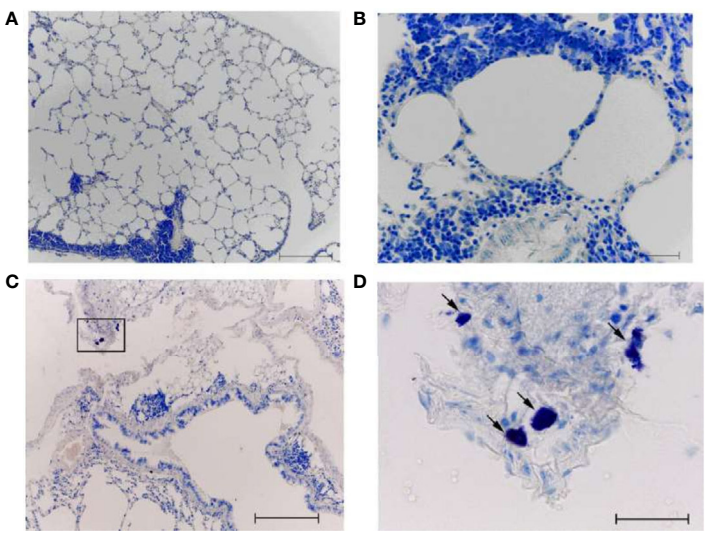


FIGURE 10 | The lungs of A/California/07/2009 (H1N1)pdm09–immunized mice on day 8 after infection with A/South Africa/3626/2013(H1N1)pdm influenza virus. With **(C, D)** and without **(A, B)** antihistamine treatment. Black frame - the area of mast cell localization (D - the same area at high magnification). The arrow indicates a mast cell. Obj. 10x **(A, C)** and 40x (B, D); scale bar = 200 μm **(A, C)** and 50 μm **(B, D)**.

receptor blockers decreased in lethality after A/H5N1 infection of vaccinated mice (31). In the present study, it was shown that after challenge with the A/H1N1 virus, protection reached 90% of the immunized animals and the administration of antihistamines did not make an additional contribution to improving survival. But as estimated by the histological features in the lungs, antihistamines still had a positive effect, since when they were administered, the signs of pneumonia decreased (**Figures 10C, D**). At the same time, the administration of antihistamines to non-immunized mice also facilitated the course of A/H1N1 influenza infection.

The data obtained in our study correlates with earlier studies. Thus, a positive effect of antihistamines on A/H5N1 infection in mice has been demonstrated previously (32). The effect of vaccination on the course of the subsequent influenza infection was also previously studied in animal experiments. The C57BL/6J mice received two doses of subunit vaccine IM 4 weeks apart. One month later, the animals were challenged with a sublethal dose of the A/Hong Kong/2/1968(H3N2) strain. Vaccinated mice showed no clinical symptoms of disease, while unvaccinated animals lost weight from 3 to 7 days after vaccination, but subsequently recovered. After infection with the A/H5N1 virus, unvaccinated animals showed moderate signs of illness for 7 days, after which they recovered. On the contrary, the vaccinated mice developed an infection, accompanied by a critical loss of body weight by 6-8 days after infection, which indicates the formation of immunopathology (33).

Currently, there is a widespread tendency to develop universal influenza vaccines with a wide spectrum of action (34). The induction of a wide range of cross-reactive immunoglobulins is fraught with the risk of an antibody-dependent enhancement of the infectious process, when antibodies that do not have neutralizing activity facilitate the penetration of the virus into cells due to FcγR-dependent endocytosis. Antibodies to M2e have low neutralizing activity, however, they provide protection through FcγR-mediated mechanisms, in particular, through antibody-dependent phagocytosis of viral particles. The formation of an immune response to a limited set of antigenic determinants of the influenza virus does not exclude the emergence of escape mutants, to which the body will not have immunity, which can lead to a large number of serious diseases. An example is previously reviewed cases of increased vulnerability to pandemic A/H1N1 strains among people who received seasonal influenza vaccine (33).

Previously, it was shown that monoclonal Abs that bound the globular head or base of the head domain of influenza HA may induced destabilization of the HA stem domain to increase infection of the macrophage-like cell line in an Fc-dependent manner. Non-neutralizing monoclonal Abs may cause enhanced respiratory disease in mice following A/Hong Kong/1/1968 (H3N2) influenza virus challenge (35). Therefore, *in vitro* tests and animal models need to be developed to confirm preclinical safety of next-generation influenza vaccines that may elicit antibodies which do not block influenza virus–receptor interaction.

CONCLUSIONS

When vaccinated with inactivated influenza vaccines, serum antibodies are the main protective component. There is a gap between vaccination and the formation of a full-fledged antibody response. In the case of viruses with H5 hemagglutinin, the immune response may occur later than in the case of immunization with seasonal influenza viruses. Non-neutralizing antibodies can interact with MCs when infected with the avian influenza virus. Our data on the positive effect of histamine receptor blockers on the course of post-vaccination infection in drifted influenza viruses can help to overcome unwanted effects. The data obtained can be useful in the implementation of vaccines against other viral infections.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The Local Ethics Committee for Animal Care and Use at the Institute of Experimental Medicine, Saint-Petersburg, Russia (protocol № 3/17 of 30.11.2017).

AUTHOR CONTRIBUTIONS

AM – *in vitro* experiments, mouse model, data analysis, manuscript preparation. IL – mouse model, serology, ELISA test, data analysis. DK – histological examination, data analysis, manuscript editing. VG –lung preparations, histological examination, manuscript editing. AP - general leadership, data analysis, manuscript editing. YD - study design, data curation, manuscript preparation, final editing. All authors contributed to the article and approved the submitted version.

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REVIEW
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Mast Cell Modulation of B Cell Responses: An Under-Appreciated Partnership in Host Defence

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Mast cells are well known to be activated *via* cross-linking of immunoglobulins bound to surface receptors. They are also recognized as key initiators and regulators of both innate and adaptive immune responses against pathogens, especially in the skin and mucosal surfaces. Substantial attention has been given to the role of mast cells in regulating T cell function either directly or indirectly through actions on dendritic cells. In contrast, the ability of mast cells to modify B cell responses has been less explored. Several lines of evidence suggest that mast cells can greatly modify B cell generation and activities. Mast cells co-localise with B cells in many tissue settings and produce substantial amounts of cytokines, such as IL-6, with profound impacts on B cell development, class-switch recombination events, and subsequent antibody production. Mast cells have also been suggested to modulate the development and functions of regulatory B cells. In this review, we discuss the critical impacts of mast cells on B cells using information from both clinical and laboratory studies and consider the implications of these findings on the host response to infections.

Keywords: infection, regulatory B cells, allergy, inflammation, CD40

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INTRODUCTION

The ability of mast cells to aid in the initiation and regulation of acquired immune responses has been demonstrated by multiple authors (1–6). As key resident sentinel cells in the skin and at mucosal surfaces, capable of detecting pathogens and tissue damage, mast cells are often one of the first cell types to be activated on pathogen invasion, tissue damage, or infection. Initial responses to bacterial pathogens often result in the production of an NF-κB-dependent cytokine cascade that includes the production of TNF-α, IL-1β and IL-6, as well as other cytokines and regulatory factors. The balance of mediators produced varies considerably depending on the tissue site and stimulus (2, 7–10). These can include immunomodulatory cytokines such as IL-10, as well as IL-1RA and a wide variety of potent chemokines which recruit appropriate effector cells. In response to several viral infections the production of chemokines, along with type 1 interferons (IFN) represent the predominant mast cell response and leads to the recruitment of NK cells and CD56+ T cells (11–20). Mast cells also respond to tissue damage *via* responses to alarmins, such as IL-33, subsequently giving rise to a further unique pattern of mediators including IL-13 and IL-5 (21–23). While degranulation is induced by certain stimuli, such as nematode parasites and select bacteria, mediator production often occurs in its absence. Lipid mediators are also selectively produced in

response to many infections and contribute to cell recruitment and vascular changes. This highly regulated and co-ordinated mast cell response can aid in the mobilisation of dendritic cells that subsequently migrate to draining lymph nodes (2, 7, 24–27), the recruitment of effector cells, and the initiation of an optimal acquired immune response including the production of neutralising antibodies.

In many cases it has been assumed that the interaction of mast cells and B cells is important, but not co-ordinated at the tissue level. Mast cells promote the overall initiation of antibody responses and at the same time mast cells are guided and enhanced in their responses by IgE or IgG subclasses bound to Fc receptors on their surface. However, increasing evidence suggests that the relationship between mast cells and B cells is much deeper and more complex, providing potential opportunities for therapeutic intervention. In this review we have selected just some of these proven and potential interactions to highlight and illustrate the complexity and importance of the mast cell-B cell relationship.

RECEPTOR-LIGAND INTERACTIONS BETWEEN MAST CELLS AND B CELLS

The potential and proven interactions between mast cells and B cells are complex and multifaceted. In considering these, it is important to distinguish between evidence obtained from human studies and those observed in rodent models. The use of mast cell lines without confirmation using primary mast cells in some studies also means that findings need to be interpreted with caution. Interactions between mast cells and B cells are summarised in **Figure 1**, including the important cell contact-dependent and mediator-dependent interactions.

CD40/CD40L

The CD40/CD40L interaction is pivotal to the regulation of antigen presentation, T cell-dependent class-switching, memory B cell development, and germinal centre formation (28). The early recognition that mast cells express CD40L suggested additional roles for mast cells in modifying B cell functions. This included the demonstration that mast cells can promote B cell class-switch to IgE production *via* a CD40/CD40L-dependent mechanism in the presence of IL-4 (29).

Signalling through CD40 was also shown to increase B cell proliferation by physical cellular contact (30). CD40L-expressing mast cells can enhance CD40/CD40L communication by promoting CD40-expression on B cells (30). Moreover, CD40L can be upregulated on mast cells through the actions of invariant NKT (iNKT) cells. iNKT cells recognise CD1d on the surface of murine mast cells and trigger the upregulation of CD40L, which can subsequently stimulate IgE class-switch by B cells to enhance allergic airways responses (31).

