Impact of LL-37 on anti-infective immunity

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Abstract: Host defense peptides (often called cationic antimicrobial peptides) have pleiotropic immunomodulatory functions. The human host defense peptide LL-37 is up-regulated at sites of infection and has little or no antimicrobial activity in tissue-culture media but under the same conditions, demonstrates immunomodulatory effects on epithelial cells, monocytes, and dendritic cells (DC). These effects include the induction of chemokine production in a mitogen-activated protein kinase-dependent manner in epithelial cell lines and monocytes and profound alterations of DC differentiation, resulting in the capacity to enhance a T helper cell type 1 response. Although the exact mechanisms of interaction between LL-37 and these cell types have not been elucidated, there is evidence for specific (i.e., receptor-mediated) and nonspecific interactions. The relative significance of the direct antimicrobial activities and immunomodulatory properties of LL-37 and other cationic host defense peptides in host defense remains unresolved. To demonstrate that antimicrobial activity was not necessarily required for protection in vivo, model peptides were synthesized and tested for antimicrobial and immunomodulatory activities. A peptide with no antimicrobial activity was found to be protective in animal models of *Staphylococcus aureus* and *Salmonella* infection, implying that a host defense peptide can protect by exerting immunomodulatory properties. *J. Leukoc. Biol.* 77: 451–459; 2005.

Key Words: *cathelicidin · host defense peptide · immunomodulator · innate immunity*

INTRODUCTION

Increasing evidence suggests that a primary role of various cationic host defense peptides (often called cationic antimicrobial peptides) in vivo may be to modulate or prime the immune response. Although there is no doubt that under some circumstances, such peptides have antimicrobial activity in vivo; e.g., at the mg/ml concentrations at which α-defensins are found in the lysosomes of neutrophils, this may not be the case for other cationic peptides in other body locations. The human cationic cathelicidin peptide LL-37, for example, has no direct antimicrobial activity in vitro under physiologically relevant salt and peptide concentrations but under the same conditions, has immunomodulatory effects in model systems of mucosal surfaces [1–6]. LL-37 is produced at mucosal surfaces by epithelial cells, up-regulated in response to infection and inflammation, and can be released by degranulation of neutrophils [7–10]. Thus, in the process of combating infection, incoming effector cells of the innate-immune response, such as monocytes and pre-dendritic cells (DC), would be expected to be exposed to gradients of LL-37, which has been demonstrated to have a wide range of immunomodulatory properties on various cell types, including epithelial cells, peripheral blood monocytes, and monocyte-derived DC (Mo-DC), at peptide concentrations and cation levels that are found at sites of infection or inflammation. In addition, certain cytokines that are present at mucosal surfaces appear to synergise with LL-37 to alter or enhance its immomodulatory activity. To test the hypothesis that the protective properties of host defense peptides in vivo might not be dependent on their direct antimicrobial activity, synthetic peptides without any antimicrobial activity were constructed. These peptides were protective in relevant animal models of *Staphylococcus aureus* and *Salmonella typhimurium* infection, respectively, demonstrating that antimicrobial activity is not absolutely required for protection against infection.

ANTIMICROBIAL ACTIVITY OF HOST DEFENSE PEPTIDES UNDER PHYSIOLOGICAL CONDITIONS

The importance of host defense peptides in the immune response has been definitively demonstrated, as animals defective in host defense peptide expression or proteolytic activation of host defense peptides are more susceptible to specific infections [11, 12]. However, the role that direct antimicrobial activity plays in this protective effect is not clear, and in fact, although the antimicrobial activities of many cationic peptides under in vitro conditions are well established, their activity under physiologically relevant conditions is not entirely clear. The antimicrobial activity of these peptides has often been

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assessed by determining the minimal inhibitory concentration (MIC) of peptide required to kill bacteria in the presence of dilute media or under low salt conditions (often ≤20 mM NaCl). For example, LL-37 has a MIC of between 1 and 30 μg/ml against a variety of common bacteria in media of low ionic strength [13, 14]. In an attempt to mimic physiological conditions found in the human body, NaCl is frequently added to the culture medium, and the presence of salt often abolishes antimicrobial activity. For example, defensins demonstrate profound salt sensitivity, and for many, their antimicrobial activity is completely lost at concentrations of 100 mM NaCl [15–17]. However, high concentrations of Na^+ ions may not sufficiently mimic physiological conditions, as we and others have demonstrated that cations such as Mg^{2+} and Ca^{2+} and polyanions tend to be more antagonistic toward antimicrobial activity than the equivalent concentrations of monovalent cations [14, 18]. In the presence of 100 mM Na^+ ions, the antimicrobial activity of LL-37 is decreased by two- to eight-fold [14], and in the presence of tissue-culture medium, LL-37 has no killing activity against *S. aureus* or *S. typhimurium* even at concentrations as high as 100 μg/ml (**Fig. 1**). In other studies of optimized α-helical peptides, 1 mM $MgCl_2$ raised the MIC for *Pseudomonas aeruginosa* as much as did 200 mM NaCl [18]. This is extremely relevant to in vivo activity, as most body fluids, including sputum [19], airway surface liquid [20], and serum/plasma [21], contain divalent cation concentrations of between 1 and 2 mM. Figure 1 demonstrates that LL-37 significantly reduces bacterial growth in phosphate buffer. However, although LL-37 had some antimicrobial activity in Mueller Hinton medium at a concentration of 100 μg/ml (data not shown), it had no observable killing activity in tissue-culture medium. Conversely, even at a concentration of 200 μg/ml, a small, synthetic peptide IMX00C1 was not antimicrobial in PBS. Although there are examples in the literature, which indicate that certain peptides may be directly antimicrobial [22], especially defensins at the high concentrations present in phagocyte lysosomes, intestinal crypts, and psoriatic skin, we are not aware of any conclusive evidence suggesting that LL-37 and other peptides (including defensins) can directly kill bacteria at mucosal surfaces. In fact, a derivative of LL-37, instilled simultaneously with *P. aeruginosa* into the mouse lung, reduced lung damage and proinflammatory cytokine production but not bacterial counts [23]. This weak antimicrobial activity under physiologically relevant conditions is observed with many cationic peptides including LL-37 [18, 24] and has led us to question whether the primary function of certain peptides is to kill bacteria directly.

