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Mechanistic insights and therapeutic opportunities of antimicrobial chemokines

Matthew A. Crawford[a], **Katie R. Margulieux**[b], **Arpita Singh**[a], **Robert K. Nakamoto**[c], and **Molly A. Hughes**[a,*]

[a]Division of infectious Diseases & International Health, Department of Medicine, University of Virginia, Charlottesville, VA, 22908, USA [b]Department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences, Bangkok 10400, Thailand [c]Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA, 22908, USA

Abstract

Chemokines are a family of small proteins best known for their ability to orchestrate immune cell trafficking and recruitment to sites of infection. Their role in promoting host defense is multiplied by a number of additional receptor-dependent biological activities, and most, but not all, chemokines have been found to mediate direct antimicrobial effects against a broad range of microorganisms. The molecular mechanism(s) by which antimicrobial chemokines kill bacteria remains unknown; however, recent observations have expanded our fundamental understanding of chemokine-mediated bactericidal activity to reveal increasingly diverse and complex actions. In the current review, we present and consider mechanistic insights of chemokine-mediated antimicrobial activity against bacteria. We also discuss how contemporary advances are reshaping traditional paradigms and opening up new and innovative avenues of research with translational implications. Towards this end, we highlight a developing framework for leveraging chemokine-mediated bactericidal and immunomodulatory effects to advance pioneering therapeutic approaches for treating bacterial infections, including those caused by multidrug-resistant pathogens.

Keywords

Antimicrobial; Mechanism; Chemokine; Peptide; Bacteria; Therapeutic

1. Introduction

Chemokines are a family of approximately 50 structurally related host-derived cytokines that, based on the number and arrangement of their N-terminal cysteine residues, are divided into four classes: CXC, CC, XC, and CX3C [1,2]. Produced by many types of cells, especially leukocytes, these small proteins (about 70–100 amino acid residues; 7–12 kDa) are typically secreted and mediate biological effects by binding a cognate G-protein-coupled

*Corresponding author at: P.O. Box 800513, Charlottesville, VA 22908, USA. mah3x@virginia.edu (M.A. Hughes).

receptors) expressed on the surface of responsive cells [3,4]. Although some chemokines are constitutively expressed and function in homeostatic processes, the majority of chemokines are inflammatory mediators produced in response to Toll-like receptor agonists and/or pro-inflammatory cytokines (e.g. IFN-γ, IL-1β, etc.) [5].

Chemokines are best known for their ability to orchestrate the residence and movement of immune cells during health and disease. This capacity impacts nearly every aspect of the immune system including, among others, hematopoiesis [6], inflammation [7], wound healing [8], immune surveillance [9], and the generation and regulation of innate and adaptive immune responses [5]. In addition to receptor-dependent functions, most, but not all, chemokines have been reported to mediate direct antimicrobial activity against microorganisms in vitro [10–13]. These observations, made by many laboratories, comprise a wide-ranging, sometimes contradictory, patchwork of considered chemokines, microbial targets, and experimental conditions. While this arrangement of inquiry limits detailed comparisons, it highlights the broad-spectrum microbicidal effects mediated by a variety of chemokines. Indeed, individual ligands from the CXC, CC, XC, and CX3C classes, representing both homeostatic and inflammatory chemokines, have been shown to kill human pathogens including Gram-negative bacteria (e.g. *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*, etc.), Gram-positive bacteria (e.g. *Staphylococcus aureus, Enterococcus faecalis, Streptococcus pyogenes*, etc.), parasites (e.g. *Leishmania mexicana* and *Plasmodium falciparum*), and/or fungi (e.g. *Candida albicans* and *Cryptococcus neoformans);* summarized in references [11,12,14].