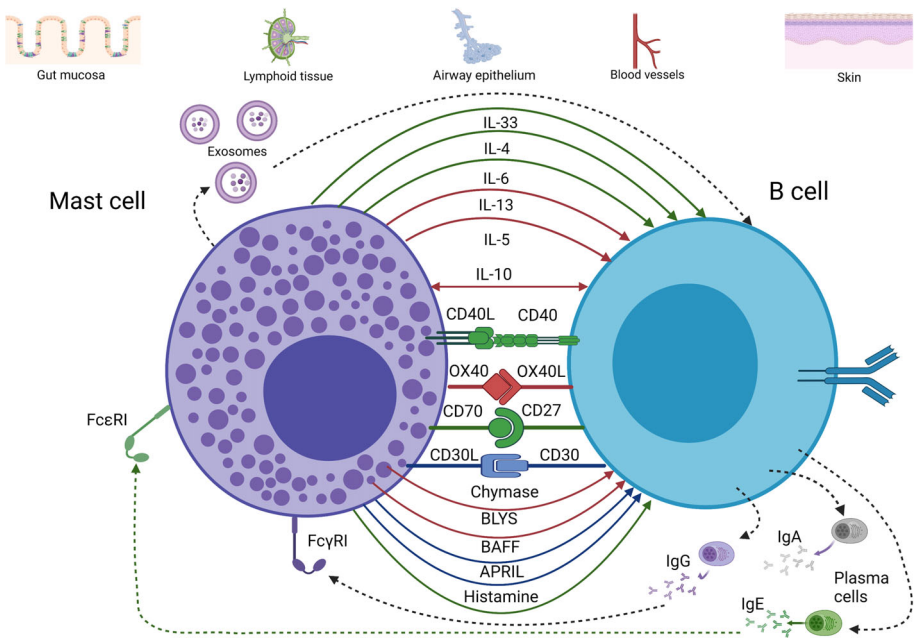

FIGURE 1 | Major pathways of communication between mast cells and B cells. Evidence of cytokine and receptor-ligand interaction between mast cells and B cells has been depicted according to the following color scheme: red for evidence found in rodents, blue for evidence found in humans, and green for rodents and humans. Interaction between mast cells and B cells can occur at mucosal sites as well as at lymphoid and vascular tissues (although less frequently than at the mucosa). This is achieved by a broad array of cytokines (mainly type 2 cytokines, IL-10, IL-6, and IL-33), membrane-bound receptors and ligands (e.g., CD40/CD40L), and granule products such as histamine and proteases. These interactions can promote B cell proliferation, survival, class-switch to IgA or IgE, among other impacts. In addition, exosomes from both mast cells and B cells may be involved in communication between these cells. (*Figure was prepared using BioRender*).

The CD40/CD40L-axis seems to play a role in local immunosuppression and immune tolerance, as it is implicated in the generation of IL-10 secreting B cells, as shown by Mion et al. (32). Indeed, the presence of mast cells enhances the development of B cells capable of producing IL-10 when appropriately stimulated, known as "IL-10 competent B cells". Mast cells do not selectively enhance IL-10 production, on a per cell basis, but have a key role in enhancing expansion of regulatory B cell (Breg) subsets producing this anti-inflammatory mediator (32). Breg cell generation could be enhanced without direct cell contact, as exosomes from mast cells contain CD40L.

The ability of mast cells to enhance Breg development *via* a CD40-dependent mechanism also appears to be dependent on the anatomical site or other microenvironmental factors. In mice, the presence of mast cells does not enhance Breg differentiation in the spleen or peritoneal cavity but is important in the colon (32). This may be related to the presence of microbial factors in the intestine that are known to activate mast cells (33–35). This type of mast cell-B cell cross-talk may be more important in reducing local inflammatory responses at sites of microbial challenge and ensuring appropriate responses to damage or infection. In the context of allergic disease, mast cells and B cells have also been shown to co-localise in the airway epithelium of ovalbumin (OVA) challenged mice. Both CD40 and CD40L expression were upregulated in this setting possibly due to upregulation of Transglutaminase 2 triggered by antibody-antigen stimulation of mast cells (36). CD40 has also been shown to be expressed by airway epithelial cells (37), where it has been implicated in promoting T cell activation. These observations suggest that blockade of CD40/CD40L interactions in the allergic airways might have multiple consequences for local immune and inflammatory regulation. Notably, mast cells do not appear to have a key role in the development of mucosal tolerance, at least in adult animals (38).

OX40/OX40L

Human mast cells from several tissues including the airways express OX40L (39), and this has been shown to provide a mechanism whereby mast cells may promote T cell responses. As mentioned previously, Hong et al. (40) have demonstrated that mast cells and B cells co-localise in the lung epithelium of OVA-sensitised mice. Moreover, expression levels of OX40/OX40L and CD40/CD40L were elevated. Inhibition of these pathways decreases the levels of OVA-specific IgA and IgE and reduces antigen-dependent mediator-release by mast cells. This model shows how B cells are activated by mast cells through the CD40/CD40L pathway as well as the OX40/OX40L-axis, in the presence of appropriate cytokines such as IL-4, IL-13, IL-6, and TGF-β. These signal through the TRAF2-MEKK1 and TRAF60-TAK1 signalling pathways, respectively, to induce B cell class-switch into IgA and IgE secreting cells. Enhanced IgE could provide positive feedback by stimulation of FcεRI in mast cells, which in turn would increase mediator production and release. In addition to these more direct interactions between mast cells and B cells through OX40-dependent mechanisms there are a host of impacts that may result from mast cell-T cell interactions through either cell-cell contact or exosomes. For example, it has been clearly demonstrated that mast cells can limit the actions of regulatory T cells (Tregs) and promote Th17 production *via* an OX40/OX40L and IL-6-dependent mechanism (41).

CD30/CD30L

Mast cells can express both CD30 and CD30L. CD30 expression on mast cells is often associated with mastocytosis or chronic inflammation. In contrast, CD30L expression by mast cells is more consistently observed in a variety of tumours and tumour-draining lymph nodes (42). Molin et al. [74] demonstrated that human mast cells interact with Reed-Sternberg lymphoma cells through the CD30/CD30L axis in Hodgkin's Lymphoma, leading to an increase in proliferation of the latter. It has also been shown that CD30L-signalling induces mast cells to produce chemokines such as CXCL8 without evidence of degranulation or lipid mediator production (43). However, the ability of this interaction to induce chemokines that would induce B cell migration has not been directly examined. Only very specific subsets of B cells in the germinal centre and extrafollicular environment normally express CD30. Notably, the human CD30+ extrafollicular B cells are a subset of active memory B cells (44). It is plausible that mast cells also interact with these B cell populations to promote proliferation under some circumstances.

CD27 and CD52

Early mast cell progenitors have been described as CD27+ (45) and mast cells ex vivo have also been described to express CD70. This was particularly studied in patients with Waldenström macroglobulinemia, a form of lymphoplasmacytic lymphoma. In this setting, soluble CD27 produced by lymphoplasmacytic cells upregulated CD40L on mast cells (30). CD40/CD40L interactions can promote the proliferation of malignant cells and have therefore been implicated as a negative factor in disease progression. It is plausible that this mechanism may extend outside of malignancy, perhaps to a subset of antibody-producing cells.

Both mast cells and B cells express the 12-amino acid GPI linked peptide CD52 (46), which is thought to have a role in retaining cell mobility. CD52 can ligate with Siglec-10 found on mast cells in addition to sialic acid. However, the precise role of this interaction is unknown. Siglec-10-signalling *via* CD24 can reduce responses to DAMPs and inhibit responses to tissue injury in humans (47). This may provide a mechanism whereby local inhibition of inflammation may result from mast cell-B cell cross-talk. However, given the wide range of cell types expressing these molecules it may be a more general method to regulate immune responses in certain tissues.

MAST CELL MEDIATOR IMPACTS ON B CELLS

In response to a variety of stimuli including pathogen-associated molecular patterns (PAMPs) or damage-associated patterns

(DAMPs), mast cells selectively produce a subset of their wide array of potential mediators. These include preformed granule-associated products such as proteases and histamine, newly formed lipid mediators such as LTC_4 and PGD_2, and over 40 different cytokines and chemokines. The combinations, timing and range of such mediator production is dependent on the nature of the acute stimulation, the microenvironment, and the mast cell subpopulation. Many mast cell mediators can directly or indirectly modify B cell recruitment, function, or differentiation. The following section will outline mediators important in the setting of acute or chronic infection.

Histamine

Histamine is a biogenic amine released by mast cell granules during allergic reactions and in response to multiple other stimuli that induce mast cell degranulation such as tissue injury, and responses to certain pathogen products. The impact of histamine on immunity has been extensively studied as reviewed by Akdis and Blaser (48). In mice, deletion of H1R resulted in suppression of IFN-γ and enhanced secretion of the type 2 cytokines, IL-4 and IL-13, with subsequent impacts on B cell responses (49). B cells express both H1 and H2 receptors that impact cellular functions. Early reports demonstrated that H1-signalling with IgM/antigen-stimulation enhanced splenic B cell proliferation (50). Kimata et al. (51) showed that B cells from healthy donors treated with anti-CD58 plus IL-4 or IL-13 enhanced IL-6 and IL-10 production when concurrently treated with histamine. This in turn, selectively increased IgE and IgG4 secretion. However, as reviewed by Merluzzi et al. (52) the impact of adding histamine in B cell culture systems has reportedly been variable, possibly due to its short half-life and the presence of histamine degrading enzymes.

H2 receptors on human cells including B cells are endogenously active, so while histamine can enhance their activity, important clues to their regulatory function can often be best obtained by using H2 antagonists or through studies of receptor-deficient cells or animals. Notably, the widely used H2 antagonists, ranitidine and famotidine have been shown to have some significant impacts on B cell activity. For example, ranitidine reduced tumor growth *via* a B cell-dependent mechanism in murine models of breast cancer (53). More recently, Meghnem et al. (54) showed that high dose ranitidine inhibited the number of circulating CD19+ B cells in 29 healthy human subjects. It is not known if chronic histamine stimulation in the context of allergic disease enhances such B cell populations.

Proteases

Mast cell proteases are released in large amounts from activated mature and immature mast cells, particularly during degranulation. They have a wide variety of important functions in aiding host defence and enhancing the function of numerous cytokines through protease-mediated activation during infection, especially at mucosal surfaces. These include TGF-β family members that play key roles in regulating B cell development and in promoting IgA class-switch essential for the appropriate immune protection of mucosal surfaces [reviewed in (55)] as well as impacts on the activity of other inflammatory cytokines such as IL-33 (56–58). Chymase enzymes from mast cells have also been shown to induce B cell secretion of IgG1 and IgE as shown by Yoshikawa et al. (59) using rat mast cell protease II, although the mechanism for this is unclear.

Tryptase production by mast cells can also influence local mediator production and the cellular microenvironment. Moreover, tryptase can activate several protease-activated receptors such as protease activated receptor 2 (PAR2). Xue et al. (60) showed that B cells constitutively express PAR2, with levels increasing in allergic rhinitis. After PAR-2 activation, signalling through Bcl2L12 leads to IL-10 transcription repression and reduced IL-10-expression by B cells from patients with allergic rhinitis. The fact that tryptase is an important activator of PAR2 adds to the important role of mast cells in an allergic setting and raises the possibility of reduced tolerogenic responses following mast cell degranulation through inhibition of IL-10 production by B cells. It remains to be discovered if this mechanism occurs in other tissue settings where mast cells and B cells co-localise such as the gut.