Studies of the immunomodulatory properties of cationic peptides are performed in standard tissue-culture media, which contain physiological concentrations of Na^+ ions above 100 mM and between 0.8 and 2 mM Mg^{2+} and Ca^{2+}. These concentrations are more relevant to those found under physiological conditions. For example, the ability of host defense peptides to induce chemokine release [4] and neutralize lipopolysaccharide (LPS)- and lipoteichoic acid (LTA)-induced stimulation of proinflammatory cytokines [25] has been demonstrated in whole human blood that contains nearly 2 mM ionized divalent cations and 200 mM Na^+. Many other immunomodulatory properties of cationic peptides, including their ability to induce chemotaxis [26, 27], to induce histamine release by mast cells [26, 28], to promote angiogenesis [2], and to modulate DC differentiation [29], have been evaluated in physiologically relevant media, indicating that these immunomodulatory properties may occur under physiological conditions.

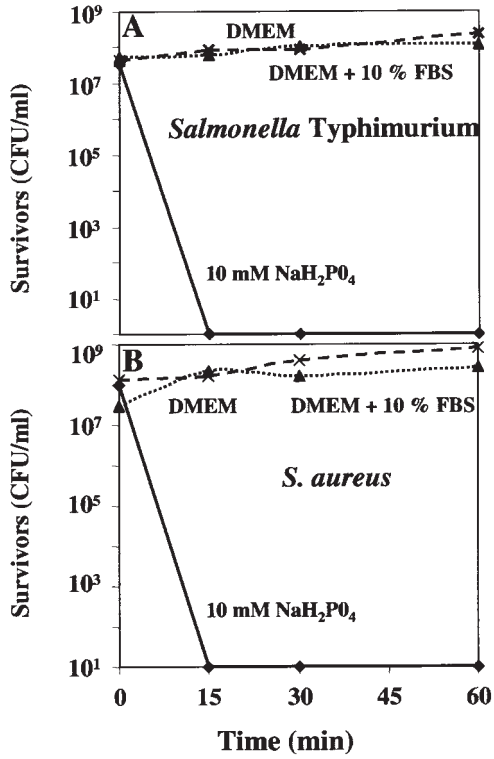

Fig. 1. Influence of tissue-culture medium on the antimicrobial activity of antimicrobial peptides. Overnight cultures of *S. typhimurium serovar enterica* or *S. aureus* American Type Culture Collection 25923 (ATCC; Manassas, VA) were diluted 50-fold in Luria broth and allowed to grow to exponential phase (optical density at 600 nm of 0.5) at 37°C. The cultures were spun down and resuspended in 10 mM phosphate buffer, pH 7.0, or in tissue-culture medium, Dulbecco's modified Eagle medium (DMEM), with or without 10% fetal bovine serum (FBS). LL-37 was added at a concentration of 100 μg/ml. Samples (100 μl) were removed at 0 min, and after 15, 30, and 60 min, viability was assessed by appropriate dilution and plating for viable bacteria on L agar (L broth containing 1% agar). (A) *S. typhimurium serovar enterica* or (B) *S. aureus* ATCC 25923 was resuspended in 10 mM phosphate buffer, pH 7.0 (◆), or in DMEM, with (×) or without (▲) 10% FBS. LL-37 was antimicrobial at 15 min in phosphate buffer; however, it lost all antimicrobial activity for both strains in tissue-culture media. In contrast to LL-37, IMX00C1 was not antimicrobial, even at a concentration of 200 μg/ml in phosphate-buffered saline (PBS; data not shown). CFU, Colony-forming units.

