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Human Lung Mast Cells Mediate Pneumococcal Cell Death in Response to Activation by Pneumolysin

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Mast cells are emerging as contributors to innate immunity. Mouse mast cells have a pivotal role in protection against bacterial infection, and human cord blood-derived mast cells reduce bacterial viability in culture. The objectives of this study were to determine whether human lung mast cells (HLMCs) might be protective against pneumococcal lung infection through direct antimicrobial activity. Tissue-derived HLMCs and the human mast cell lines HMC-1 and LAD2 were cocultured with wild-type and mutant pneumococci, and viability and functional assays were performed. Mast cells were also stimulated with purified pneumolysin. HLMCs killed wild-type serotype-2 (D39) pneumococci in coculture but had no effect on an isogenic pneumolysin-deficient (PLN-A) pneumococcus. D39 wild-type, but not PLN-A pneumococci, induced the release of leukotriene C4 from human mast cells in a dose-dependent manner, which was not accompanied by histamine release. Stimulation of mast cells with sublytic concentrations of purified pneumolysin replicated this effect. Furthermore, pneumolysin induced the release of the cathelicidin LL-37 from HLMCs, purified LL-37 reduced pneumococcal viability, and neutralizing Ab to LL-37 attenuated mast cell-dependent pneumococcal killing. In addition, at high concentrations, all pneumococcal strains tested reduced HLMC viability through a combination of pneumolysin and H2O2-dependent mechanisms. HLMCs exhibit direct antimicrobial activity to pneumococci through their activation by pneumolysin. This antimicrobial activity is mediated, in part, by the release of leukotriene C4 and LL-37. This suggests that mast cells provide an early warning system and potentially limit pneumococcal dissemination early in the course of invasive pulmonary pneumococcal disease.

Studies in mice demonstrated that mast cells play a critical role in the host response to acute bacterial infection (7, 8). For example, Echtenacher et al. (7) demonstrated that mast cell-deficient mice (c-kit mutant WBB6F1-KitW/KitWv mice) succumbed to a model of acute septic peritonitis within 5 d, whereas wild-type (WT) mice were able to clear the infection. In addition, they also demonstrated that this effect was due, at least in part, to mast cell-derived TNF-α (7). Indeed, several studies from different laboratories showed that the release of TNF-α from resident mast cells at various tissue sites is an essential prerequisite for the recruitment and activation of neutrophils required to control the infection (8–10).

Mast cells also exert direct antimicrobial activity that is dependent on several mechanisms (11–14). For example, mast cells can internalize bacteria opsonized by circulating Abs or complement components, which trigger mast cell phagocytosis by binding to IgGRs or complement receptors (C3aR), respectively (13). Once internalized, the pathogen is destroyed by oxidative (sequestering oxygen radicals to produce an oxidative burst) and nonoxidative (acidifying the endocytosed capsule by fusing with lysosomes) bactericidal systems (13, 14), in much the same way as professional phagocytes. In addition, mast cells kill bacteria through the release of antimicrobial peptides, known as cathelicids (12), with the generation of extracellular traps (11).

Streptococcus pneumoniae (the pneumococcus) is the most common bacterial respiratory pathogen in the United Kingdom, frequently causing community-acquired pneumonia and resulting in mortality >20% for those with concurrent pneumococcal septicemia (15, 16). Worldwide, the situation is worse; pneumococcal septicemia is the major cause of infant mortality in developing countries, causing ∼25% of all preventable deaths in children younger than 5 y and >1.2 million infant deaths annually (17, 18).

The pneumococcal toxin pneumolysin is a major pneumococcal virulence factor that is expressed by virtually all clinical isolates.
of the bacterium. Pneumolysin is a cholesterol-dependent cytolytin, with the ability to create transmembrane pores in cholesterol-containing membranes and, thereby, cause cell lysis (19). Interestingly, however, at sublytic concentrations, pneumolysin can cause a range of immunomodulatory effects, including activation of host complement (20), potentiation of neutrophil activity (21, 22), activation and chemotaxis of CD4+ T cells (23, 24), and enhanced production of proinflammatory cytokines in macrophages and monocytes (25, 26). The importance of pneumolysin as a pneumococcal virulence factor was reported in several in vivo studies that showed reduced pathogenesis in mice infected with pneumolysin-deficient strains of S. pneumoniae compared with isogenic toxin-producing strains (27–30). Furthermore, application of purified pneumolysin directly into the lungs of rats induced an acute inflammatory response similar to that observed during pneumococcal pneumonia (31).