IL-6

Mast cells can be a rich source of IL-6 in response to certain infections. For example, when activated with high doses of *Escherichia coli* lipopolysaccharides (LPS), rodent mast cells have been reported to produce more IL-6 on a per cell basis than similarly treated macrophages. IL-6 has several roles in regulating B cell and plasma cell development and was originally described as a B cell differentiation factor. IL-6 is crucial for development of immunoglobulin-producing plasma cells and in some cases, class-switching. Merluzzi et al. (30) have shown that mast cells promote B cells differentiation into plasma cells with an IgA isotype through IL-6 secretion, which suggests that B cells can class-switch to IgA without T cell help. This process may be particularly important in the context of mucosal infections and host defence in airways. Another IL-6 family member, leukemia inhibitory factor, produced by mast cells (61), has been shown to selectively activate B1a cells in mice (62). It has also been suggested that in the presence of microbial stimulation IL-6 can promote the generation of Bregs (63). This adds to the complexity of the potential impact of mast cell IL-6 production following microbial breach of the epithelial barrier or mast cell contact with bacterial products.

Mast cells can also impact tissue remodelling events through IL-6. As described by Breitling et al. (64) in a murine pulmonary hypertension model, mast cells promoted vascular remodelling of the pulmonary artery of rats. A genetic analysis of lung samples from these rats revealed increased immunoglobulin gene products relative to controls, indicating a link between mast cells and immunoglobulin production. Upon further investigation, mast cell-derived IL-6 proved important. Pulmonary hypertension was improved by IL-6 inhibition, although increased mast cell density persisted when IL-6 was diminished. The depletion of B cells with anti-CD20 as well as the use of B cell-deficient mice also improved pulmonary hypertension. Along those lines, autoantibody levels as well as vascular remodeling decreased after oral ketotifen treatment, a mast cell stabiliser. Further information on mast cell/B cell

communication will be crucial for devising novel strategies to treat pulmonary hypertension.

Interferons and B Cell Chemoattractants

Mast cells are not thought to be a significant source of type 2 IFNs (e.g., IFN-γ), although they can produce it under some circumstances (65). Mast cells are, however, an important source of type 1 IFNs following viral infection (11, 12, 18). The IFN response of human mast cells to viral infection can be enhanced by IL-4 (66) with potential to modify B cell activity. It has been demonstrated that type 1 IFN-signalling in B cells can lead to the loss of tolerance and the development of autoreactive B cells (67). Type 1 IFN responses by mast cells are only one of a number of possible routes by which they may enhance antibody generation in autoimmune disease, such as their enhancement of anti-citrullinated protein antibodies in rheumatoid arthritis (68). This role for IFNs is best studied in the context of systemic lupus but could potentially also enhance responses to environmental allergens at sites such as the nasal mucosa or intestine which are prone to viral infection.

Human mast cells are also an important source of type 3 IFNs such as IL-29 during select viral infections and upon activation by specific viral-associated products such as double stranded RNA. IFN-λ has been demonstrated to enhance the differentiation of naïve B cells into plasmablasts *via* the mTORC1 pathway (69). While the contribution of mast cells to this and other IFN-dependent modulation activities on B cells is likely small within classical draining lymph node sites, it may be of greater importance at sites of mast cell-B cell co-localisation and viral exposure, such as the nasal mucosa, intestinal lamina propria, or inflamed skin.

In environments with high levels of IFNs such as sites of viral infections, mast cells can produce large amounts of several lymphocyte chemoattractants (70). Indeed, mast cells can produce CXCL10 in the context of reovirus, dengue virus, influenza, and RSV infection (71). CXCL10 has B cell and T cell chemoattractant abilities in addition to multiple other impacts. CXCL13 is also recognised as a critical B cell chemoattractant and although not widely studied from mast cells has also been reported to be produced following reovirus infection of human cord blood-derived mast cells (13).

IL-33 and Type 2 Cytokines

Mast cells can be a significant source of type 2 cytokines that impact B cell development and function. For example, the production of IL-4 by mucosal mast cells in allergic rhinitis may enhance IgE class-switch by B cells, albeit to a lesser degree than IL-13 (72). Local microbial stimulation likely contributes to such mast cell activation at mucosal settings. IL-33, a member of the IL-1 receptor family, has also been reported to strongly influence B cells. IL-33 has been reported to induce activation of murine B1 cells through the ST2 receptor and drive the production of CCL2 and CCL3, chemokines involved in the trafficking of monocytes, macrophages, and other effector cells. Stimulation of B1 cells with IL-33 also generates the production of angiogenic factors such as VEGF and GM-CSF (73). IL-33 also stimulates IL-5 production and secretion in both mast cells and B cells, which leads to paracrine and autocrine stimulation through the IL-5 receptor to promote B cell proliferation, maintenance of Bregs, and antibody production (52). Overall, mast cells both produce IL-33 and respond to this alarmin through the production of similar mediators. Therefore, it possible that mast cell/B cell interaction at mucosal sites can perpetuate local inflammatory responses in specific settings through production of cytokines and angiogenic factors.

BAFF and APRIL

Mast cells not only influence B cells in terms of class-switching and differentiation but also produce soluble mediators from the TNF ligand family that enhance B cell survival and limit apoptosis. As shown by Wang et al. (74), among many cell types, mast cells produce B cell activating factor (BAFF) in the ectopic lymphoid tissue of nasal polyps. Increased BAFF production may promote B cell survival to potentially promote ectopic lymphoid tissue formation. In support of this, another related member of the TNF ligand family, A Proliferation-Inducing Ligand (APRIL) along with B-Lymphocyte Stimulator factor (BLYS) can promote the survival of lymphoplasmacytic cells in Waldenström macroglobulinemia (75). Mast cells produced APRIL in response to CD70 stimulation through CD27 (75). The activities of these molecules, together with CD40/CD40L interactions support the importance of a range of TNF family members in mast cell/B cell interaction.

Exosomes

Mast cells may also modulate B cells through transfer of exosomes. Mast cell-derived exosomes can harbour multiple molecules highlighted herein (e.g., CD40 and CD40L) and others such as CD86, MHC II, LFA-1, and ICAM-1 (76). Exosome secretion may be dependent on IL-4 and mast cell maturity. Paradoxically, exosomes from mast cells have been shown to induce secretion of IL-2, IFN-γ and IL-12, skewing the immune response to type 1 cytokine polarisation. This indicates that mast cells can broadly shape immune responses (e.g., allergic reactions) including the intensity through their exosome contents. Despite strong evidence of exosome importance for modifying B and T cell activities *in vitro* (77–79), the role of mast cell exosomes has yet to be conclusively demonstrated *in vivo*, particularly in settings of infection.

MAST CELL/B CELL INTERACTION AND CO-LOCALISATION

Mast cells are resident tissue cells observed in high density at mucosal surfaces and in the skin. However, they are also found throughout the body, mainly in association with blood vessels. Mast cells are present in lymphoid tissue, but usually not as a major resident population. However, mast cell migration to inguinal lymph nodes has been reported in the context of early inflammatory responses (e.g., following UV exposure) (80–82). Mast cells are also found in close-proximity to B cells in tonsil and Peyer's patches (81–83) (**Figure 2**). At sites of ectopic

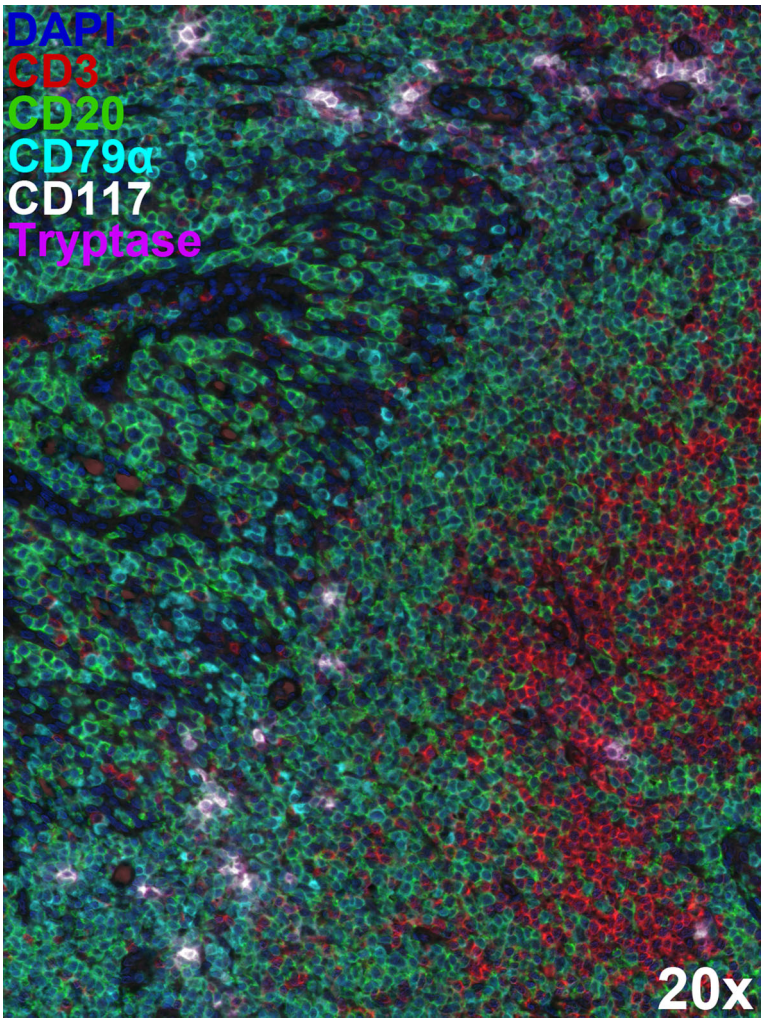

FIGURE 2 | Extensive mast cell/B cell co-localisation within lymphoid tissue. Multiplex immunostaining using Opal™ technology (Akoya Biosciences) identified $CD20^{+}CD79\alpha^{+}$ B cells (green and cyan), $tryptase^{+}CD117^{+}$ mast cells (magenta and white), and $CD20^{-}CD3^{+}$ T cells (red) within formalin-fixed, paraffin-embedded 5 µm-thick human tonsil sections. The 6-plex panel (including DAPI counterstaining) scans were acquired on the Mantra 2™ Quantitative Pathology Workstation using pre-defined parameters at 20x original magnification and spectrally unmixed using inForm® software (Akoya Biosciences). Representative image illustrating close-proximity between mast cells and B cells within tonsil architecture. Mast cells appear less in T cell dense areas.

lymphoid tissue development, such as in the airways during chronic allergic disease (84) and in the joints during rheumatoid arthritis (68, 85), mast cells and B cells are also found within the same microenvironment at high density, potentially as a result of the disease (**Table 1**).