IMMUNOMODULATOR ACTIVITY OF LL-37 AT EPITHELIAL SURFACES

LL-37 is the proteolytically processed form of a protein, hCAP-18, which is found at extremely high concentrations in the specific granules of neutrophils (~630 μg per 10^9 cells) [30]. The hCAP-18 precursor protein is also found at more modest concentrations in lymphocytes, macrophages, and a range of epithelial cells [13, 31]. Upon encountering invading microorganisms, neutrophils release the contents of azurophilic

and specific granules into the resultant phagolysosome (by phagosome-lysosome fusion) or to the cell exterior. LL-37, the 37-residue C-terminal peptide of hCAP-18, is cleaved by proteinase-3 after exocytosis from the neutrophils [32]. Thus, during infection and inflammation, high concentrations of LL-37 will be released at sites of neutrophil accumulation. This has been demonstrated to occur in gingival tissues and in the saliva, where there is no evidence that LL-37 is produced by these tissues, but instead, it is presumed to be deposited by the homeostatic degranulation of neutrophils [33, 34].

The precursor hCAP-18 is also produced by a variety of epithelial cell types. Its expression is moderately inducible upon stimulation with proinflammatory cytokines such as interleukin (IL)-1α [8] or bacterial components [35]. Although it is not yet clear how hCAP-18 is released from these cell types and processed, the processed peptide LL-37 is found at increased concentrations at cell surfaces in a number of inflammatory conditions, including psoriasis [36] and inflammatory lung conditions [37]. Thus, in the context of infection and inflammation, LL-37 can be derived from release by neutrophils recruited to the site of infection and production and release by epithelial cells (**Fig. 2**). LL-37 has been demonstrated to be a chemoattractant for neutrophils, monocytes, and subsets of T cells [27]. Consequently, it is expected to contribute to further recruitment of these cells to the site of infection or inflammation. LL-37 has also been shown to induce IL-8 release by lung epithelial cell lines [1, 4]. IL-8 is a potent chemokine for neutrophils and monocytes and is found at high concentrations at sites of inflammation. At the physiological concentrations of LL-37 found at sites of infection and inflammation (5 μg/ml), LL-37 would thus be anticipated to stimulate epithelial cells to produce chemokines such as IL-8, which we hypothesize would in turn increase the recruitment of effector cells of the innate-immune response (Fig. 2).

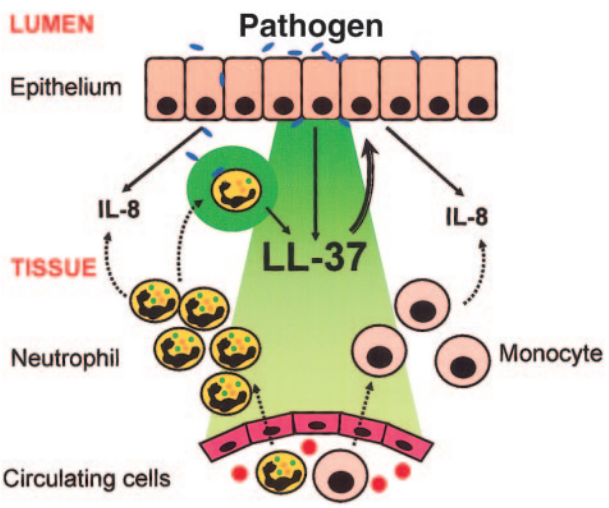

Fig. 2. LL-37 is a potent immunomodulator at epithelial surfaces. Upon detection of infection or stimulation with inflammatory mediators, resident or recruited neutrophils release granule contents including high concentrations of antimicrobial peptides such as LL-37. Epithelial cells respond to inflammatory stimuli such as the proinflammatory cytokine IL-1α and induce transcription of LL-37. A concentration gradient of LL-37 would occur at the site of infection. This concentration gradient would lead directly to chemotaxis of neutrophils, monocytes, and other cell types, with LL-37 acting as a chemotactic agent for many cell types of the innate-immune response. In addition, LL-37 can induce epithelial cells to produce IL-8 and other chemokines. Increased concentrations of IL-8 would lead to increased infiltration of neutrophils and monocytes. These cells would be arriving at sites with relatively high concentrations of LL-37, which would promote a variety of immunomodulatory effects.

LL-37 has pleiotropic effects on epithelial cells. It has been shown to induce chemokine production in a mitogen-activated protein kinase (MAPK)-dependent manner [1, 5] and to promote re-epithelization [3], although it is not entirely clear whether LL-37 initiates these activities in a specific (i.e., receptor-mediated) or nonspecific manner. Studies have indicated that there are a variety of receptors on different cell types [1, 27, 38]. Recent data from our laboratory accepted for publication indicate that LL-37 binds directly to epithelial cell surfaces and to become internalized by endocytosis [39]. Transcription and release of IL-8 are inhibited by blocking the internalization of LL-37 with an inhibitor of endocytosis, brefeldin A [39], although brefeldin A did not inhibit the transcriptional up-regulation of the IL-8 gene in response to LPS, an important control as a result of the known pleiotropy of brefeldin A (**Fig. 3**). These studies indicate that LL-37 binds to and is internalized by an active process, which is not a consequence of nonspecific effects such as membrane permeabilization or disruption. Internalization of this peptide is a requirement for at least one downstream biological activity. Further work needs to be done to better characterize this interaction and to determine if it is linked to other LL-37-mediated immunomodulatory events.