The host factors regulating the control or progression of invasive pneumococcal disease remain poorly defined. Whether human mast cells interact with the pneumococcus is unknown. However, we previously showed more mast cells in the airways of mice that successfully cleared pneumococci from their lungs following intranasal infection compared with those that did not (32).

In this study, we demonstrate for the first time that primary human lung mast cells (HLMCs) and the mast cell line HMC-1 exhibit direct antimicrobial activity against S. pneumoniae in vitro, which is dependent on the key pneumococcal virulence factor pneumolysin. This suggests that HLMCs play an important role in the host response to pneumococcal infection.

Materials and Methods

HLMC purification and culture

All human subjects gave written informed consent, and the study was approved by the Leicestershire Research Ethics Committee. HLMCs were dispersed and purified from macroscopically normal lung, obtained within 1 h of resection for lung cancer, using immunoaffinity magnetic selection, as described previously (33). The final HLMC purity was >98%, with cell viability >97% (monitored by exclusion of trypan blue).

Cell culture

The human mast cell line HMC-1 was a generous gift from Dr. J. Butterfield (Mayo Clinic, Rochester, MN). The cells were cultured, as described previously (34), in Iscove’s medium containing 10% iron-supplemented FCS and 1.2 mM thiglycollate. Cells were split 1:10 every 7 d and resuspended in fresh medium. LAD-2 cells were obtained from Dr. D. Metcalfe (National Institutes of Health, Bethesda, MD). The cells were cultured as described previously (35).

Bacterial strains

S. pneumoniae serotype 2, strain D39 was obtained from the National Collection of Type Cultures, London, U.K. (NCTC 7466). The phenotypic characteristics of the D39 isogenic pneumolysin-deficient mutant (PLN-A) and the H2O2-deficient mutant (SpxB) were described in detail previously (27, 29, 36). The SpxB mutant was kindly provided by Prof. K. Muhlemann (University of Bern, Bern, Switzerland). WT bacteria were identified as pneumococci prior to experiments by Gram stain, catalase test, α-hemolysis on blood agar plates, and optochin sensitivity. Isogenic mutants PLN-A and SpxB were identified by growth on erythromycin and chloramphenicol-supplemented blood agar plates, as previously described (27, 29, 36).

Mast cell and bacteria coculture

S. pneumoniae serotype 2, strain D39 and its two isogenic mutants were incubated with mast cells for 1, 2, 3, or 6 h, and mast cell viability was assessed using trypan blue. Pneumococcal viability was assessed using CFU counts from the coculture supernatant with serial dilutions. Adherent and intracellular pneumococci were assessed by electron microscopy and confocal microscopy. For some experiments, 0.4-μm culture inserts were added to separate the mast cells and pneumococci. Anti-LT-37 neutralizing Ab clone 3D11 (Hyctul Biotechnology, Uden, The Netherlands) or IgG1 isotype control was used in some experiments.

Mast cell challenge with pneumolysin

For the pneumolysin-challenge assays, media was prewarmed to 37°C, and plating of cells was carried out on a plate warmer set to 37°C. A total of 1 × 10² (or 1 × 10³ for LL-37) mast cells in 50 μl DMEM were seeded in 96-well plates. Serial dilutions of purified pneumolysin were made to 2× final concentration in DMEM, and 50 μl was added immediately to the cells in triplicate. Plates were incubated for 1 h at 37°C in a humidified incubator flushed with 5% CO₂. After the incubation, well contents were transferred to a 96-well V-bottom plate and centrifuged at 250 × g for 5 min. Supernatants were removed and frozen at −20°C for later measurement of histamine, leukotriene C4 (LTC4), and LL-37. Cell pellets were resuspended in 10 μl DMEM followed by 10 μl trypan blue. Cell counts and viability were performed using a hemocytometer 10 min after the addition of trypan blue. For the LL-37 assay, cell pellets for control cells were resuspended in 100 μl ultrapure water and frozen for measurement of total LL-37.

To confirm the results from the trypan blue-exclusion counts, a lactate dehydrogenase (LDH) cytotoxicity assay was performed on a subset of experiments according to the manufacturer’s instructions (Biovision, Cambridge Biosciences, Cambridge, U.K.).