This co-localisation may be functionally important. Early studies demonstrated that mast cells could effectively promote B cell function and proliferation (86, 87). There is evidence for interaction between mast cells and B cells at the nasal mucosa of patients with allergic rhinitis. Mast cells have been shown to induce IgE synthesis by purified tonsillar B cells in response to antigens without the need for exogenous IL-4 or IL-13. Mast cells in this context were reported to have enhanced expression of FcεR1, CD40L, IL-4, and IL-13 (72). *In vitro*, Palm et al. (88) demonstrated in mice that degranulated (and to some extent resting) mast cells enhanced B cell activation including elevated expression of CD19, MHC II, CD86, and L-selectin. Such activated B cells also secreted greater amounts of IgG and IgM. In the intestine, a different impact of mast cell-B cell interaction has been suggested. Mast cells and B cells co-localise in the lamina propria of the intestine of individuals with inflammatory bowel disease and an association has been reported between the presence of mast cells and elevated IgA secretion (30). These authors also clearly demonstrated the presence of mast cells and B cells in human lymph nodes undergoing reactive hyperplasia. Mast cells were particularly localised surrounding follicles and in the paracortical and medullary regions of the lymph nodes, raising the possibility of interaction with recirculating naïve B

TABLE 1 | Sites of close-proximity between mast cells and B cells in multiple hosts.

Sites of close-proximity	Mast cell identifier(s)	B cell identifier(s)	Host	Citation(s)
Gut mucosa	Tryptase$^+$	CD20$^+$	Human	Merluzzi et al., 2010 (30)
Airway epithelia	CD117 (c-Kit)$^+$	CD23$^+$	Mouse	Hong et al., 2013 (36)
Lungs	Tryptase$^+$	CD45RA$^+$	Rat	Breitling et al., 2017 (64)
Inguinal lymph node	Toluidine blue$^+$	CD19$^+$	Mouse	Byrne et al., 2008 (80)
	Tryptase$^+$	CD20$^+$	Human	Merluzzi et al., 2010 (30)
Tonsil	CD117$^+$	CD20$^+$	Human	Rivellese et al., 2018 (68)
	Alcian blue$^+$		Human	He and Xie 2005; He et al., 2005 (81, 82)
Ectopic lymphoid tissue	Tryptase$^+$	CD19$^+$	Human	Zhai et al., 2018 (84)
	CD117$^+$	CD20$^+$	Human	Rivellese et al., 2018 (68)

cells and activated post germinal centre B cells. Using a co-culture of primary mast cells and B cells, the presence of mast cells promoted the survival and proliferation of B cells through an IL-6-dependent mechanism which also required cell-cell interactions such as CD40/CD40L (30). In contrast to these findings, it has also been suggested that mast cells within lymph nodes may have more suppressive immunomodulatory functions which limit B cell responses. For example, Chacon Salinas et al. (89) have demonstrated that the production of IL-10 by mast cells can indirectly disrupt germinal centre formation *via* impacts on T follicular helper cells. The production of IL-10 and IgA by B cells has also been implicated in reducing neuroinflammation (90) and inflammatory responses at mucosal surfaces (91). Thus, any impact of mast cells in modifying such responses could have critical downstream impacts on the mucosal immune environment.

Outside of secondary and ectopic lymphoid tissues there are other environments where mast cells and B cells may be concentrated. These include the previously mentioned lamina propria of the intestine as well as several tumour settings. Bone marrow studies in patients with Waldenström macroglobulinemia have shown an increased number of mast cells, where they are thought to contribute to tumour growth as well as angiogenesis, as shown by Ahn et al. (92). In addition to an increase in mast cell density, the proportion of CD40L+ mast cells correlated with poor tumour prognosis, illustrating the potential cross-talk between B cells (albeit abnormal ones) and mast cells through the CD40/CD40L axis. A similar link has been suggested in the setting of multiple myeloma as shown by Pappa et al. (93). Neoplastic B cells may also modify the activities of mast cells such as recruitment and activation. This is in addition to a multitude of less B cell specific interactions between mast cells and tumours that occur in tumour settings (94–97). For example, Fischer et al. (98) have shown that neoplastic cells from Hodgkin's Lymphoma secrete CCL5 at sites of infiltration to recruit mast cells. Mast cells may also impact other aspects of the tumour microenvironment. For example, increased numbers of mast cells are found in nodular sclerosing forms of Hodgkin's lymphoma, associated with tissue fibrosis and the presence of IL-13 expressing Reed Sternberg cells. Moreover, mast cells were associated with greater disease progression and increased micro-vessel density in primary cutaneous B cell lymphomas and have potential as a prognostic marker (99).

MAST CELLS AND CHRONIC INFECTION

Parasite Infections

Mast cells have important roles in many globally important parasite infections, although their contribution differs with the infecting microorganism. Helminth gastrointestinal infections are associated with increased mast cell density and activation at the site of infection, which contributes to epithelial sloughing and increased motility (100–102). Mast cells may also contribute to tissue repair through impacts on fibroblast activation and tissue remodeling. Mast cell proteases are directly toxic to many helminths (103) and the granule product chondroitin-sulphate has also been reported to be active (104) in preventing nematode adhesion and penetration of the intestinal mucosa. Helminth infection-associated mast cell activation likely also modifies the responses of local B cells and plasma cells through mediator impacts, some of which are detailed below.

Mast cells participate in shaping type 2 immune responses to many nematodes through the release of IL-33, IL-25, and thymic stromal lymphopoietin. As shown also by Hepworth et al. (100, 105, 106), mast cell-deficient mice had an impaired type 2 immune response against helminths. Enhanced type 2 immune responses could indirectly alter B cell responses and contribute to the high IgE levels observed in many such infections. In secondary infections, the presence of specific IgE may enhance local mast cell responses enabling more rapid infection clearance (100, 106).

Infections by *Schistosoma mansoni*, a parasite mainly found in Africa and South America, are known to induce polyclonal B cell responses. *S. mansoni* produces glycoproteins recognised by galectin-3 that induce strong antibody responses. In a chronic schistosomiasis murine model developed by Oliveira et al. (107), mast cell degranulation was suggested to be one of the drivers for IgA class-switch and subsequent antibody production by peritoneal B1 cells. This process was regulated by galectin-3. Even though these findings are not yet completely understood, they highlight complex interactions between mast cells and B cells in parasite infections.

It is important to stress that many mast cell effector functions in response to chronic or repeated infection require the presence of B cells and antibodies. This was formally demonstrated by Matsumoto et al. (108) using activation induced cytidine deaminase (AID)-deficient mice, which despite the presence of

intact T and mast cell compartments demonstrated delayed elimination of *Strongyloides venezuelensis*. Enhanced expulsion was restored with the addition of IgG1 and IgE to AID-deficient mice indicating the importance of antibody production. Antibody enhanced expulsion was shown to be mediated by mast cells, which underscores the importance of mast cell stimulation through their Fcγ and Fcε receptors (108).

While the role of mast cells and B cells in helminth infections is well established, their role in protozoan infections is less clear. In infections by *Plasmodium* spp., mast cell-derived TNF-α was shown to be important for the clearance of infection and protection against cerebral involvement. However, mast cell degranulation, increased concentrations of histamine and higher levels of IgE against *Plasmodium falciparum* were associated with worse outcomes. Similarly, in the setting of cerebral malaria, mast cell degranulation involved histamine release, increased vascular permeability, enhanced endothelial damage, and lead to the release of VEGF, which associated with worse outcome (109).

In infections by *Leishmania* spp., the participation of mast cells seems to depend on the species of the parasite as a first line of defence, without a clear interaction with B cells. As shown by Naqvi et al. (110), mast cells can phagocytose *L. tropica* which causes cutaneous leishmaniasis but not *L. donovani*, which causes visceral leishmaniasis. Both species are susceptible to killing by extracellular traps created by mast cells, but *L. tropica* is more vulnerable. These observations highlight differential roles for mast cells depending on the type of leishmaniasis.

In Chagas disease, there have been descriptions of co-existence of mast cells and B cells in heart biopsies of patients with dilated myocardiopathy. The presence of mast cells in the heart and intestine has been associated with worse prognosis, possibly implying ongoing inflammation and fibrotic processes involving mast cells (111–113).

Viral Infections

Mast cells have been implicated in the host response to multiple viral infections. Multiple viral associated stimuli can induce the production of type I and III IFNs, chemokines, inflammatory cytokines as well as factors involved in tissue remodelling such as VEGF (11, 17, 70). Such interactions occur in the absence of B cells or antibody in many cases. For example, infections with reovirus, respiratory syncytial virus, adenovirus, and influenza of human mast cells have been reported, leading to substantial mediator release (11, 13, 16, 114–116). These types of responses are often the result of signalling through RNA or DNA sensors such as TLR 3, 7, 8 or 9, RIG-I, MDA-5, and STING pathways (12, 117, 118). Mediators produced can include both B cell chemoattractants and cytokines which act on B cells, such as IL-6. However, antibody mediated events can also play a key role in these processes for some viruses.

Antibody-dependent enhancement occurs when sub-neutralising levels of antibody facilitate the entry of virus into the cell *via* Fc receptors. For mast cells this process has been described for Dengue virus infection, mediated by low concentrations of IgG *via* FcγRII (15, 119) where it can induce cytokine and chemokine production, and mast cell apoptosis (120). IL-1β and TNF-α which have been implicated in vascular damage and endothelial dysfunction (121) are also produced by infected human mast cells. Mast cell activation in this clinical context has been implicated in Dengue Hemorrhagic Fever and Dengue Shock Syndrome (122–124).

Antibody-dependent enhancement of infection of mast cells may also be a factor in response to subsequent infections with other viruses such as Zika virus (116) and has most recently been suggested as a factor in SARS-CoV-2 infection (125, 126). It likely also occurs for other viruses under specific circumstances where antibody is not sufficient for neutralisation.

Antibody-mediated processes in viral infections can also activate mast cells *via* Fc receptor cross-linking leading to degranulation, lipid mediator production, and subsequent longer-term cytokine and chemokine production. The nature of the responses is highly dependent on the class and subclass profile of antibodies produced in response to infection. In many cases the type 1 cytokine response to viruses does not support a strong specific IgE response, but given the long half-life of such antibodies on mast cells in sites such as the skin and airways there is potential for IgE mediated events to enhance events such as the mobilisation of dendritic cells (24, 26) leading to enhanced B and T cell responses in draining lymph nodes. During HIV infections, mast cells and their precursors may also harbour virus which can be reactivated through either TLR- or antibody/Fc receptor-pathways (114, 127, 128). Indirect processes such as complement product (C3a, C5a) mediated activation of mast cells may also occur in response to antibody complexes with viral products.

