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

**Glenn Cruse, Vitor E. Fernandes, Jose de Salort, Depesh Pankhania, Marta S. Marinas, Hannah Brewin, Peter W. Andrew, Peter Bradding,[1] and Aras Kadioglu[1]**

**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 $C_4$ 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 $H_2O_2$-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 LL-37 from HLMCs. This suggests that mast cells provide an early warning system and potentially limit pneumococcal dissemination early in the course of invasive pulmonary pneumococcal disease.** ***The Journal of Immunology*****, 2010, 184: 7108–7115.**

Mast cells are major effector cells in allergic and inflammatory diseases and are synonymous with allergy (reviewed in Refs. 1–3). Thus, mast cell research is often centered on the deleterious pathological effects of these cells. However, mast cells have many important functions in homeostatic physiology and may play a significant role in the healing of wounds and defense against bacterial and parasitic infection, participating in innate and adaptive immunity (reviewed in Refs. 3–5). Mast cells are ubiquitous throughout connective tissues and mucosal surfaces, particularly at the interface with the external environment, such as the skin, respiratory tract, and gastrointestinal tract (reviewed in Refs. 3, 5, 6). At these sites, they are well placed and well equipped to deal with a multitude of tissue insults. Therefore, it is likely that their primary role is to act as sentinel cells, sensing the external environment and ready to respond to a variety of diverse tissue insults with an early and appropriate program of gene expression and mediator release aimed at initiating inflammation and then repair.

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 $WBB6F_1$-$Kit^W/Kit^{Wv}$ 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 cathelicidins (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

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Abbreviations used in this paper: HLMC, human lung mast cell; LDH, lactate dehydrogenase; $LTC_4$, leukotriene $C_4$; WT, wild-type.



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of the bacterium. Pneumolysin is a cholesterol-dependent cytolysin, 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 thioglycerol. 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 $H_2O_2$-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–LL-37 neutralizing Ab clone 3D11 (Hycult Biotechnology, Uden, The Netherlands) or $IgG_1$ 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 $1 \times 10^4$ (or $1 \times 10^5$ 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% $CO_2$. After the incubation, well contents were transferred to a 96-well V-bottom plate and centrifuged at $250 \times g$ for 5 min. Supernatants were removed and frozen at −20°C for later measurement of histamine, leukotriene $C_4$ ($LTC_4$), 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). $LTC_4$ (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 (Hycult 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 ($10^8$ CFU) of pneumococci, $10^4$ HLMCs were able to reduce pneumococcal numbers from log $1 \times 10^8$ CFU to log $4.4 \times 10^7$ CFU after 1 h of coculture and from log $1 \times 10^8$ CFU to log $4.1 \times 10^6$ 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. 1*A*).

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. 1*B*), although to a slightly lesser degree than with the HLMCs. HMC-1 cells significantly reduced pneumococcal viability at CFU ranges of $10^6$–$10^2$ but not at higher CFU of $10^8$. 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. 1*C*, 1*D*), suggesting that pneumolysin was key to the antimicrobial activity of human mast cells. Pneumococcal viability was stable in pneumococcal monocultures (Fig. 1*E*), and there was no significant difference between the number of pneumococci adherent to the mast cell surface in any of the