Mediator assays

Histamine was measured by radioenzymatic assay, as described previously (33). LTC4 (Cayman Chemical, Ann Arbor, MI) was measured by ELISA, according to the manufacturer’s instructions. Cathelicidin (LL-37) was measured by ELISA, according to the manufacturer’s instructions (Hyctul Biotechnology, Uden, The Netherlands). Cytokines were measured at Millipore (St. Charles, MO) using Milliplex technology.

Statistical analyses

Unless otherwise stated, data are presented as the mean ± SEM from individual experiments performed in triplicate. Pneumococcal viability data were normalized to control and are expressed as the log of the calculated CFU. Each experiment using HLMCs was performed with a different cell donor. Differences between data sets were analyzed using the Student two-tailed t test; a p value < 0.05 was considered statistically significant.

Results

Mast cells reduce WT pneumococcal viability in coculture, which is dependent upon pneumolysin

HLMCs markedly reduced the number of viable WT serotype-2 (WT D39) pneumococci in coculture at all pneumococcal CFU ranges tested. This reduction in pneumococcal viability was evident at 1 h but was more marked after 6 h of coculture. Thus, with the addition of high numbers (10⁸ CFU) of pneumococci, 10⁴ HLMCs were able to reduce pneumococcal numbers from log 1 × 10⁶ CFU to log 4.4 × 10⁶ CFU after 1 h of coculture and from log 1 × 10⁸ CFU to log 4.1 × 10⁶ CFU after 6 h of coculture (n = 5; p = 0.0387 and p = 0.0007, respectively). Similar results were seen with lower CFU of pneumococci (Fig. 1A).

We next tested the effects of pneumococcal coculture with the human mast cell line HMC-1. These cells also demonstrated direct antimicrobial activity to pneumococci (Fig. 1B), although to a slightly lesser degree than with the HLMCs. HMC-1 cells significantly reduced pneumococcal viability at CFU ranges of 10⁵–10⁶ but not at higher CFU of 10⁸. Interestingly, however, when HLMCs and HMC-1 cells were cocultured with an isogenic pneumolysin-deficient (PLN-A) pneumococcus, there was no effect on pneumococcal viability at any CFU range, even after 6 h of coculture (Fig. 1C, 1D), suggesting that pneumolysin was key to the antimicrobial activity of human mast cells. Pneumococcal viability was stable in pneumococcal monocultures (Fig. 1E), and there was no significant difference between the number of pneumococci adherent to the mast cell surface in any of the
Pneumococci induce LTC4 synthesis without histamine release

Mediator release from HMC-1 cells cocultured with WT D39 was comparable to HLMCs with regard to the release of LTC4 but not histamine (Fig. 2C, 2D). However, in contrast to the WT D39 pneumococci, the pneumolysin-deficient PLN-A serotype of pneumococci did not induce any LTC4 synthesis from HMC-1 cells at 1 or 6 h of coculture (n = 5) (Fig. 2E, 2F).

Pneumolysin alone induces mast cell LTC4 synthesis but not histamine release

To confirm that the activation of mast cells to synthesize and release LTC4 was mediated by pneumolysin alone, we next examined the effects of purified pneumolysin challenge on mast cells. We first determined the concentrations of pneumolysin required for mast cell lysis. Surprisingly, we found that human mast cells were remarkably resistant to pneumolysin-induced lysis at the concentrations we tested (Fig. 3A). Thus, HLMC and HMC-1 and LAD2 cell viability after 1 h of incubation with 10 μg/ml purified pneumolysin was only reduced to 74.4% ± 4.3% (n = 3; p = 0.0271), 89.3% ± 3.4% (n = 5; p = 0.0336), and 83.1% ± 3.3% (n = 5; p = 0.007), respectively (Fig. 3A). We confirmed these results with an LDH cytotoxicity assay and found them to be comparable (Fig. 3B). In contrast, airway smooth muscle cells and erythrocytes were far more sensitive to pneumolysin-induced cell lysis at equivalent concentrations of pneumolysin (Fig. 3A).