Atopy and Bacterial Infections

Mast cells and B cells are both important players in effective responses against bacterial pathogens. Mast cells function as critical sentinel cells during the early stages of infection and promote the recruitment of effector cells to sites of infection. On the other hand, the B cell mediated antibody response is key to combatting longer-term and secondary infections. Interplay between these cell types can therefore generally be seen as positive for anti-bacterial host defence. However, this is not always the case. The skin of individuals with atopic dermatitis is frequently colonised by *Staphylococcus aureus*. This suggests that *S. aureus* may contribute to the pathophysiological events that culminates in atopic dermatitis. Interestingly, mast cells and B cells may partner in driving this disease. A report using an atopic dermatitis mouse model showed that mast cell-deficient mice inoculated with wild-type *S. aureus* and challenged with ovalbumin had reduced skin disease and serum IgE than wild-type mice. Mast cell contribution may be through their degranulation products, as skin-derived murine mast cells degranulate in response to *S. aureus*-derived δ-toxin, which is enhanced if mast cells are first primed with B cell-derived IgE (129). Other Gram-positive bacterial-derived products promote mast cell production of type 2 cytokines known to induce atopic dermatitis features such as IL-13, as well as proinflammatory

TNF-α and IL-6. Patients with atopic dermatitis have increased blood IgE production signatures in blood and higher frequency of blood and lesional B cells compared to control groups (130) but the precise contribution of mast cells to this response is unknown.

In related studies considering superantigens as triggers for atopic disease, Schlievert et al. (131) analysed the response of keratinocytes to bacterial superantigens and found an enhanced production of chemokines and cytokines. Among them, IL-33 was notably increased. Mast cells can also respond to IL-33 producing a number of type 2 cytokines which could promote class-switch to IgE and antibody generation. Taken together, these studies indicate an interplay between staphylococci colonisation, superantigen stimulation, mast cell stimulation and therefore B cell stimulation, and production of IgE which in turn could produce a positive feedback on mast cell degranulation.

Hypersensitivity and Fungal Infections

Interaction between B cell and mast cells could also be an influencing factor in immunity and inflammation in response to several fungal infections. Histamine release from mast cells is a frequent feature of cutaneous and mucosal fungal infections. Allergic bronchopulmonary aspergillosis (ABPA) has been mainly reported in patients with asthma and cystic fibrosis where the production of IgE towards *Aspergillus* spp. spores by B cells leads to the activation of mast cells (132). *A. fumigatus* antigen Af1 is presented through MHC II to Th2 lymphocytes (132) eliciting production of IL-4 and IL-13. This type 2 cytokine rich environment promotes class-switch to IgE. As with other pathogens, IgE bound to mast cells can mediate mast cell activation to *Aspergillu*s spp. antigens in secondary or chronic infections (133). It is also worth noting that *Aspergillus* spp. can induce mast cell degranulation independently of IgE in rodents, without causing any damage to the hyphae (134) but more damage to the airway mucosa.

While the fungus itself produces damage in the airway epithelium, the release of mast cell proteases and the recruitment of eosinophils contribute substantially to remodelling of the airway in response to such infection (135). Eosinophils release their toxic granular proteins while mast cells release tryptase and both of them activate and promote the production of TGF-β. This cytokine induces bronchial fibroblasts to differentiate to myofibroblasts that directly induce in the remodelling of the airway wall (135). Further corroborating the involvement of mast cells driven by IgE in ABPA, treatment with omalizumab (a monoclonal antibody targeting the high-affinity receptor binding site on human IgE and thereby reducing mast cell sensitisation) has been shown to be effective for patients with severe allergic asthma and ABPA (136).

Cross-talk between mast cells and B cells has also been shown to be important in *Malassezia* spp. infections. *Malassezia* spp. are a group of opportunistic fungi that grow mainly in skin areas with abundant sebaceous glands. They have been implicated in the pathogenesis of atopic eczema, seborrheic dermatitis and pityriasis versicolor (133). Selander et al. have shown, using a rodent model, that *Malassezia sympodialis* can activate both non-sensitised and IgE-sensitised mast cells (137). While IgE-sensitised mast cells degranulate, release cysteinyl leukotrienes, cytokines and chemokines when stimulated with extracts of *M. sympodialis*, non-sensitised mast cells selectively release leukotrienes without degranulation (137). This activation is induced through the TLR2/Myd88 and MAPK pathway. Although the *in vivo* impact of such responses is not well studied, cysteinyl leukotrienes can act through Cys LT1 receptors on B cells to enhance immunoglobulin production *in vitro* (138). Taken together, these findings support the idea of a mast cell response to *Malassezia* spp. enhanced by IgE produced by B cells in atopic dermatitis. This would be expected to both promote local inflammation and effector cell recruitment and potentially enhance the development and maintenance of an acquired immune response to infection through impacts on dendritic cells and draining lymph nodes.

Some similar mast cell responses have also been observed in response to *Candida albicans*. Mast cells can respond directly to this pathogen through both TLR and dectin-1 mediated pathways, giving rise to both lipid mediator and cytokine responses, often without degranulation. These responses may promote the generation of acquired immunity. A link has also been established between *Candida* spp. colonisation of the skin and exacerbation of atopic dermatitis (139, 140). As shown by various reports (139, 140), *Candida* spp. can induce IgE-mediated mast cell degranulation and subsequent responses in humans who have been previously sensitised and both promote inflammation and exacerbate histaminergic symptoms of patients. Taken together, these findings suggest a dual role of mast cells in the interaction with *Candida* spp.: they act as sentinels and first line of defence, but their mediators can become detrimental for the host and perpetuate inflammation.

CONCLUSION

This short description of some of the most crucial known and potential interactions between B cells and mast cells raises many questions. Mast cells are tissue resident cells, often in limited numbers in lymph nodes. However, they are more prominent in tissues such as the tonsils and Peyer's patches where they are in close-proximity to B cells as they are in the respiratory and intestinal mucosa. Traditionally, B cells in the skin have often been overlooked but both B1 and B2 cells are present and B cell populations in these sites are increased during inflammation. It is in the skin and other mucosal tissues where mast cells play the most important role as sentinel cells against infection; it is also in these sites where interaction between mast cells and B cells might be most critical to local infection and inflammation regulation. Given the key role of mast cells in promoting the selective recruitment and activation of other effector cells, it seems likely that mast cells play an early role in B cell responses. However, the

complex interactions between mast cells and B cells persist longer-term. Current data suggests a number of mechanisms whereby mast cells can support or limit Breg development. Most of these can occur without direct cell contact. Similarly, the production of antibodies and cytokines such as IL-10 by the B cell lineage can dramatically alter mast cell function and provide a long-term mechanism for heightened responses to secondary infection. There is a wide menu of potential mechanisms whereby mast cells can modify B cell populations and function. Each tissue site and pathogen response will likely access only a subset of such mechanisms. Understanding and modifying these mast cell-dependent pathways shows promise for enhancing responses in chronic infection, limiting the development of autoreactive B cells and combating local immune suppression in some tumour settings. Critical to this process is direct analysis of the nature and function of local resident B cell populations and their resident mast cell neighbors in normal tissues and sites of disease.

AUTHOR CONTRIBUTIONS

AMP contributed the literature search, evaluation of material, **Figure 2** and a substantial portion of the written manuscript. MRH contributed **Figure 1**, evaluation of key sections of literature scientific insights and review. AMP contributed **Figure 1** and MRH contributed **Figure 2**. JSM provided substantial writing and editing of the manuscript and conceptualization of the review. All authors contributed to the article and approved the submitted version.

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Mast Cells Are Identified in the Lung Parenchyma of Wild Mice, Which Can Be Recapitulated in Naturalized Laboratory Mice

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Background: It is well documented that laboratory mice bred and maintained in ultra-hygienic specific pathogen-free (SPF) barriers display reduced richness and complexity of microbiota compared with wild mice. The laboratory mice profoundly lack lung parenchymal mast cells. Hence, we aimed to investigate the lung distribution of mast cells in free-living wild mice.

Methods: Wild house mice were trapped in South-Eastern Norway and Hemtabad, West Bengal, India. C57BL/6 laboratory mice were bred in a purposefully built, closed environment with bedding material obtained from the natural environment in order to normalize the gut microbiota of these laboratory mice to that of the wild mice, and the offspring were collected for study at eight weeks of age.

Results: Mast cells were easily identified at a substantial density in the lung parenchymal tissues of wild mice from both Norway and India, which stands in clear contrast to the rare distribution of lung parenchymal mast cells in the conventional laboratory SPF mice. Consistently, wild mice also expressed higher pulmonary levels of stem cell factor, a critical growth factor for mast cell survival. Higher levels of histamine were recorded in the lung tissues of the wild mice. Interestingly, "naturalized" C57BL/6 laboratory mice which spent their entire life in a semi-natural environment developed lung parenchymal mast cells at an appreciable density.

Conclusion: Our observations support that environmental factors, possibly through modulation of microbiota, may impact the tissue distribution of mast cells in mouse lung parenchyma.

Keywords: laboratory mouse, mast cell, wild mouse, lung, mouse naturalization

INTRODUCTION

Because of their anatomical and physiological similarity to humans, as well as a number of other advantages, such as the ease of maintenance and breeding in a laboratory setting, house mice (*Mus musculus*) have long been the model species of choice to mimic human diseases in experimental studies (1). However, limitations of translating mouse research in areas where humans are possibly different from mice are also evident. There is a growing concern that laboratory mice do not truthfully mirror relevant aspects of human physiology and pathology, including immune responses, and hence it is quite often difficult to extrapolate results derived from mouse studies to human treatments (2). Lack of precision in data extrapolation from mice to humans may be due to their fundamental divergence at the genetic level over a long evolutionary history. Alternatively, this may also reflect a more recent artefact arising from the creation and breeding of laboratory mouse strains (1). Accumulating experimental evidence suggests that traditional laboratory mice that are bred and maintained in an ultra-hygienic specific pathogen-free (SPF) barrier display reduced richness and complexity of microbiota compared with wild house mice. Discrepant immunological features have been well documented between SPF laboratory mice and wild-caught mice (3–7). Wild populations of house mice are predicted to be a more relevant model to reflect human immune responses (8).

Asthma, a chronic disease characterized by airway inflammation and respiratory symptoms, is one of the most prevalent human diseases affecting the quality of life of more than 300 million people across the globe (9). There is an urgent need for improved treatment plans to tackle this chronic disease. Animal models are critical for elucidating the immunological mechanisms in asthma and developing therapeutic strategies. To date, the established mouse asthma models have relied on the use of laboratory-adapted, inbred strains of mice (e.g., C57BL/6 and BALB/c). Of particular relevance to allergy and asthma, these laboratory strains profoundly lack lung parenchymal mast cells (10), which stands in clear contrast to the presence of abundant mast cell populations in human lungs (11). This observation challenges the relevance of mouse asthma models for understanding human asthma (12).