The extent to which chemokine-mediated antimicrobial activity promotes host defense in vivo is unclear. Employing murine models of infection, a number of groups have demonstrated that mice deficient in a particular chemokine are more susceptible to bacterial challenge [15]. For example, CXCL14 (BRAK), a mucosa-associated chemokine whose corresponding receptor(s) remains unsettled [16], is broadly bactericidal and its genetic ablation is associated with the impaired clearance of *Streptococcus pneumoniae*, but not *P. aeruginosa*, during pulmonary infection [17]. In the total absence of a chemokine, however, it is not possible to distinguish direct chemokine-mediated antimicrobial effects and indirect receptor-dependent effects such as immune cell recruitment. Perhaps the best evidence that antimicrobial chemokines contribute directly to host defense derives from a series of investigations examining the effects of CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC) against *Bacillus anthracis.* These three CXC chemokines each exert antimicrobial effects against *B. anthracis* in vitro, and their induction within the lungs of a murine host is associated with increased resistance to inhalational anthrax [18]. Moreover, antibody-mediated neutralization of these CXC ligands, but not their shared cellular receptor CXCR3, markedly increases host susceptibility to *B. anthracis* infection [19], thus supporting the notion of a direct, receptor-independent antimicrobial function for host chemokines in vivo. Using a similar approach, CXCL9 has also been shown to directly contribute to protection of the gut during *Citrobacter rodentium* infection independent of chemokine-receptor signaling [20].

The molecular mechanism(s) by which antimicrobial chemokines kill microorganisms remains unknown; however, recent observations have expanded our fundamental

understanding of chemokine-mediated bactericidal activity to reveal increasingly diverse and complex actions. In the current review, we present and consider mechanistic insights of chemokine-mediated antimicrobial activity against bacterial pathogens. We also discuss how contemporary advances are reshaping traditional paradigms and opening up new and innovative avenues of research with translational implications for the development of novel approaches for treating infections caused by pathogenic, potentially multidrug-resistant, bacteria.

2. Antimicrobial regions of chemokines

Despite low sequence identity, chemokines maintain well-conserved tertiary structures that include a disordered N-terminus, an N-loop, three antiparallel β-sheets, and a C-terminal α-helix [21,22]. Structure-function studies aimed at identifying elements responsible for chemokine-mediated antimicrobial activity have found that bactericidal chemokines typically possess amphi-pathic structures that contain discrete “patches” of cationic and hydrophobic amino acid residues [10,23]. In many chemokines, amphipathicity is a particularly distinct feature of the C-terminal α-helix [24], indicating that antimicrobial effects are exerted by this region. Indeed, the C-terminal α-helix generally shares many characteristics with antimicrobial peptides (AMPs) that function in host defense (e.g. defensins, cathelicidins, and histatins) [12]. Moreover, peptides derived from the C-terminal helical region of CCL13 (MCP-4) [25,26], CCL20 (MIP-3α) [27], CXCL4 (PF4) [28], and CXCL9 [29], but not the respective N-terminus, have been reported to be capable of killing bacteria. In each case, these C-terminal peptides exhibit bactericidal activity comparable to the full-length chemokine against the microorganism(s) examined.

Although the C-terminal α-helix appears to comprise antimicrobial activity in some chemokines, it is not wholly sufficient for full antimicrobial activity in others. For example, while peptides derived from the C-terminal region of CCL28 (MEC) are capable of killing Gram-negative bacteria, none of the derived peptides are as potent as the full-length chemokine [30,31]. This, and a similar observation regarding CXCL6 (GCP-2) [32], suggests a role for the balance of the parent molecule in exerting antimicrobial activity. One reasonable explanation is that the integrity, orientation, and/or physiochemical properties of the C-terminal α-helix may be affected by other portions of the chemokine, and that these intra-molecular interactions critically promote bactericidal activity by the helical region. This possibility is supported by experimental observations and in silico analyses demonstrating that C-terminal chemokine-derived peptides are unstructured and require the presence of a membrane mimetic to reestablish helical secondary structure [26,27]. An equivalent conclusion is evidenced by our laboratory’s examination of a C-terminal peptide derived from CXCL10 (Fig. 1). Of note, unstructured C-terminal peptides do not necessarily lack antimicrobial activity as membrane lipid bilayers presumably facilitate the formation of amphipathic structures at the bacterial surface [33,34].