Because human mast cells were resistant to lysis by purified pneumolysin at the concentrations we tested, we next examined the response of mast cells to pneumolysin challenge by measuring the mediators released acutely in vitro. As with the mast cell and pneumococcal cocultures, pneumolysin at sublytic concentrations induced the release of LTC4 into the supernatants of LAD2 cells. Pneumolysin at the concentrations we tested, we next examined the effects of purified pneumolysin challenge on mast cells. We first determined the concentrations of pneumolysin required for mast cell lysis. Surprisingly, we found that human mast cells were remarkably resistant to pneumolysin-induced lysis at the concentrations we tested (Fig. 3A). Thus, HLMC and HMC-1 and LAD2 cell viability after 1 h of incubation with 10 μg/ml purified pneumolysin was only reduced to 74.4% ± 4.3% (n = 3; p = 0.0271), 89.3% ± 3.4% (n = 5; p = 0.0336), and 83.1% ± 3.3% (n = 5; p = 0.007), respectively (Fig. 3A). We confirmed these results with an LDH cytotoxicity assay and found them to be comparable (Fig. 3B). In contrast, airway smooth muscle cells and erythrocytes were far more sensitive to pneumolysin-induced cell lysis at equivalent concentrations of pneumolysin (Fig. 3A).

Because human mast cells were resistant to lysis by purified pneumolysin at the concentrations we tested, we next examined the response of mast cells to pneumolysin challenge by measuring the mediators released acutely in vitro. As with the mast cell and pneumococcal cocultures, pneumolysin at sublytic concentrations induced the release of LTC4 into the supernatants of LAD2 and HMC-1 cells, without accompanying histamine release (Fig. 3C, 3D). The amount of LTC4 released with pneumolysin challenge was comparable to that of the mast cell pneumococcal cocultures, supporting the notion that the synthesis of LTC4 from mast cells in coculture was also mediated by pneumolysin (Fig. 3C, 3D).
Pneumolysin does not induce cytokine synthesis

Because mast cells selectively release LTC₄ with pneumolysin stimulation, we next measured the cytokine release from human mast cells following stimulation with pneumolysin. Because we were looking for a direct effect of pneumolysin, the mast cells were not activated by antigenic stimulation. We were unable to identify any difference between the controls and the pneumolysin-stimulated mast cells for any of the cytokines measured (Fig. 4). IL-1β, -2, -4, and -12p70 and TNF-α were not detectable in any of the cell types and are not included in the figure. GM-CSF and IL-5 were only detectable in the HLMCs (Fig. 4). Notably, CXCL8 and CCL2 were the only detectable cytokines in the HMC-1 cells; the latter was greatly elevated compared with levels in the HLMCs and LAD2 cells (Fig. 4), but no effect of pneumolysin was evident.

Mast cell-induced pneumococcal cytotoxicity is due to LL-37 and does not require cell contact

Because the HMC-1 mast cell line is known to express the cathelicidin LL-37, which was shown to be cytotoxic to Streptococcus pyogenes (11), and because we showed that pneumolysin is required for HLMC cytotoxicity to pneumococci, we next tested whether purified pneumolysin could induce LL-37 release from HLMCs. Lysed HLMCs contained 16.6 ± 2.0 ng of LL-37 per 10⁶ cells. With the addition of pneumolysin at 1 or 10 µg/ml for 1 h, HLMCs released 5.3 ± 2.2 ng/10⁶ cells of LL-37 (32% of cell content) and 8.5 ± 4.9 ng/10⁶ cells of LL-37 (51% of cell content), respectively (Fig. 5A).

To confirm that LL-37 was cytotoxic to pneumococci, we next incubated WT pneumococci with and without LL-37. Thus, following a 2-h incubation with 50 ng/ml of LL-37, pneumococcal CFU was reduced by 95% compared with controls incubated without LL-37: 1.1 ± 10³ CFU versus 2.2 ± 10⁴ CFU, respectively (Fig. 5B). Therefore, the reduction in pneumococcal viability by mast cells is most likely due to LL-37 release.

To confirm the functional activity of LL-37, we next examined the effects of neutralizing Ab on HLMC and pneumococcal cocultures. After 3 h of incubation, there was a highly significant reduction in WT D39 pneumococci cocultured with HLMCs. The ability of mast cells to kill pneumococci was significantly attenuated using an LL-37 neutralizing Ab (Fig. 5C). No effect was seen with the IgG1 isotype control.