Mast cells have been described as one of the major types of cells that are involved in the development of asthma and allergy by virtue of their potential to secrete a variety of allergic mediators (13). Mast cells are derived from hematopoietic progenitors in the bone marrow and these progenitors migrate to the vascularized tissues where they further differentiate into mature mast cells. Mast cells are enriched in the skin, around blood vessels, and in mucosal membranes such as the respiratory and gastrointestinal tracts. Tissue-specific distribution of mast cells is dependent on various mediators. It has been shown that after allergen-mediated sensitization in the respiratory tract, CCL2 is locally produced and recruits mast cell progenitors which express CCR2 (14). Stem cell factor (SCF) is one of the major growth and differentiation factors for mast cells (15). In addition to SCF, mast cell growth and differentiation can be facilitated by several other cytokines including IL-3 (16). Tissue mast cells are capable of further differentiating both phenotypically and functionally as a consequence of tissue-specific stimulation under defined microenvironmental conditions. For example, inflamed human lungs are reported to have more tryptase/chymase-producing mast cells compared with non-inflamed lung tissue in which tryptase-producing mast cells are dominant (17, 18). The number of mast cells is increased at sites of allergic inflammation, and there is a correlation between mast cell density in the tissue and the severity of allergic symptoms (19). In allergy, plurivalent antigens bind and crosslink IgE molecules bound to the high-affinity IgE-receptor (FcεRI) expressed on mast cells, resulting in cell degranulation and release of proinflammatory mediator molecules.

Given the literature reports revealing the discrepancies between conventional laboratory mice and wild mice with respect to various immunological features (20), we were intrigued to investigate lung mast cell distribution in free-living wild mice. We captured a number of free-living mice near or in farm-houses in South-Eastern Norway and Hemtabad, West Bengal, India. We examined mast cell distribution in these wild mice and observed substantial numbers of mast cells in the lung parenchyma of these wild mice. All the laboratory control mice, either C57BL/6 or BALB/c, examined in parallel, lacked mast cells in lung parenchyma. We also show that laboratory mice born and raised in a semi-natural environment for rodents could develop lung parenchymal mast cells. Our aim was to understand mast cell biology in relation to environmental and genetic influences, and potentially to improve the mouse model for asthma and allergy research.

MATERIALS AND METHODS

Live-Trapping of Wild Mice and the Source of Laboratory Mice

Wild house mice were caught using our previously described practical methodology on mouse capture (6). Free-living mice were captured in Hemtabad, West Bengal, India. Furthermore, wild-caught and control laboratory mice from Norway were obtained from a previously reported material (6, 21). The *Mus musculus* identity of the wild mice were confirmed as previously described (22) or by PCR genotyping based on the *Mus musculus*-specific GAPDH gene sequences using two sets of primers (forward 5'-TGGCCGGATACCTAGTTCCA-3'; reverse 5'-AGGTGAATCAGGGAAGCAGC-3', and forward 5'- AACAACTGGCTTTCCACCCA -3'; reverse 5'-ACTGCCTGGTAAAGGTCACG-3'). The specific pathogen-free (SPF) C57BL/6 and BALB/c laboratory mouse controls were obtained from Charles River/Scanbur, Norway, Huafukang Bioscience, Beijing, or bred in-house at the Centralized Animal Facilities at the Hong Kong Polytechnic University. The animal protocols were approved by the Ethics Committee of the Guizhou Medical University. Animal material from Norway was collected under approval from the Norwegian Food Safety Authority (FOTS ID 4788, 6801, 8080 and 8198) and the Norwegian Environment Agency (ref. 2012/693 and 2014/7215).

Housing and Breeding of Laboratory Mice in a Semi-Natural Environment

Both female and male C57BL/6 mice were housed in mouse pens designed with a naturalistic farm-like environment as previously reported (21), however, without the direct presence of wild-caught mice. Briefly, a purposefully built, closed environment was prepared with bedding material regularly brought in from domestic animal farmhouses, as well as sawdust, soil, compost, twigs and hay. This would recapture a common habitat for the free-living house mouse, aiming to normalize the C57BL/6 mice in terms of the gut microbiota and cellular immunology. Mice were allowed to breed in this closed environment and their progeny were collected for study at 8 weeks of age.

Formalin-Fixed Paraffin-Embedded Lung Tissue Block Preparation

The lung tissues were fixed in 10% neutral buffered formalin for 24 h at room temperature followed by embedding in paraffin wax. The lung sections were taken from lung lobes avoiding the central airways. Tissue processing was carried out using a Thermo Scientific Excelsior AS Tissue Processor. Consecutive 5-µm sections were generated from each of the FFPE tissue blocks using a standard microtome blade and the sections were fixed onto glass microscope slides.

Microscopic Examination

Deparaffinized FFPE sections were rehydrated using xylene and downgraded concentrations of alcohol. The slides were stained with 0.1% toluidine blue (Sigma; 89640-5G) for 30 sec, followed by rinsing in 96% ethanol for a few seconds and then dehydrating in absolute ethanol. For tryptase, c-Kit and histamine staining by immunohistochemistry, slides were stained with a rabbit monoclonal antibody against mast cell tryptase (Huabio; ET1610-64), a rat monoclonal antibody against c-Kit (Biolegend; 105822), or rabbit polyclonal antibody against histamine (Abcam; ab37088), followed by staining with a rabbit IgG-specific (Abcam; ab64261) or a rat IgG-specific (Cell Signaling Technology; 7077S) HRP-linked secondary antibody, using the HRP/DAB Detection IHC kit (Abcam; ab64261) according to the manufacturer's instruction. For H&E staining, slides were stained with hematoxylin (Thermo Scientific; 72711) and eosin (Pioneer Research Chemicals; PRC/66/1) using a standard methodology. Slides were mounted with LAMB DPX mounting medium (Thermo Scientific), and microscopic images were acquired using a Nikon Eclipse Ci-L upright clinical microscope or using a Nikon Ti2-E wide-field microscope. Mast cell density was determined by counting the number of positively stained cells in high power fields (400 ×) per mm^2. For counting the mast cell, we focused on the lung parenchymal tissues and tried to avoid the area close to the bronchi. For quantitative analysis of histamine expression, optical density (OD) was obtained and processed using ImageJ. Histamine levels were expressed as OD per area. Depending on the density of the cells identified or the frequency of the positive staining, the whole sectioned tissues (toluidine blue), or three (histamine) or five (tryptase and c-Kit) randomly selected high power fields were scanned for enumeration or quantification, or otherwise as indicated.

Real-Time Quantitative Reverse Transcription PCR

All the FFPE lung blocks and 5-µm sections were stored at room temperature until RNA extraction. Following deparaffinization, total RNA was extracted and purified using an RNeasy FFPE Kit (Qiagen; 73504) according to the manufacturer's instruction. cDNA was synthesized using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher; K1622). qRT-PCR was performed with the ViiA 7 Real-Time PCR System (Applied Biosystems™) using the Power SYBR™ Green PCR Master Mix (Thermo Fisher; A25776). PCR was carried out with an initial incubation at 50°C for 2 min, and 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The specificity of the reaction was verified by melt curve analysis. The relative expression values of each gene were normalized to GAPDH expression and were calculated by the 2-ΔΔCT method. The PCR primer sequences are displayed in **Table S1**.

Statistical Analysis

A Mann-Whitney *U* test was used to calculate statistical differences between the two comparisons. Where applicable, data are expressed as median with individual mouse data points shown. Outliers were removed based on Grubbs' test.

RESULTS

Wild Mice Exhibit Lung Parenchymal Mast Cells

We trapped free-living wild mice in South-Eastern Norway and Hemtabad, West Bengal, India. Mouse lungs were embedded in paraffin and sectioned for microscopy. Mast cells could be easily identified in the lung parenchyma of wild mice from India according to staining with toluidine blue, a dye that stains mast cell metachromatic granule content, which stands in clear contrast to the rare distribution of lung parenchymal mast cells in the conventional C57BL/6 laboratory mice (**Figure 1A**). Statistically significantly different distributions of lung parenchymal mast cells were observed between wild mice and control laboratory mice (**Figure 1A**). Furthermore, identification of mast cells based on staining for mast cell tryptase (**Figure 1B**) or expression of c-Kit (**Figure 1C**) also supported increased distribution of mast cells in wild mice. A similar trend towards increased lung mast cell numbers was noted in mice trapped in Norway, based on toluidine blue staining (**Figure S1A**) and tryptase expression (**Figure S1B**), although the number of animals was too low to validate statistically. We noted that tryptase and c-Kit staining seemed to be more sensitive than toluidine blue for identifying lung mast cells in wild mice obtained in India. In contrast, tryptase and toluidine blue staining seemed to be consistent for staining lung mast cells in wild mice obtained in Norway. Differential staining sensitivity of toluidine blue for mast cells in different tissues or different

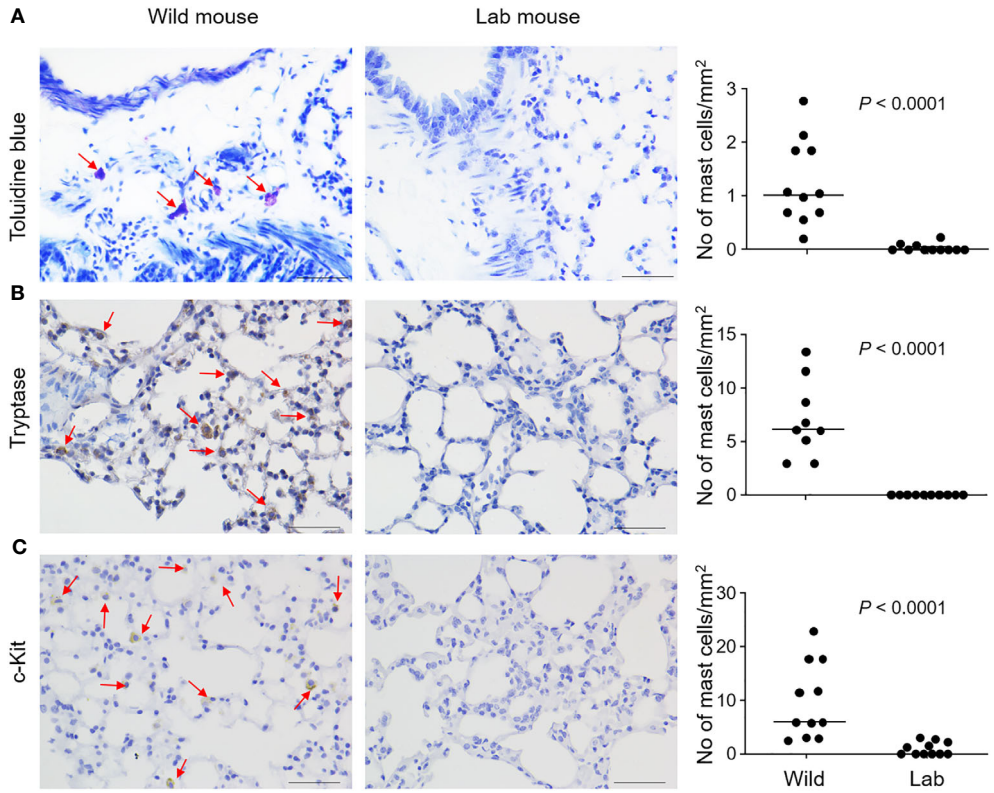

FIGURE 1 | Mast cells are identified in the lung parenchyma of wild free-living mice. Free-living wild mice were trapped at Hemtabad, India (n = 11). Their lung tissues, together with those from C57BL/6 laboratory (lab) mice (n = 11), were processed and sectioned, followed by staining with the mast cell-specific dye toluidine blue **(A)**, or peroxidase-based immunostaining using an anti-mouse tryptase antibody **(B)** or an anti-mouse c-Kit antibody **(C)**. Arrows indicate mast cells which were stained purple (for toluidine blue) or brown (for immunostaining). Mast cell density was quantified and shown as number of cells per unit area (right panels). Each dot represents an individual mouse and horizontal lines indicate the median. Scale bar: 50 μm. Two wild mice and one lab mouse, which had extremely high values were removed (**B**, right panel) as outliers based on Grubbs' test (P < 0.05).

species has been observed previously. For example, guinea pig lung mast cells could not be clearly revealed by toluidine blue [(23); and personal communication with Dr Mikael Adner, Stockholm]. Furthermore, we could find more densely populated mast cells around the bronchi in both the laboratory mice and the wild mice (**Figure S2**), which is consistent with a previous study (10). Therefore, in our cell number quantification, we tried to focus on mast cells in the lung parenchyma and avoid the areas around bronchi. The average density of lung parenchymal mast cells in C57BL/6 mice, which was minimal, is also consistent with this previous report (10).