The first cells that are recruited to sites of infection and inflammation are neutrophils. If the infection is not resolved in minutes to hours, a second wave of cells, including monocytes, will be conscripted to the site of infection [40]. These monocytes will arrive at sites of elevated local concentrations of LL-37. We have previously demonstrated that upon exposure to LL-37, peripheral blood-derived monocytes produce chemokines such as IL-8, monocyte chemoattractant protein-1 (MCP-1), and MCP-3 in a MAPK-dependent manner [5]. These chemokines would collectively attract neutrophils, monocytes, and macrophages, respectively. Thus, when the first line response is insufficient, LL-37-activated monocytes are induced to produce chemokines, which we hypothesize will lead to the recruitment of important immune response effector cells to assist in the resolution of infection. It is interesting that LL-37 induces the transcription and release of chemokines but not proinflammatory cytokines in peripheral blood-derived monocytes or in animal models (D. M. E. Bowdish and R. E. W. Hancock, unpublished observations; [4]). Indeed, LL-37 inhibits the production of proinflammatory cytokines in response to LPS, LTA, or other pathogen-associated molecular patterns in monocyte- or macrophage-like cell lines [25, 41, 42]. Thus, LL-37 is probably involved in the resolution of inflammation by blocking the production of proinflammatory cytokines and by recruiting cells of the immune response, which are required to remove invading microorganisms.

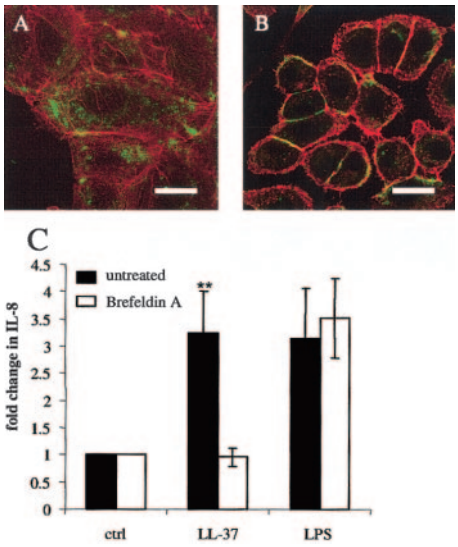

Fig. 3. LL-37 interaction with a lung epithelial cell line leading to internalization and IL-8 synthesis. A biotinylated version of LL-37 was created by synthesizing LL-37 with an additional cysteine at the C-terminal (LL-37C). LL-37C was biotinylated at the C-terminal cysteine side-chain with N-α-(3-maleimidylpropionyl) biocytin (Molecular Probes, Eugene, OR). LL-37C (2 mmol) and N-α-(3-maleimidylpropionyl) biocytin (23 mmol) were dissolved separately (430 mM and 1 mM, respectively) in 50 mM Tris buffer, pH 7, and mixed. The mixture was incubated for 2 h at room temperature with shaking. Excess N-α-(3-maleimidylpropionyl) biocytin was quenched using 2-mercaptoethanol (BioRad, Montreal, QC). Biotinylated LL-37 (LL-37B) was purified using a reverse-phase fast protein liquid chromatography column (Resource 15RPC, 3 ml, Pharmacia, Piscataway, NJ) with a water/acetonitrile gradient containing 0.1% trifluoroacetic acid. Peptide purity was confirmed by high-pressure liquid chromatography and mass spectrometry analysis. (A) LL-37 binding to and internalization by the A549 lung epithelial cell line. A549 cells were seeded onto glass coverslips and incubated overnight at 37°C, 5% CO_2. After a 24-h incubation, cells were stimulated with 10 μg/ml LL-37B for 2 h. The coverslips were washed extensively after fixing, and the cells were permeabilized using 0.1% Trition X-100. LL-37B (green) was probed using streptavidin and then detected with biotin-Oregon green. Actin (red) was detected using Alexa-conjugated phalloidin. Biocytin was used as a negative control for all conditions. Cells were then washed extensively with PBS, and the coverslips were mounted in Vectashield. Coverslips were viewed using a BioRad radiance confocal microscope. (B) Dependence of internalization on endocytosis. To determine if endocytosis was required for trafficking of LL-37B, A549 cells were prepared as described above for immunofluorescence, and the cells were incubated with an inhibitor of endocytosis, brefeldin A (1 h, 5 μg/ml, Sigma Chemical Co., St. Louis, MO), prior to a 2-h incubation with 10 μg/ml LL-37B (2.2 μM). In the presence of brefeldin A, LL-37B does not enter the cells. Original bar symbolizes 20 μm. (C) Dependence of LL-37-induced IL-8 gene expression on endocytosis. A549 cells were incubated with brefeldin A (1 h, 5 μg/ml) or were left untreated prior to the addition of 10 μg/ml LL-37 (2.2 μM) or 100 ng/ml *P. aeruginosa* PAO1 LPS for up to 4 h. RNA collection was performed using standard methods. LL-37-induced IL-8 production was inhibited by brefeldin A pretreatment, whereas LPS-induced IL-8 production was not. Semiquantitative reverse transcriptase-polymerase chain reaction was performed using primers to IL-8 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control (ctrl). The fold change was normalized to GAPDH. A ratio of one indicates no increase in expression over the control. Results are expressed as mean ± SE of three separate experiments. Student's two-tailed *t*-test was performed. **, $P < 0.01$.