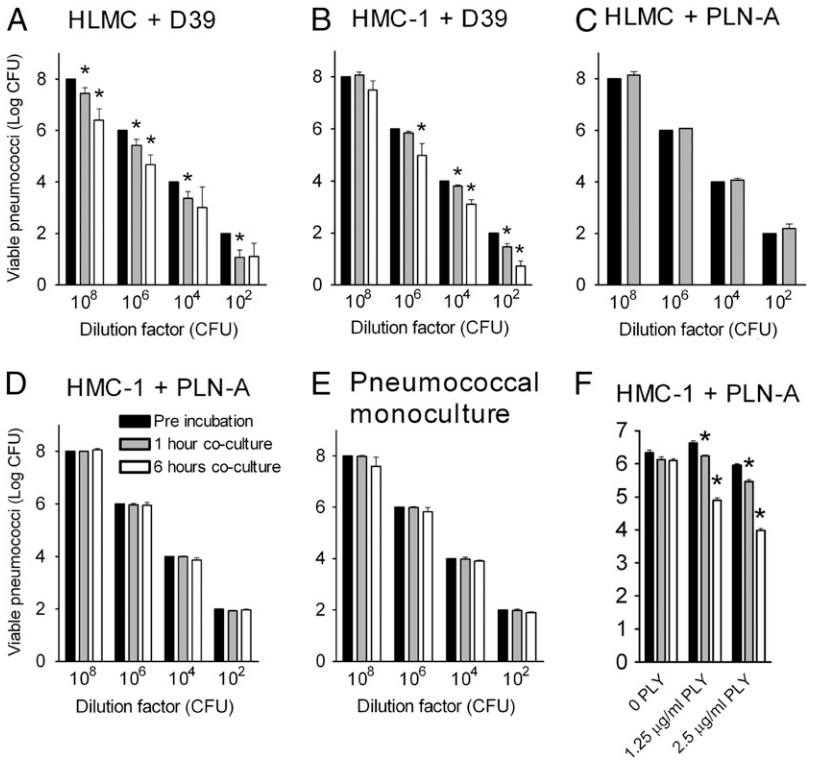


**FIGURE 1.** Human mast cells display anti pneumococcal cytotoxicity. *A*, HLMCs kill WT D39 pneumococci in coculture, which is evident after 1 h but more marked after 6 h. *B*, HMC-1 cells also kill WT D39 pneumococci in coculture, although to a lesser degree than HLMCs. *C*, HLMCs have no effect on pneumococcal viability in the absence of pneumolysin, as shown using the pneumolysin-deficient serotype PLN-A. *D*, HMC-1 cells also have no effect on PLN-A cells. *E*, Pneumococci in monocultures are unaffected by the culture conditions. *F*, Addition of exogenous pneumolysin to pneumolysin-deficient PLN-A and HMC-1 cocultures replicates the effects of WT pneumococci. $*p < 0.05$.

conditions (data not shown). Additionally, there was no evidence of intracellular pneumococci in any condition using confocal and/or electron microscopy in HLMCs or HMC-1 or LAD-2 cells (data not shown). There was no cytotoxicity of pneumococci evident when cocultured with human airway smooth muscle cells (data not shown).

To confirm that mast cell-mediated cytotoxicity of pneumococci was dependent on pneumolysin, we next examined the effects of PLN-A and HMC-1 cocultures with the addition of exogenous pneumolysin. Using dose ranges of pneumolysin and mast cell viability assays, we determined that 1.25 or 2.5 μg/ml pneumolysin was an appropriate dose to use without affecting mast cell viability. The addition of 1.25 or 2.5 μg/ml pneumolysin to the HMC-1 + PLN-A cocultures markedly reduced the number of viable pneumococci at 1 and 6 h (Fig. 1*F*). Thus, with the addition of 2.5 μg/ml pneumolysin, viable pneumococci were reduced from $9.6 \times 10^5$ CFU at 0 h to $4.6 \times 10^5$ CFU at 1 h (68% reduction; $n = 4$, $p = 0.0057$) and $9.8 \times 10^3$ CFU at 6 h (99% reduction; $n = 4$; $p < 0.0001$) (Fig. 1*F*), confirming that pneumolysin was critical for mast cell-dependent pneumococcal cytotoxicity. The addition of exogenous pneumolysin had no deleterious effect on mast cells alone or pneumococci-alone control incubations at 1.25 or 2.5 μg/ml concentration or for durations of 1 or 6 h (data not shown).

### *Pneumococci induce $LTC_4$ synthesis without histamine release in human mast cells, which is dependent on pneumolysin*

Because pneumolysin was required for the antimicrobial activity of mast cells to pneumococci, we next studied the effects of pneumolysin-sufficient WT D39 pneumococci on mast cell mediator release. WT D39 pneumococci did not induce histamine release from HLMCs, even at high CFU. However, HLMCs synthesized and released $LTC_4$ in a dose-dependent manner in these cocultures. Thus in the 1-h control, HLMCs released 581.0 ± 132.3 pg/$10^6$ cells compared with 1778.8 ± 523.7 pg/$10^6$ cells with $10^8$ CFU pneumococci ($n = 5$; $p = 0.0386$) (Fig. 2*A*). This $LTC_4$ release was still evident, although less marked, after 6 h of coculture (Fig. 2*B*).