Wild Mouse Lungs Express Higher Levels of Histamine

We next examined the lung tissue histamine levels, which correlates with mast cell distribution (24), using immunostaining. Wild mouse lungs were observed to express higher levels of histamine, as confirmed directly from the visual comparison of the microscopic images (**Figures 2A, B**) and based on the staining intensity quantification (**Figure 2C**).

Wild Mice Express Higher Levels of SCF and Have Similar Overall Lung Tissue Histology as Laboratory Mice

Next, we investigated factors underlying the emergence of lung mast cells in the wild mice. We measured a group of cytokines and molecules that are involved in mast cell differentiation and migration, which included SCF, IL-3, IL-4, IL-6, IL-9, TGF-β, VCAM-1, CXCR2 and CCL2 (25, 26). Except for SCF, which demonstrated a modest enhancement in the wild mice (**Figure 3A**), none of the others were found to be enhanced at appreciable levels (data not shown). It has been reported that enriched microbiota can upregulate the production of SCF (27). To investigate whether wild mice developed enhanced lung inflammation as a result of persistent exposure to various types of microbes including pathogens, we compared the overall lung histological features between the wild mice and laboratory mice. Lung inflammation has been reported to be an inducer for recruitment of pulmonary mast cells (28). However, no obvious differences were observed between the laboratory mice and the wild mice trapped in India using H&E staining (**Figure 3B**).

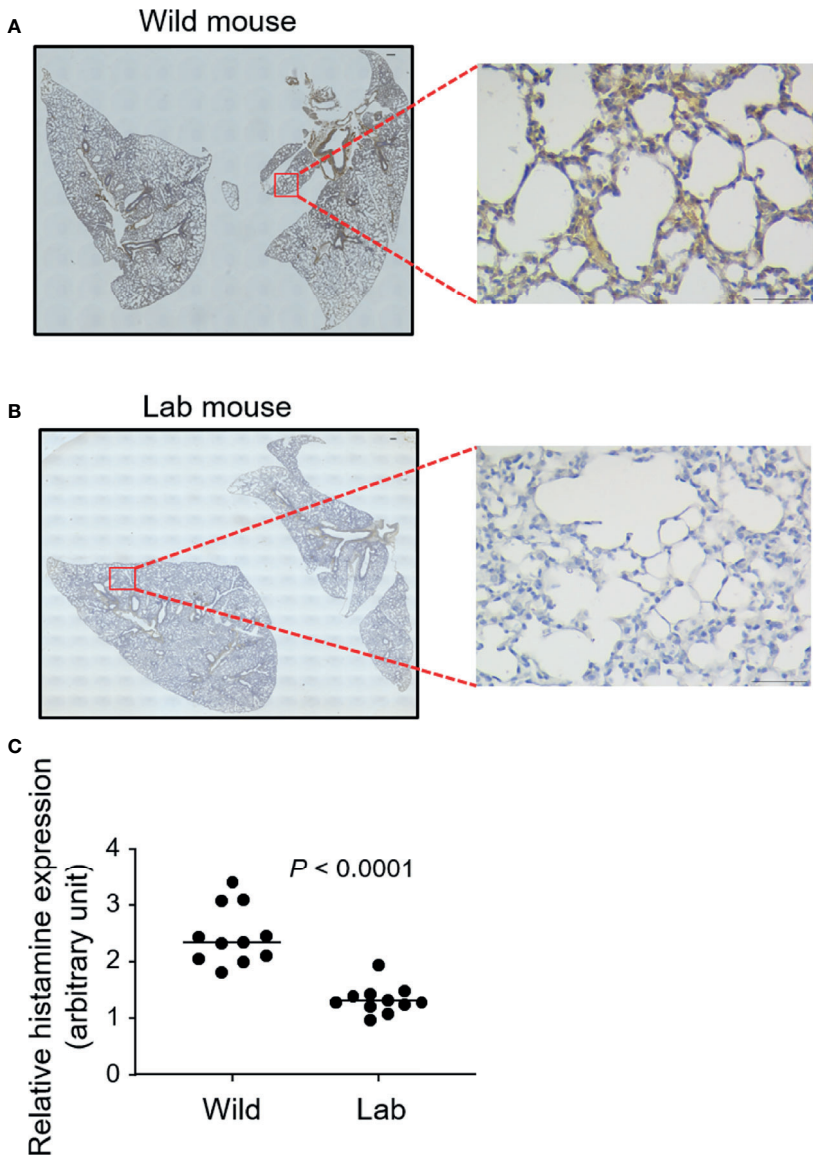

FIGURE 2 | Wild mouse lungs express higher levels of histamine. Free-living wild mice were trapped at Hemtabad, India (n = 11) and their lung tissues **(A)**, together with laboratory (lab) mouse (C57BL/6) controls (n = 11) **(B)**, were processed and sectioned, followed by peroxidase-based immunostaining using an antihistamine antibody. Magnified views of the highlighted regions are shown. At least three randomly chosen areas were scanned for determining the intensity of histamine which was expressed as optical density (OD) per area **(C)**. Each dot represents an individual mouse and horizontal lines indicate the median. Scale bar: 200 μm or 50 μm (magnified view).

Laboratory Mice Born and Raised in a Semi-Natural Environment Develop Lung Parenchymal Mast Cells

It is generally assumed that immunological phenotypic adaptation can arise owing to environmental impacts by living in a dirty, natural environment (20). We therefore explored whether it was possible to repopulate lung mast cells in the laboratory mice by exposing them to a natural environment mimicking the natural habitat of wild mice. Laboratory mice were bred in a closed area with bedding material obtained from the natural rodent living environment (**Figure 4A**) (21). We collected offspring mice who spent their entire life in this semi-natural, dirty environment when they were eight weeks old for analysis. Interestingly, these mice showed a significantly higher density of lung parenchymal mast cells compared to the barrier facility-bred and -reared mice as confirmed using toluidine blue staining (**Figures 4B–D**) as well as tryptase staining (**Figure S3**), thus demonstrating that exposure to the natural living environment early in life was associated with the recruitment of mast cells in the lung parenchymal tissues.

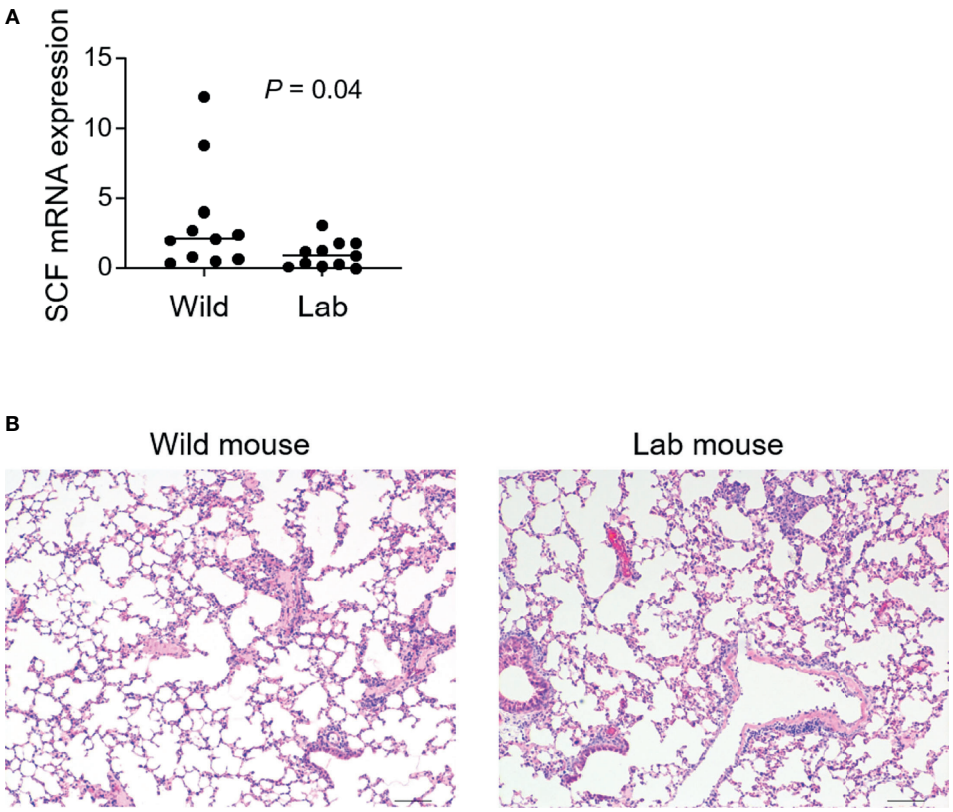

FIGURE 3 | Higher levels of SCF are identified in wild mouse lungs which show similar overall histological features as laboratory mice. Free-living wild mice were trapped at Hemtabad, India. **(A)** RNA was purified from formalin-fixed paraffin-embedded (FFPE) lung tissues of both wild mice (n = 11) and C57BL/6 laboratory (lab) mice (n = 11), followed by cDNA synthesis. Levels of mRNA expression of SCF were assessed using quantitative reverse transcription PCR. SCF expression was normalized relative to the expression of GAPDH. Each dot represents an individual mouse and horizontal lines indicate the median. **(B)** Lung tissues from wild mice together with lab control mice were processed and sectioned for H&E staining. Shown are the representative images. Scale bar: 100 μm.

DISCUSSION

We have provided evidence showing that wild mice contained substantially greater numbers of lung parenchymal mast cells compared with the commonly used laboratory mice, which almost completely lacked lung parenchymal mast cells. Our aim was to understand mast cell biology in relation to environmental and genetic influences, and potentially to provide implication for refining relevant mouse models whereby the function of lung mast cells is crucial, e.g., relevant models for asthma research.