LL-37 MODULATION OF DC DIFFERENTIATION

Resident immature DC (iDC) in mucosal and epithelial tissues are highly effective antigen-capturing cells, which are derived from pre-DC populations (monocytes and plasmacytoid cells) and hematopoietic precursor cells [43]. The primary function of iDC is to sample antigen. Following antigen uptake and recognition of pathogens by pattern recognition receptors, iDC become activated into antigen-processing and -presenting mature DCs (mDC). These mDC migrate to the lymph nodes, where they present sample antigen to naïve T cells [44]. To replenish these populations of iDC, extensive recruitment of pre-DC populations to the peripheral tissues and concurrent differentiation occurs, the net result of which is the replacement of resident DCs with a "second line" of newly recruited iDCs [45]. In the above model of DC differentiation and maturation, these second-line DC would be arriving at areas of high LL-37 concentration. We have demonstrated that the presence of LL-37 has profound effects on the phenotype and function of all stages of DC development [46]. A short exposure to LL-37 early in the differentiation process of peripheral blood monocytes to iDC leads to a wide spectrum of phenotypic changes at concentrations that are expected to be present at sites of inflammation [36, 47, 48]. These LL-37-primed Mo-DC displayed altered morphology, significantly enhanced endocytic capacity, and altered phagocytic capacity, possibly as a result of the increased expression of α_2 integrins and the opsonic and nonopsonic receptors CR3 and CR4 [46].

Although early LL-37 treatment had profound effects on the downstream expression of numerous cell-surface markers, it did not induce maturation of the DC. LL-37-derived iDC did not show altered expression of CD80, human leukocyte antigen-DR, CD83, or other markers of maturation when compared with control iDC nor produce cytokines in the absence of additional maturational stimuli. This is in contrast with one study [49], where it was found that a fusion product of the murine cationic host defense defensin with a tumor antigen induced maturation of DC through Toll-like receptor 4 (TLR4). Exposure to LL-37 during differentiation did not induce maturation of DCs. However, it is intriguing that LL-37-primed iDC did have increased expression of CD86, a costimulatory molecule, which amplifies DC stimulation of T cells, and this increased expression of CD86 was observed in LPS-matured, LL-37-primed DC, suggesting that these DC would have increased T cell stimulatory capacity. It is interesting that other modifiers of iDC maturation, such as prostaglandin E_2 and IL-10, inhibit the maturation process and IL-12 production and thereby, promote tolerogenic or T helper cell type 2 (Th2) responses [50, 51]. In contrast, LL-37-primed DC underwent normal maturational processes, produced significantly more Th1 stimulatory cytokines, and significantly enhanced T cell interferon-γ responses, thus demonstrating an enhanced Th1 response. These data suggest that precursor DC, arriving at sites of high LL-37 concentration, will undergo significant phenotypic and functional changes, which enhance antigen-sampling capacity. As these DC mature and move to T cell areas, their ability to promote a Th1 response could have an

important impact on the development of the adaptive-immune response (**Fig. 4**).

ENHANCEMENT OF LL-37-INDUCED IMMUNOMODULATORY EFFECTS

A major criticism of host defense peptide research is that many of the antimicrobial and immunomodulatory effects observed in culture occur at concentrations that are much higher than would be expected in vivo or that would only be expected to occur at sites of severe, chronic inflammation. Determining the exact concentrations of cationic peptides in vivo is technically difficult [6]. With this caveat, LL-37 can be detected at concentrations of 1 μM (~5 μg/ml) in the bronchoalveolar lavage fluid of healthy infants [47], and its concentration is increased by two- to threefold in the bronchoalveolar lavage fluid from infants with systemic or pulmonary inflammation [47]. However, accurately assessing the dilution factor for the airway surface liquid, which has an estimated depth of only 1 μl per cm^2 tissue [52], and accounting for any secretory response stimulated during lavage procedure mean that bronchoalveolar lavage estimations may have low accuracy. Also, this method measures the average increase in peptide concentration across a wide surface area, and any localized increases, which we hypothesize, could be much greater than are currently estimated, would be missed. Thus, it seems possible that under some physiological conditions, the concentrations of host defense peptides and specifically, concentrations of LL-37 might be quite high at localized sites of infection.