Mediator release from HMC-1 cells cocultured with WT D39 was comparable to HLMCs with regard to the release of $LTC_4$ but not histamine (Fig. 2*C*, 2*D*). However, in contrast to the WT D39 pneumococci, the pneumolysin-deficient PLN-A serotype of pneumococci did not induce any $LTC_4$ synthesis from HMC-1 cells at 1 or 6 h of coculture ($n = 5$) (Fig. 2*E*, 2*F*).

### *Pneumolysin alone induces mast cell $LTC_4$ synthesis but not histamine release*

To confirm that the activation of mast cells to synthesize and release $LTC_4$ 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. 3*A*). 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. 3*A*). We confirmed these results with an LDH cytotoxicity assay and found them to be comparable (Fig. 3*B*). In contrast, airway smooth muscle cells and erythrocytes were far more sensitive to pneumolysin-induced cell lysis at equivalent concentrations of pneumolysin (Fig. 3*A*).

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 $LTC_4$ into the supernatants of LAD2 and HMC-1 cells, without accompanying histamine release (Fig. 3*C*, 3*D*). The amount of $LTC_4$ released with pneumolysin challenge was comparable to that of the mast cell pneumococcal cocultures, supporting the notion that the synthesis of $LTC_4$ from mast cells in coculture was also mediated by pneumolysin (Fig. 3*C*, 3*D*).

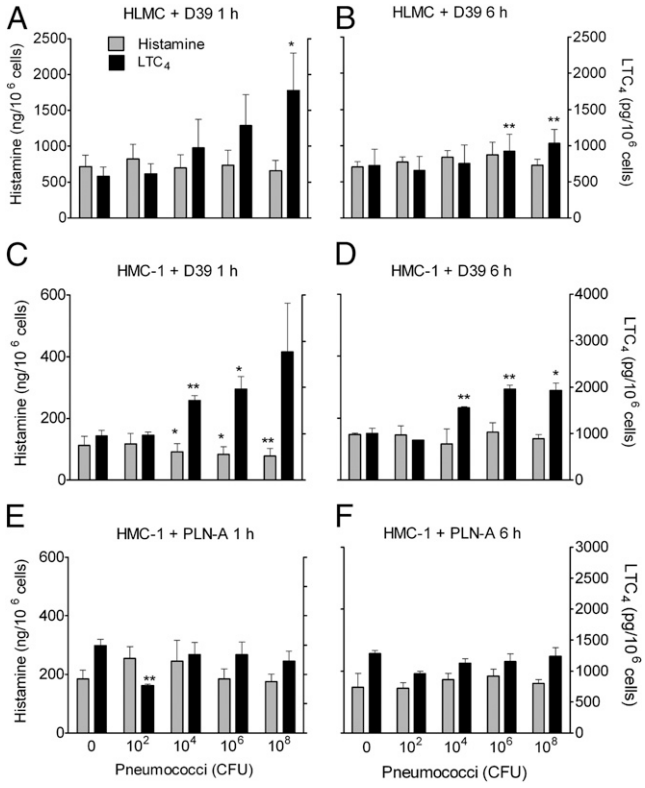


**FIGURE 2.** WT D39, but not pneumolysin-deficient PLN-A, pneumococci induce $LTC_4$ production without histamine release from human mast cells. *A*, WT D39 pneumococci induce the release of $LTC_4$ from HLMCs in a dose-dependent manner, without accompanied histamine release. *B*, This effect was still evident after 6 h of coculture. *C* and *D*, HMC-1 cells also release $LTC_4$, but not histamine, when cocultured with WT D39 pneumococci. The pneumolysin-deficient PLN-A pneumococci did not induce $LTC_4$ release at 1 h (*E*) or 6 h (*F*). $*p < 0.05$; $**p < 0.01$.

### *Pneumolysin does not induce cytokine synthesis*

Because mast cells selectively release $LTC_4$ 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 $10^6$ cells. With the addition of pneumolysin at 1 or 10 μg/ml for 1 h, HLMCs released 5.3 ± 2.2 ng/$10^6$ cells of LL-37 (32% of cell content) and 8.5 ± 4.9 ng/$10^6$ cells of LL-37 (51% of cell content), respectively (Fig. 5*A*).