The importance of the C-terminal helical region in chemokine-mediated antimicrobial activity is well founded, but not exclusive, as several recent studies have reported the N-terminal region of certain chemokines to exhibit antimicrobial effects. For instance, peptide derivatives of CXCL6 [32], CXCL7 [35], and CXCL10[36], that lack the C-terminal α-

helix, each retain marked bactericidal activity; however, none are as active as the parent chemokine. Moreover, a short N-terminal fragment derived from CXCL14 demonstrates concentration-dependent bacterial killing that is indistinguishable from full-length CXCL14 [17]. Of interest, an overlapping peptide is generated during proteolytic processing in vitro. Considering that chemokines are substrates for host proteases, it is especially appealing to speculate that proteolytic cleavage may uniquely tailor chemokine-mediated antimicrobial activity within the host, much like its influence on chemotaxis and receptor selectivity [35,37]. As the domains accounting for chemokine-mediated bactericidal effects have become increasingly well defined, so too have their surprising breadth and variety. Additional studies are needed to more precisely delineate the regions and intra-molecular interactions responsible for supporting antimicrobial activity, and determining the extent to which these areas overlap with those crucial for host-targeted, receptor-dependent functions such as eliciting chemotaxis.

3. Chemokine-mediated effects against the bacterial cell envelope

Bacterial cell envelopes are dynamic, multilayered-structures that protect from, and interact with, the extracellular environment. The cell envelopes of Gram-negative and Gram-positive bacteria share basic qualities, but are distinguished through key architectural variations [38]. Briefly, Gram-negative bacteria are enclosed by two concentric membranes, an outer and inner membrane, which are separated by a thin layer of peptidoglycan and the periplasmic space. In contrast, Gram-positive bacteria are defined by a thick peptidoglycan layer that surrounds a single cytoplasmic membrane. In each case, the cell envelope provides shape, structure, and strength to the bacterial cell, as well as a scaffold for the assembly of protein complexes central to physiological processes including, among others, energy generation, cell growth and division, nutrient/small molecule transport, sensing of environmental cues, and administering adaptive changes to the cell surface [38–40]. Disruption of cell envelope integrity and, consequently, its central role in organism viability, has the potential to result in bacterial lysis and death [41–43]. As such, the lipid bilayers and other constituents/processes that comprise bacterial cell envelope function are common targets of antimicrobial agents produced by competing microorganisms and host immune defenses.

The surface of Gram-negative and Gram-positive bacteria possesses a net negative charge due primarily to the presence of lipopolysaccharide (LPS) and teichoic acid moieties, respectively [38,44]. Taken together with the existence of positively-charged topological patches on the surface of antimicrobial chemokines [10,24], it is generally presumed that chemokines localize to the bacterial cell envelope through electrostatic interactions and, subsequently, disrupt membrane barrier function resulting in a myriad of deleterious effects (e.g. collapse of the bacterial electrochemical membrane potential, cessation of macromolecule biosynthesis, loss of osmotic regulation, etc.) sufficient to cause cell death. This fundamental mechanism of bactericidal action is analogous to the classical framework proposed for a number of AMPs that function in host defense, and is in agreement with many experimental results [45]. However, as discussed in the following sections, this relatively non-specific model is not adequate to explain emerging distinctions amongst the effects of individual chemokines nor account for several contemporary insights.

3.1. Chemokine localization to the bacterial cell envelope

Many well-described AMPs, and a number of antimicrobial chemokines, localize to the bacterial surface through electrostatic interactions [44,46–49]. Presumably, these cationic effector molecules interact with anionic constituents present in the outer membrane of Gram-negative bacteria or the cell wall of Gram-positive organisms. The importance of charge-based interactions in chemokine-mediated antimicrobial activity is evidenced primarily by reduced or abolished microbial killing in high salt solutions [11]. Accordingly, the bactericidal activity of several chemokines including CCL20, CCL28, and CXCL6 is disrupted when assessed in solutions containing high Na^+ concentrations [10,30,32,50]. Similarly, some antimicrobial chemokines have been reported to lose bactericidal activity when positively-charged amino acids are substituted with neutral or negatively-charged residues. For example, substitution of arginine 108 within the cationic C-terminal region of CCL28 with either alanine or aspartate significantly limits direct killing of Gram-negative and Gram-positive bacteria [31]. Thus, in many instances, the disruption of electrostatic interactions is sufficient to preclude chemokine-mediated antimicrobial activity.