To assess whether mast cell-induced cytotoxicity required cell–cell contact, we tested the effects of mast cell–pneumococcal

**FIGURE 2.** WT D39, but not pneumolysin-deficient PLN-A, pneumococci induce LTC₄ production without histamine release from human mast cells. A, WT D39 pneumococci induce the release of LTC₄ from HLMCs in a dose-dependent manner, without accompanied histamine release. B, This effect was still evident after 6 h of coculture. C and D, HMC-1 cells also release LTC₄, but not histamine, when cocultured with WT D39 pneumococci. The pneumolysin-deficient PLN-A pneumococci did not induce LTC₄ release at 1 h (E) or 6 h (F). **p < 0.05; ***p < 0.01.

**FIGURE 3.** Mast cells are resistant to lysis from pneumolysin, which induces LTC₄ production but not histamine release. A, Human mast cells are resistant to lysis by purified pneumolysin compared with human airway smooth muscle cells and erythrocytes. Assessed by trypan blue staining. B, Comparison of mast cell lysis using trypan blue staining and LDH release. Pneumolysin at sublytic concentrations induced the release of LTC₄, but not histamine, in HMC-1 cells (C) and LAD-2 cells (D). **p < 0.05; ***p < 0.01; ****p < 0.001.
coculture with the addition of cell-culture inserts (0.4 μm pore size) to separate the cells. With the addition of the inserts, viable WT pneumococcal numbers decreased by 31% over the 2-h incubation period in the pneumococcal monoculture (from $9.6 \times 10^8$ CFU at 0 h to $6.6 \times 10^8$ CFU at 2 h; $n = 4$; $p = 0.004$) (Fig. 5D). However, in the HLMC–D39 pneumococcal cocultures, the decrease in viable pneumococci number was much greater (88% reduction), decreasing from $9.5 \times 10^8$ CFU at 0 h to $1.1 \times 10^8$ CFU at 2 h ($n = 4$; $p = 0.002$) (Fig. 5D). Thus at 2 h, HLMCs had significantly reduced pneumococcal viability compared with pneumococci monocultures ($p = 0.004$), even in the absence of cell contact. This supports a role for a soluble HLMC-derived mediator in reducing pneumococcal viability.

**Pneumococci reduce human mast cell survival in coculture**

We also examined the effects of whole pneumococci on mast cell viability. WT D39 pneumococci reduced HLMC viability only at the greatest CFU ($1 \times 10^8$). Thus, with $10^8$ CFU pneumococci, HLMC viability was $89.0\% \pm 1.3\%$ of control after 1 h of coculture ($n = 5$; $p = 0.0012$) and $55.3\% \pm 2.9\%$ of control after 6 h of coculture ($n = 5$; $p = 0.0006$) (Fig. 6A). WT D39 pneumococci were more effective at killing HMC-1 cells at equivalent CFU, with HMC-1 viability of $63.4\% \pm 9.0\%$ at 1 h ($n = 5$; $p = 0.0099$) and $16.6\% \pm 5.8\%$ at 6 h ($n = 5$; $p = 0.042$) (Fig. 6B).

Because we had demonstrated that human mast cells were resistant to lysis from pneumolysin at concentrations up to 10 μg/ml, we next examined whether pneumolysin-deficient pneumococci had any effect on mast cell viability in coculture. PLN-A pneumococci cocultured with HMC-1 cells only decreased mast cell viability at the greatest CFU ($10^8$) of pneumococci and at the longest (6 h) incubation period. Hence, with $10^8$ CFU PLN-A pneumococci, HMC-1 viability was significantly reduced after a 6-h incubation to $46.5\% \pm 10.2\%$ ($n = 3$; $p = 0.0298$) (Fig. 6C), suggesting that although pneumolysin was partly respon-
whereas the addition of 200 U/ml of catalase to the mast cell line HMC-1 cells. The H₂O₂ killed HMC-1 cells. PLN-A pneumococci were less effective at killing HMC-1 cells in the presence of catalase, confirming that pneumolysin and H₂O₂ contribute to mast cell cytotoxicity.

A. Human mast cells exhibit direct antimicrobial activity against the important human respiratory pathogen *S. pneumoniae*. Human mast cells elicit this antimicrobial response in the absence of cell–cell contact through their activation by the key pneumococcal virulence factor pneumolysin. We showed that pneumolysin induces the release of the cathelicidin antimicrobial peptide LL-37 from HLMCs, which contributes to mast cell–dependent pneumococcal killing. Furthermore, human mast cells respond to pneumococcal exposure with the release of the proinflammatory mediator LTC₄, which has the potential to recruit professional phagocytes to sites of infection.