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: $1.1 \times 10^3$ CFU versus $2.2 \times 10^4$ CFU, respectively (Fig. 5*B*). 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. 5*C*). No effect was seen with the $IgG_1$ isotype control.

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

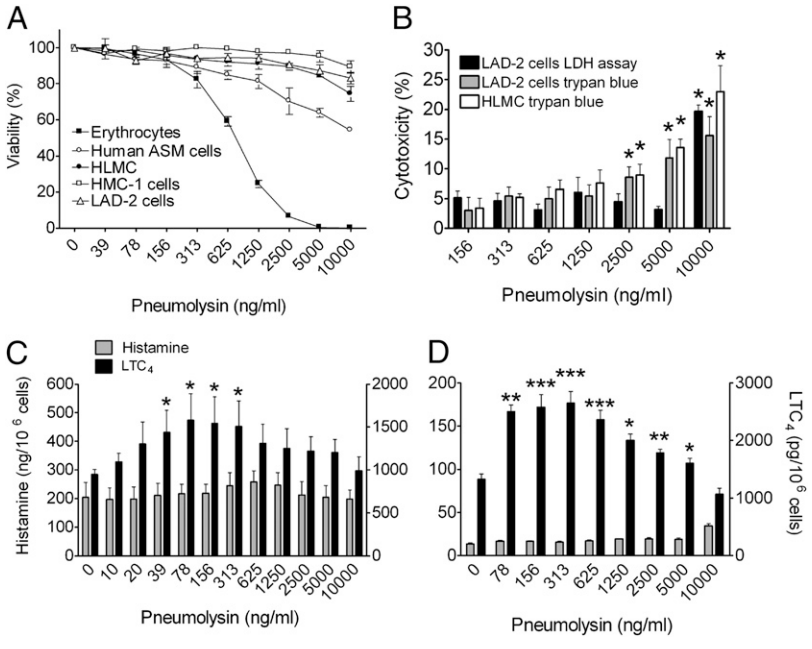


**FIGURE 3.** Mast cells are resistant to lysis from pneumolysin, which induces $LTC_4$ production but not histamine release. *A*, Human mast cells are resistant to lysis by purified pneumolysin compared with human airway smooth muscle cells and erythrocytes. Assessed by trypan blue staining. *B*, Comparison of mast cell lysis using trypan blue staining and LDH release. Pneumolysin at sublytic concentrations induced the release of $LTC_4$, but not histamine, in HMC-1 cells (*C*) and LAD-2 cells (*D*). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

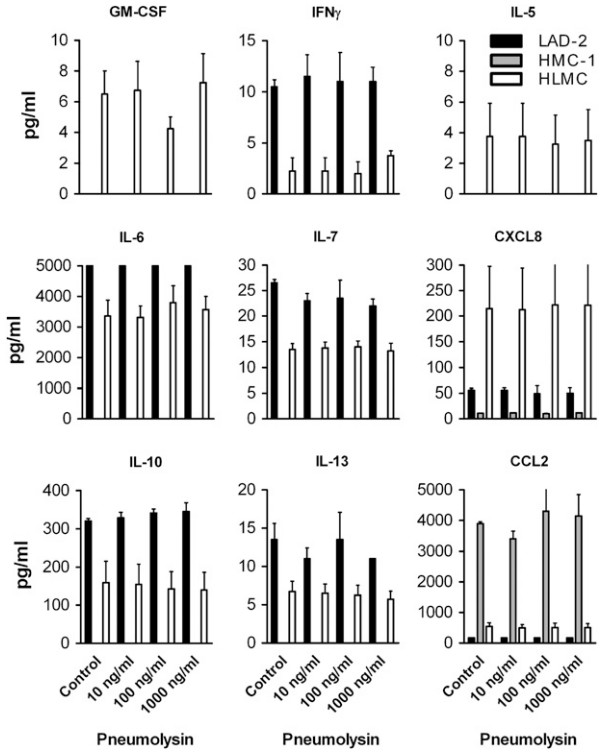


**FIGURE 4.** Primary HLMCs and the human mast cell lines HMC-1 and LAD-2 have different cytokine profiles, but none was affected by the addition of pneumolysin at sublytic doses.