It can be assumed that a concentration gradient of LL-37 would form in the lung as a result of release of the peptide by neutrophils and de novo production by epithelial cells. However, many other cytokines and inflammatory mediators would also be present, and the involvement of these other inflammatory mediators in peptide-mediated immunomodulation has not been fully assessed. To date, synergies between host defense peptides and components of the inflammatory milieu, including larger proteins, such as lactoferrin, elastase, and secretory leukocyte protease inhibitor, have been tested with regard to antimicrobial activity [13, 53]. However, synergies with regard to other immunomodulatory activities have not been investigated.

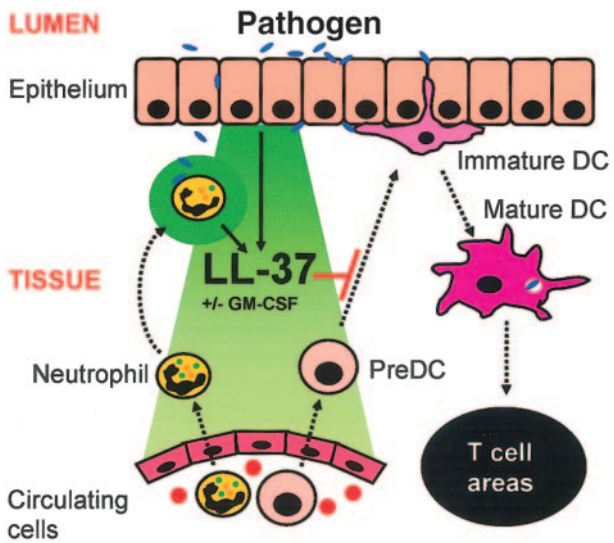

Fig. 4. The cytokine milieu may affect LL-37 responses. Circulating effector cells of innate and adaptive immunity arrive at sites of high LL-37 concentration. LL-37, produced by epithelial cells and neutrophils at sites of infection and inflammation, modulates the differentiation of iDC from precursor cells. These LL-37-derived, second-line DC have altered phenotypes with increased antigen-capture capacity and enhance a more robust Th1 response. Synergy between granulocyte macrophage-colony stimulating factor (GM-CSF) and LL-37 may be important in this process.

As described above, we have demonstrated that LL-37 induces activation of the MAPK, extracellular signal-regulated kinase 1/2, and p38 in peripheral blood-derived monocytes at high concentrations of LL-37 (50 μg/ml). It is interesting that the presence of GM-CSF increases the magnitude of this activation and decreases to between 5 and 10 μg/ml the threshold amount of LL-37 required to induce activation [5]. This synergy was found to be specific to GM-CSF, as the structurally related peptides IL-4 and M-CSF did not have the same ability to enhance LL-37-induced signaling.

GM-CSF is a cytokine that is produced by macrophages and T lymphocytes [54] and is also produced by lung epithelial cell lines in response to proinflammatory cytokines or exposure to bacteria [55, 56]. GM-CSF promotes the survival, proliferation, differentiation, and activation of hematopoietic cells, predominantly in the macrophage and neutrophil lineages [54]. GM-CSF has a number of other immunomodulatory properties, including enhancement of antigen presentation, promotion of phagocytosis and antibody-dependent killing, induction of chemotaxis, and induction of the release of reactive oxygen intermediates and histamines [57–60]. It is interesting that GM-CSF is known to work synergistically with other cytokines. For example, addition of GM-CSF with IL-10 leads to increased expression the CC chemokine receptor 1 [61]. In bronchial epithelial cells, GM-CSF is produced upon stimulation with TLR agonists and proinflammatory cytokines [55, 62, 63]. Thus, we propose that stimulation by proinflammatory components of GM-CSF production at local infection sites would serve to magnify the local immunomodulatory effects of LL-37. Incoming monocytes and pre-DC would then be attracted to sites containing higher concentrations of LL-37 and GM-CSF.

We believe that the modest levels of LL-37 found at a variety of sites throughout the body [30, 47] may not be sufficient to initiate all of the immunomodulatory events ascribed to this molecule per se. However, in the presence of a secondary signal such as GM-CSF, the threshold for immunomodulation would be decreased. There are many precedents in the adaptive-immune response for the requirement of secondary signals [64, 65]. For example, the combination of GM-CSF and IL-4 is required to induce differentiation of peripheral blood-derived monocytes into iDC. Thus, in the studies described above, DC were exposed to GM-CSF and LL-37. Statistically significant changes in the size and surface complexity of the cells and of CD18 and CD11b expression were observed at 5 μg/ml LL-37, and it seems likely that this is, in part, a result of the presence of GM-CSF [46].

HOW DOES LL-37 INDUCE ITS IMMUNOMODULATORY EFFECTS?

It is not entirely clear whether LL-37 mediates its effects through specific receptor-mediated interactions or through more nonspecific interactions. In individual studies, LL-37 and other host defense peptides have often been compared with chemokines. However, unlike the majority of chemokines, LL-37 has been demonstrated to have cross-species reactivity. Whereas most chemokines are restricted to the species expressing the appropriate receptor, LL-37 has been shown to exert its immunomodulatory effects across a wide range of species, including rat [26, 66], mouse [4], rabbit [2], and human. Although this does not eliminate the possibility of a nonspecific receptor, it is an unusual phenomenon that requires more investigation.