There is increasing evidence that mast cells exhibit important antimicrobial activity and play a key role in the defense against bacterial and viral infections (11–14, 37). Mouse mast cells play a critical role in the host response to infection caused by many bacteria, effects that are particularly dependent upon the release of mast cell–derived TNF-α and LTC₄ (7, 38). However, the role of human mast cells in the defense against bacterial infection is poorly defined, and the direct effects of ex vivo primary human mast cells have not been investigated. Human cord blood–derived mast cells can phagocytose and kill opsonized bacteria, including *Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus faecalis* (39), and the human mast cell line HMC-1 can induce the death of *S. pyogenes* through the release of the antimicrobial peptide LL-37, a member of the cathelicidin family (11).

Our observation that HLMCs are conferred with direct antimicrobial activity against the important respiratory pathogen *S. pneumoniae* is the first to show the antibacterial properties of mature tissue-dwelling human mast cells and helps us to understand the role of mast cells in the defense against respiratory infection. Pneumococcal disease is one of the leading causes of morbidity and mortality worldwide, and the organism is the principal cause of pneumonia in children and adults (29). Importantly, pneumolysin is a key virulence factor involved in invasive disease, which is produced by virtually all clinical isolates of the pneumococcus (40). In this study, we demonstrated that pneumolysin is critical for the activation of HLMCs and HMC-1 cells, leading to the release of LTC₄ and LL-37 and, subsequently, associated pneumococcal cell death.

Pneumolysin is a member of the family of cholesterol-dependent cytolytic toxins that are synthesized by Gram⁺ bacteria (29). At sublytic concentrations, pneumolysin was shown to induce a wide range of cell–modulatory activities, such as inhibition of ciliary beating on respiratory epithelium, inhibition of phagocytic respiratory burst, induction of cytokine synthesis, and CD4⁺ T cell activation and chemotaxis (29). At high concentrations, pneumolysin leads to lysis and cell death. Interestingly, human mast cells were relatively resistant to the cytolytic effects of this toxin, compared with other airway cells, and to previously tested immune cells, which may enhance their ability to respond to pneumococcal infection compared with other cells. The mechanism through which pneumolysin activates HLMCs is not known, but pneumolysin was shown to activate TLR4 (41). This is also expressed on human mast cells (42, 43), and pneumolysin-dependent activation of TLR4 enhances resistance to pneumococcal infection in mouse models (41).

The ability of pneumococci and purified pneumolysin to induce the differential release of LTC₄ over histamine is intriguing and may be TLR related. The differential release of mediators from mast cells is well described (3, 44); however, stimuli that selectively induce LTC₄ release, but not histamine release, are relatively rare. Interestingly, McCurdy et al. (45) reported that activation of TLR2 with the agonists peptidoglycan and zymosan induced LTC₄ release, but not degranulation, in human cord blood–derived mast cells, whereas the synthetic TLR1/TLR2 agonist Pam₃CSK₄ induced degranulation, but not LTC₄ production (45). Furthermore, in a recent study by Sur et al. (46), it was showed that inhibition of the protein tyrosine kinase JAK2, using specific inhibitors and small interfering RNA knockdown, markedly reduced the production of LTC₄ in anti-IgE–stimulated mast cells, without the associated inhibition of histamine release (measured by β hexosaminidase release) or PGD₂ synthesis (46). This suggests that JAK2 plays an important role in the specific production of LTC₄. Importantly, TLR4 activation by LPS induces immediate phosphorylation of JAK2, which is blocked by an anti–TLR4 neutralizing Ab (47).

The absence of histamine release in our experiments is in direct contradiction to a previous study, using the rat basophil leukemia mast cell line RBL-2H3 and pneumococcal cocultures, which reported significant degranulation. Barbuti et al. (48) failed to show any degranulation before 1 h and reported a slow increase in degranulation over a 4-h period with high concentrations of pneumococci. This is consistent with pneumococcal–induced mast cell cytolysis, which we reported to occur after 1 h with high concentrations of pneumococci and that increased with time. In support of this, we also showed that high concentrations of pneumolysin induced mast cell cytotoxicity with accompanying
LDH leakage that mirrored histamine release (Fig. 3B, 3D). Thus, it was important that we examined the effects of pneumolysin at sublytic doses at which leakage of cellular content was not a contributing factor. In keeping with our work, Barbuti et al. (48) failed to identify the release of TNF-α.