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. 5*D*). 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. 5*D*). 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% ± 1.3% of control after 1 h of coculture ($n = 5$; $p = 0.0012$) and 55.3% ± 2.9% of control after 6 h of coculture ($n = 5$; $p = 0.0006$) (Fig. 6*A*). WT D39 pneumococci were more effective at killing HMC-1 cells at equivalent CFU, with HMC-1 viability of 63.4% ± 9.0% at 1 h ($n = 5$; $p = 0.0099$) and 16.6% ± 5.8% at 6 h ($n = 5$; $p = 0.042$) (Fig. 6*B*).

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% ± 10.2% ($n = 3$, $p = 0.0298$) (Fig. 6*C*), suggesting that although pneumolysin was partly responsible for the reduction of mast cell viability in these assays, other factors were involved.

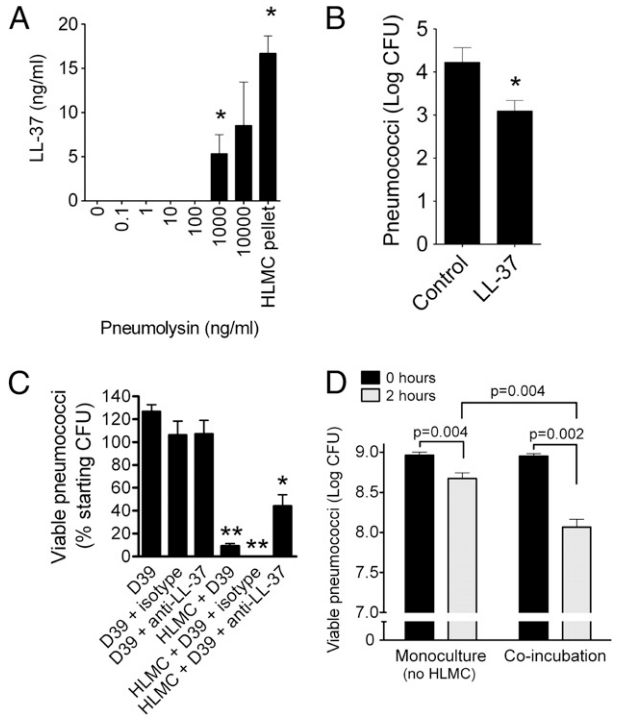


**FIGURE 5.** HLMC cytotoxicity to pneumococci is due to LL-37 and is independent of cell contact. *A*, HLMCs store the cathelicidin LL-37 and release it within 1 h when stimulated with pneumolysin. *B*, LL-37 reduces pneumococcal viability with similar efficacy as HLMCs. *C*, The addition of LL-37 neutralizing antiserum reduces the cytotoxicity of D39 pneumococci induced by HLMCs. *D*, HLMCs kill WT D39 pneumococci, even when separated by a 0.4-μm membrane eliminating cellular contact. $*p < 0.05$; $**p < 0.01$.

*Pneumococcal-induced reduction of mast cell viability involves pneumococcal $H_2O_2$ and pneumolysin*

To elucidate the mechanism of mast cell cytotoxicity induced by pneumococci, we next examined the effects of HMC-1 coculture with the $H_2O_2^-$pneumolysin$^+$ WT D39 isogenic pneumococcal mutant SpxD39. Importantly, we found that after 6 h of coculture with HMC-1 cells, the $H_2O_2^-$ mutant SpxD39 was comparatively ineffective at inducing mast cell death. Thus, with the addition of $10^8$ CFU SpxD39, mast cell viability was reduced to only 73.4% ± 8.1% after 6 h ($n = 3$; $p = 0.0815$) (Fig. 7*A*), which did not quite reach statistical significance. Compared with the WT D39 pneumococci, there was significantly less mast cell cytotoxicity

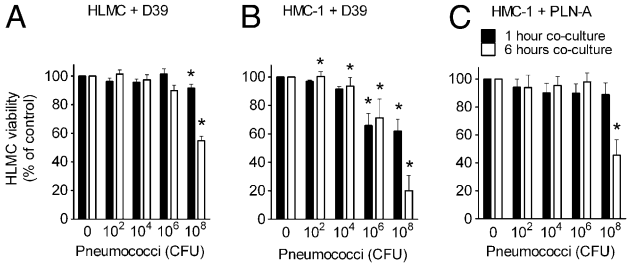


**FIGURE 6.** Pneumococci kill human mast cells at high CFU. *A*, WT D39 pneumococci kill HLMCs after 6 h of coculture but only at high CFU. *B*, This cytotoxicity was more evident in HMC-1 cells. *C*, Pneumolysin-deficient PLN-A pneumococci were less effective at killing HMC-1 cells. $*p < 0.05$.