To date, there have been a number of receptors associated with LL-37-induced immunomodulation including N-formyl-peptide receptor-like-1 (FPRL-1), $P2X_7$, epidermal growth factor receptor (EGFR), and as of yet, unidentified high- and low-affinity receptors [1, 26, 27]. Two of these putative receptors, $P2X_7$ and EGFR, are not proposed to be direct receptors for LL-37 per se. For example, although inhibitors of the $P2X_7$ receptor can block LL-37-mediated IL-1β processing, other effects, such as an increase in membrane permeability and size and shape changes, cannot be inhibited. This indicates that LL-37 induces an upstream activation event, which results in activation of $P2X_7$ and appears to be independent of other identified receptors [38]. This upstream event must occur very rapidly, as altered membrane permeability is observed as soon as 1 min after LL-37 stimulation. Similarly, it is not proposed that LL-37 binds the EGFR directly but rather, that it induces cleavage of surface-bound metalloproteases in an undefined manner, the result of which is transactivation of the EGFR [1].

To date, the only receptor to which LL-37 has been proposed to bind directly is the pertussis toxin-sensitive, G-protein-coupled receptor FPRL-1 [27]. LL-37-mediated chemotaxis can be inhibited by an agonist of this receptor or by pertussis toxin. However, other LL-37-mediated effects such as MAPK activation, mast cell chemotaxis, and IL-8 production are not pertussis toxin-sensitive, indicating that LL-37 may mediate these events through other receptors or other mechanisms of action.

HOST DEFENSE PEPTIDES AS THERAPEUTIC AGENTS

It is challenging to demonstrate the role of host defense peptides in host immune responses, although a number of elegant animal experiments have been performed [22]. A number of completely novel peptide families were designed by us to remove antimicrobial activity but retain select immunomodulatory effects. These peptides were screened in a number of cell-based assays and in vivo infection models. They do not stimulate harmful inflammatory responses (e.g., stimulation of