Of great relevance to pneumolysin, mast cell LTC₄ generation is critical for the recruitment of neutrophils in mouse models of bacterial infection (38), and it is well established that neutrophils are the major effector cells in host pulmonary defense against pneumococcal infection (49–52). Thus, the ability of HLMCs to respond to pneumolysin with the generation of LTC₄ may be a critical factor in the human response to pulmonary pneumococcal infection.

The ability of HLMCs to kill pneumococci is mediated, at least in part, by the cathelicidin LL-37. We showed that HLMCs store preformed LL-37 that is released rapidly on exposure to pneumolysin, that LL-37 reduces pneumococcal viability, and that LL-37 neutralization in HLMC–pneumococcal coculture attenuated mast cell–dependent pneumococcal killing. Cathelicidins, such as LL-37, are small antimicrobial peptides that kill bacteria by membrane integration, forming pores or even ion channels that disrupt microbial function (53). Mouse mast cells deficient in CRAMP, another member of the cathelicidin family, are less able to kill group A Streptococcus (12). In addition, LL-37 can modulate TLR (54) and immune responses. In a recent study, von Köckritz-Blickwede et al. (11) demonstrated that the human mast cell line HMC-1 elicited antimicrobial activity that was independent of phagocytosis. They demonstrated that LL-37 is caught in extracellular traps, which may explain our inability to measure it in mast cell–pneumococcal coculture supernatants. However, their cocultures of HMC-1 cells and S. pyogenes demonstrated that although HMC-1 cells directly killed S. pyogenes without phagocytosis, cellular contact was required (11). In contrast, we demonstrated that although cell contact may increase the cytotoxicity of mast cells to pneumococci, HMC-1 cells can elicit antimicrobial activity to pneumococci in the absence of cell contact, as demonstrated by separation with 0.4-µm membranes. This novel phenomenon is likely to be unique to pneumococci, because the antimicrobial activity of HLMCs and HMC-1 cells to pneumococci requires activation of mast cells with the pneumococcal-specific pneumolysin.

Mouse and cord blood-derived human mast cells are also able to phagocytose bacteria (13, 14, 55), but they are relatively inefficient at phagocytosis compared with professional phagocytes; under certain conditions, they may even harbor pathogens from the immune system (56). However, we could not identify intracellular pneumococci in our experiments. Therefore, it is unlikely that phagocytosis of pneumococci by mast cells is a major route for the elimination of bacteria.

In addition to the antimicrobial activity of mast cells, we showed that pneumococci could reduce mast cell viability at greater CPU. HLMCs were less sensitive in this respect than HMC-1 cells. We showed that H₂O₂ is an important virulence factor for pneumococcal-induced mast cell cytotoxicity. However, von Köckritz-Blickwede et al. (11) demonstrated that H₂O₂ was required for the extracellular trap formation that was a critical requirement for the antimicrobial activity of HMC-1 cells to S. pyogenes. Interestingly, the formation of these traps was at the cost of the HMC-1 cells’ viability, because the cells seemed to have to undergo nuclear degradation to form these traps: a process referred to as “NEToxis” by the investigators (11). This could account, in part, for the loss in cell viability that we observed in our cocultures.

In summary, we showed for the first time that HLMCs resident in the airways and lung parenchyma exhibit direct antimicrobial activity to the key respiratory pathogen S. pneumoniae. In response to pneumolysin, they rapidly release the antimicrobial peptide LL-37 from preformed stores, which is capable of inducing pneumococcal cell death, and the proinflammatory mediator LTC₄, which has the potential to recruit professional phagocytes. Thus, we propose that HLMCs exert a two-pronged attack against the pneumococcus, with early and direct antipneumococcal killing mediated by LL-37, coupled with the initiation and amplification of the innate immune response to invading bacteria. Our data suggest that mast cells are an underestimated, yet important, factor for the defense against pneumococcal infection in the lung.

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Disclosures

The authors have no financial conflicts of interest.

References


45. Theoharis, T. C., D. Kempurar, M. Tagen, P. Conti, and D. Kalogeromitros. 2007. Differential release of mast cell mediators and the pathogenesis of in-


50. Barbuti, G., M. Moschioni, S. Censini, A. Covacci, C. Montecucco, and P. Montemurro. 2006. Streptococcus pneumoniae induces mast cell de-

51. Beiter, K., F. Wartha, B. Albiger, S. Normark, A. Zychlinsky, and B. Henriques-