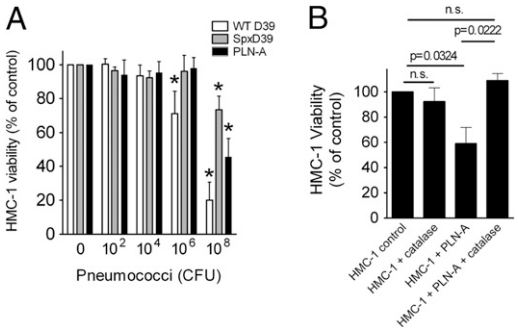


**FIGURE 7.** Pneumolysin and $H_2O_2$ contribute to pneumococcal-mediated mast cell cytotoxicity. *A*, WT D39 pneumococci effectively killed HMC-1 cells. PLN-A pneumococci were less effective at killing HMC-1 cells. The $H_2O_2^-$ pneumococci (SpxD39) were also less effective at killing HMC-1 cells than were WT D39 and PLN-A pneumococci. *B*, Pneumolysin-deficient pneumococci were unable to kill HMC-1 cells in the presence of catalase, confirming that pneumolysin and $H_2O_2$ contribute to mast cell cytotoxicity. $*p < 0.05$.

(HMC-1 viability was 73.4% ± 8.1% [SpxD39] versus 20.1% ± 10.8% [WT D39]; $n = 3$; $p = 0.0098$). The equivalent CFU of PLN-A and WT D39 pneumococci reduced HMC-1 viability to 46.5% ± 10.2% ($n = 3$; $p = 0.0298$) and 20.1% ± 10.8% ($n = 3$; $p = 0.0358$), respectively (Fig. 7*A*).

To confirm that pneumolysin and $H_2O_2$ were the virulence factors involved in mast cell cytotoxicity, we next used the PLN-A pneumococci and HMC-1 cocultures with and without catalase to remove $H_2O_2$ from the cocultures. In these experiments, PLN-A pneumococci reduced HMC-1 viability to 59.1% ± 12.7% ($n = 5$; $p = 0.0324$) after 3 h of coculture. The addition of 200 U/ml of catalase to the PLN-A/HMC-1 cocultures completely abolished the cytotoxicity induced by the pneumolysin$^-H_2O_2^+$ pneumococci, whereas the addition of 200 U/ml of catalase to the mast cell monocultures had no significant effect (Fig. 7*B*). This confirmed that $H_2O_2$ in conjunction with pneumolysin were the primary virulence factors involved in the pneumococcal-induced cytotoxicity of human mast cells.

## Discussion

We demonstrated for the first time that ex vivo HLMCs and the mast cell line HMC-1 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 $LTC_4$, 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 $LTC_4$ (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 $LTC_4$ and LL-37 and, subsequently, associated pneumococcal cell death.

Pneumolysin is a member of the family of cholesterol-dependent cytolysins 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 $LTC_4$ 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 $LTC_4$ 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 $LTC_4$ release, but not degranulation, in human cord blood-derived mast cells, whereas the synthetic TLR1/TLR2 agonist $Pam_3CSK_4$ induced degranulation, but not $LTC_4$ production (45). Furthermore, in a recent study by Sur et al. (46), it was shown that inhibition of the protein tyrosine kinase JAK2, using specific inhibitors and small interfering RNA knockdown, markedly reduced the production of $LTC_4$ in anti-IgE–stimulated mast cells, without the associated inhibition of histamine release (measured by β hexosaminidase release) or $PGD_2$ synthesis (46). This suggests that JAK2 plays an important role in the specific production of $LTC_4$. 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. 3*B*, 3*D*). 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 $LTC_4$ 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 $LTC_4$ 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 CFU. HLMCs were less sensitive in this respect than HMC-1 cells. We showed that $H_2O_2$ is an important virulence factor for pneumococcal-induced mast cell cytotoxicity. However, von Köckritz-Blickwede et al. (11) demonstrated that $H_2O_2$ 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 "NETosis" 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 $LTC_4$, 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.

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