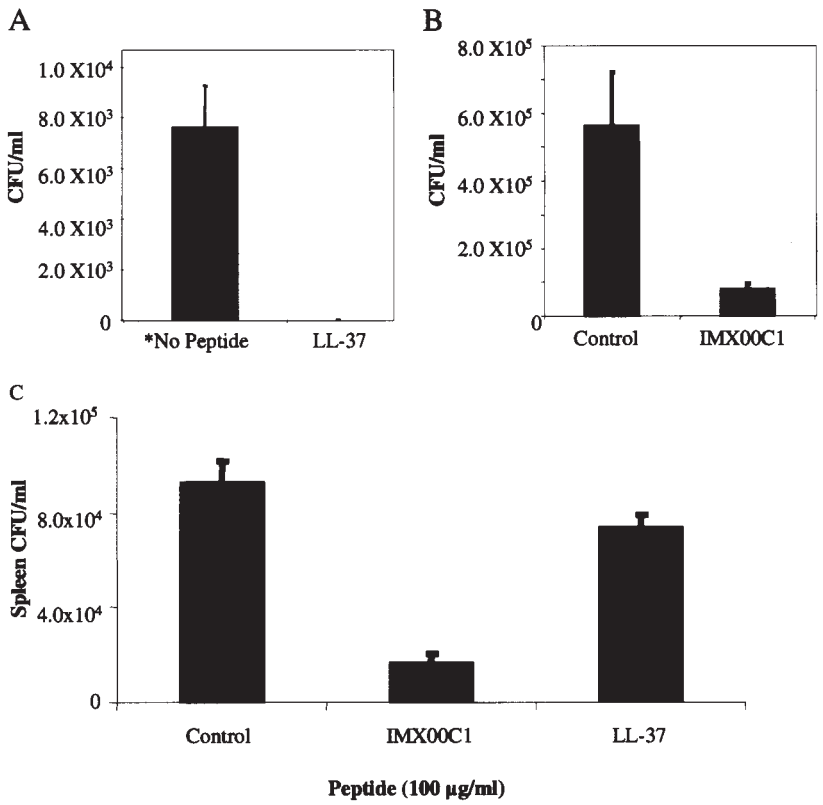

Fig. 5. Protection by selected peptides in murine models of infectious disease. It is important to note that as different bacteria have different virulence and pathogenesis mechanisms, different models and conditions were chosen to assess each bacterial species. Three- to 6-week-old CD-1 mice and BALB/c from Charles River Laboratories (Wilmington, MA) were used in this study. The mice were housed in the animal facility at the University of British Columbia (Vancouver, Canada) in direct accordance with guidelines drafted by the University of British Columbia's Animal Care Committee and the Canadian Council on the Use of Laboratory Animals. Mice were monitored every day, and when any mouse showed significant distress or became moribund, it was immediately killed. For inoculations, bacteria were grown overnight with shaking in Luria broth at 37°C. (A) CD-1 mice were given 1×10^8 *S. aureus* in 5% porcine mucin intraperitoneal (IP). Peptide (200 μg) was also given IP. Mice were monitored for 3 days, then killed, blood removed, and plated for viable counts. *Mice (two of eight) died in the No Peptide group. The average CFU count and SE is shown. LL-37 was found to significantly reduce bacterial load in the murine infection model. (B) CD-1 mice (10/group) were given 3×10^7 *S. aureus* in 5% porcine mucin via intraperitoneal injection. IMX00C1 (50 μg, 2.2 mg/kg) was given via a separate intraperitoneal injection 6 h after *S. aureus* injection. Mice were killed 24 h later, blood removed, and plated for viable counts. (An earlier time-point for measurement of blood counts was chosen as a result of the death of two animals in the experiment in A.) The average CFU ± SE is shown. The peptide treatment group was significantly different from the control group ($P<0.05$). This experiment was repeated a minimum of three times. (C) BALB/c mice (eight mice/group) were injected intraperitoneally with 100 μg (5 mg/kg) test compound IP and the same time as injection of 4.5×10^5 *S. typhimurium*. Twenty-four hours later, the animals were killed, the spleen removed, homogenized, diluted in PBS, and plated for viable bacterial counts. Counts in mice treated with IMX00C1 were statistically different ($P<0.01$) from the control (untreated) group, whereas LL-37 was not significantly different as measured by ANOVA.

tumor necrosis factor α), and unlike LL-37, they are not cytotoxic at concentrations up to 1 mg/ml (higher concentrations not tested). One particular family of synthetic peptides (12–13 amino acids) with extended structures and a net charge of between +1 and +3 was found to have no significant, direct antimicrobial activity in vitro, but it is significant that several variants were able to reduce the bacterial load when administered in an in vivo infection model. One example of this peptide family, IMX00C1, was able to significantly reduce bacterial load in systemic *S. aureus*- and *S. typhimurium*-infection models (**Fig. 5**). LL-37 has MIC values in Mueller Hinton medium of 32 and 64 μg/ml against these strains of *S. aureus* and *Salmonella*, respectively, and is unable to mediate direct killing of these strains in tissue-culture medium at 100 μg/ml (Fig. 1), and IMX00C1 was completely inactive as an antimicrobial (MIC ≥128 μg/ml), even in phosphate buffer. The synthetic peptide and LL-37 were effective in the Gram-positive infection model using *S. aureus*. However, in the Gram-negative infection model with *S. typhimurium*, IMX00C1 significantly reduced bacterial load in the spleen, but LL-37 did not. The exact mechanism of the protective effect has not yet been elucidated, and the possibility of direct antimicrobial effects in vivo has not been formally excluded. As a result of the total lack of antimicrobial activity of these synthetic peptides under a variety of in vitro conditions and the loss of antimicrobial activity by LL-37 under physiologic concentrations of cations, it appears likely that the protective effect seen in these experiments is primarily a result of their immunomodulatory properties.

It is worth considering what would permit one to conclusively test the alternative hypotheses whether, e.g., LL-37 is acting as an immunomodulator or an antimicrobial in vivo. As mentioned above, a derivative of LL-37, instilled simultaneously with *P. aeruginosa* into the mouse lung, reduced lung damage and proinflammatory cytokine production but not bacterial counts [23]. In an analogous manner, it is well established that LL-37 treatment can reverse endotoxaemia, even in the absence of infection. Therefore, we consider that the general thesis that LL-37 can act as an immunomodulator is well established. What is not clear is what function of LL-37 dominates in clearing infection. One possibility would be to create peptides with similar character to LL-37 that were not antimicrobial in vitro but were still protective in vivo. In a sense, we have tried to do this in the experiments in Figure 5, but these peptides are different sequentially from LL-37 and thus, are not really definitive. We would propose that the best experiment (suggested to us by Dr. Tom Ganz, University of California, Los Angeles) would be to create an immunomodulatory, nonantimicrobial version of the cathelicidin-related antimicrobial peptide (CRAMP) and test if when introduced in into CRAMP$^{-/-}$ mice, it could complement back the activities of CRAMP in antagonizing infection by *Streptococcus* [12]. It remains possible that protection by LL-37 against infection is influenced by antimicrobial and immunomodulatory properties.

In conclusion, these data demonstrate the potential for the therapeutic use of immunomodulatory, anti-infective peptides, which do not eliminate bacteria directly and therefore, circumvent issues of antimicrobial resistance. We have demonstrated that host defense or therapeutic synthetic peptides, with little or no antimicrobial activity under physiologically relevant conditions, can modulate the immune response and provide in vivo protection. We have also demonstrated that this could occur by modulating the responses of a variety of effector cells of the innate-immune response, including epithelial cells, monocytes, and DC. These peptides selectively modulate innate-immune response changes that do not involve a vigorous inflammatory component but effectively protect the host from bacterial infection. These agents should find broad use in a variety of modes of administration and treatment regimens and against a range of pathogens.

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