Compared with the abundant expression of mast cells in human lungs, absence of lung parenchymal mast cells in conventional laboratory mice may arise from both genetic and environmental factors. In evolutionary terms, mice and humans diverged between 80 and 90 million years ago (29). An ever growing body of evidence also indicates that gut microbiota can effectively contribute to the shaping of the immune signatures of individuals including mice (30). Therefore, humans and mice, harboring quite different microorganisms, could have developed unique features and functionality of their respective immune system. Our data showing the presence of lung parenchymal mast cells in the wild-caught house mice, to some lesser extent in naturalized laboratory mice, but not in SPF laboratory mice, argues for a strong environmental impact on mast cell tissue distribution (**Figure 5**).

Free-living wild mice inhabit environments that are drastically different from laboratory SPF mice, and mice from these two populations have evolved to demonstrate profoundly different patterns of microbiota (31). We have previously shown that co-housing of SPF laboratory mice with wild mice leads to substantial changes in the fecal microbiota of the laboratory strain after 8-12 weeks (21, 32). Co-housing of laboratory mice with pet store mice can convert their immune phenotypes from originally reflecting human neonates to bearing immune signatures of adult humans (4). Providing laboratory mice with a history of infections that mice normally encounter in the wild changes their blood immune signatures akin to wild mice and adult humans (33). A seminal study showed that breeding laboratory mouse progeny in a wild surrogate mother generates so-called 'wildling' mice with a purely inbred genotype but with the microbiota and many of the immune phenotypes of wild mice (34). Recently, wildling mice were shown

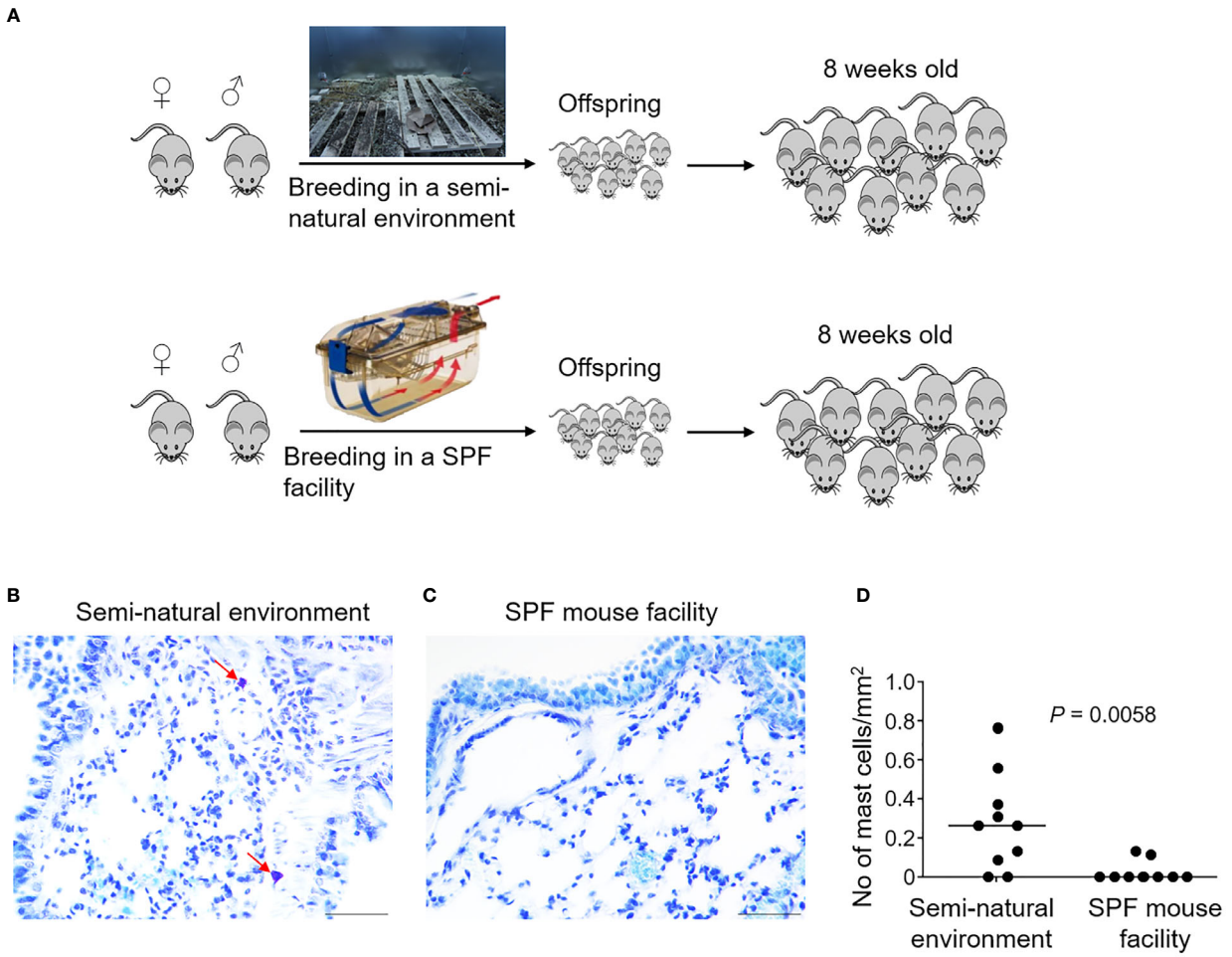

FIGURE 4 | Laboratory mice born and raised in a semi-natural environment develop lung parenchymal mast cells. C57BL/6 laboratory mice were either bred in a purposefully built, closed environment with bedding material from the natural environment at Oslo, Norway, or bred in a conventional specific pathogen-free (SPF) animal facility as indicated **(A)**. Lung tissues were collected from the mice born and raised in the semi-natural environment [**(B)**, $n = 10$] or the SPF facility [**(C)**, $n = 10$] at 8 weeks old. Lung tissue sections were stained with the mast cell-specific dye toluidine blue. Arrows indicate mast cells which were stained purple. Mast cell density was quantified by enumerating toluidine blue-positive mast cells per unit area **(D)**. Each dot represents an individual mouse and horizontal lines indicate the median. Scale bar: 50 µm. One mouse from the SPF group which had an extremely high value was removed as an outlier based on Grubbs' test ($P < 0.05$).

to develop stronger asthmatic inflammation compared with SPF mice (bioRxiv preprint doi: https://doi.org/10.1101/2021.03.28.437143). Mast cell numbers in the lungs were not reported in these studies, and it would be of interest to investigate if these may be altered in wildlings, which would provide an explanation to their stronger asthmatic responses, and strengthen the link between enriched microbiota and mast cell presence.

Since laboratory mice normally harbor few lung mast cells and poorly respond in asthma models, various approaches have been employed to induce pulmonary recruitment of mast cells in mice. Adjuvant-free sensitization with ovalbumin (OVA) followed by chronic intranasal OVA challenge leads to the recruitment of mast cells in the lung tissue (35). Sensitization with OVA admixed to alum followed by exposing mice to daily challenges with aerosolized OVA for one week (36), intranasal OVA challenges twice weekly for at least one month (37), or three consecutive daily intratracheal OVA challenges (38) induces lung recruitment of mast cells. In the model presented here, sensitization to natural allergens could have similarly recruited mast cells to the lung parenchyma, as an alternative or additional causative factor for our findings. The mode of exposure in naturalized and wild mice may arguably be more in accord with exposures in humans compared to repeated OVA/alum aerosols, but how these models might comparatively play out in translational studies remains to be investigated.

Mice have served and will continue to serve as a valuable research tool for the study of immunology including mast cell biology. Indeed, research based on the use of mouse models have contributed substantially to our knowledge in understanding the roles of mast cells in asthma and allergy. However, preclinical

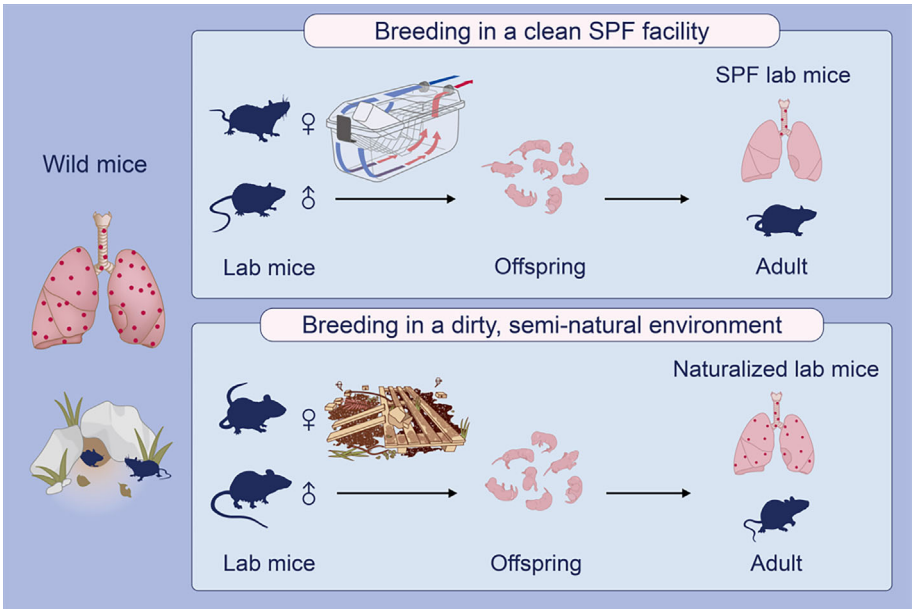

FIGURE 5 | A graphical representation explains a possible environmental impact on the development of lung parenchymal mast cells in mice. Laboratory (lab) mice maintained in ultra-hygienic, specific pathogen-free (SPF) conditions profoundly lack lung parenchymal mast cells, in contrast to the rich presence of mast cells in human lungs (not depicted). Interestingly, free-living wild house mice are found to express lung parenchymal mast cells. Support for an environmental impact on the development of lung mast cells in these mice comes from the fact that mast cells do appear in the lung tissues of 'naturalized' lab mice bred in a semi-natural environment with farm-derived bedding materials.

research using animal models that are different from human immunology may account for the discrepancies between predictions of animal models and clinical trial outcomes. From a translational medicine point of view, establishing clinically relevant mouse asthma models may make preclinical research extrapolatable, which can avoid waste of time and research resources. Indeed, the majority of asthma drugs that pass preclinical testing never survive clinical trials. Among the reasons for the high failure rate of drug development, limitations of appropriate animal models used for drug testing obviously constitute a major one (1). Our naturalization approach may provide an alternative practical solution to the establishment of mouse models with resident lung mast cells.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee of the Guizhou Medical University.

AUTHOR CONTRIBUTIONS

YF, PB, and ZX conceived and designed the study. Y-WY, AC, and LZ performed research. All authors contributed to data analysis and interpretation. Y-WY, PB, and ZX wrote the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.736692/full#supplementary-material

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