Interestingly, electrostatic interactions do not appear central to the ability of all chemokines to exert bactericidal effects. This conclusion is distinctly illustrated in the context of protective bacterial adaptations conferring colistin resistance. Colistin is a cationic, peptide antibiotic that targets Gram-negative bacteria via electrostatic interactions with the anionic bacterial outer membrane [51]. Chemical modifications of the lipid A component of LPS that mask the negative charge of the bacterial outer membrane (e.g. addition of phosphoethanolamine to lipid A phosphate moieties), preclude colistin localization and associated killing [52]; however, CXCL9 and CXCL10 retain bactericidal activity against colistin-resistant *E. coli* and *K. pneumoniae* possessing modified lipid A [53]. This finding is consistent with the related observations that CCL25, CCL28, and CXCL14 do not interact with LPS [17,54], and that neither CXCL9, CXCL10, nor CXCL14 exhibit reduced antimicrobial effects against Gram-positive bacteria in the presence of increased salt concentrations [17–19]. These experimental determinations reasonably suggest that factors other than charge-based interactions are important for the antimicrobial action of at least several chemokines.

The abundance of exceptions to the notion that electrostatic interactions are responsible for targeting antimicrobial chemokines to the bacterial cell envelope indicate that charge-based considerations are only one aspect of chemokine localization and/or bactericidal activity, or may be unnecessary to a particular subset of chemokines. One intriguing alternative is that select chemokines localize to the bacterial surface by interacting directly with specific, conserved bacterial proteins. Indeed, FtsX, an integral membrane protein important in cellular division and cell wall peptidoglycan processing, has been shown to interact with CXCL10 and promote localization to the cell envelope of *B. anthracis* [55]. Moreover, an extracellular region of FtsX displays homology to the chemokine-binding domain of CXCR3 (the cellular receptor of CXCL10), and a peptide comprising this similar region protects *B. anthracis* from CXCL10-mediated killing [36,55]. Additional studies are warranted to determine if analogous interactions occur more commonly between antimicrobial chemokines and bacterial components of the cell envelope.

3.2. Chemokine-mediated disruption of membrane integrity

As judged by electron microscopy, it is clear that many antimicrobial chemokines cause considerable damage to bacterial membranes. For example, CXCL9-treated *S. pyogenes*, as well as CXCL10-treated *B. anthracis* and *E. coli* demonstrate a loss of bacterial cell integrity characterized by extensive disruption of the cytoplasmic membrane [18,29,55–57]. CCL13, CCL28, CXCL6, and CXCL14 have also each been observed to cause membrane rupture and leakage of intrabacterial content in a range of Gram-negative and Gram-positive bacteria including *E. coli, P. aeruginosa*, and *S. aureus* [17,25,30,32,58]. Although these assessments effectively illustrate the consequences of chemokine-mediated antimicrobial activity, they do not establish membrane disruption as causing bactericidal effects. Towards this end, several chemokines have been shown to rapidly mediate bacterial membrane depolarization (e.g. CXCL10 and CXCL14) and/or permeabilization (e.g. CXCL6 and CXCL9), indicating that the disruption of membrane barrier function is an early, causative event in bacterial killing by a number of antimicrobial chemokines [17,20,32,36]. On balance, peptides derived from CCL20, CXCL7, and CXCL8 (IL-8) do not perturb bacterial membranes despite killing bacteria [27,59,60]. Thus, while bacterial membranes appear to be central targets of chemokine-mediated bactericidal activity, other key bacterial components/processes are also likely to be important.

There are currently few detailed insights into the molecular mechanism(s) by which membrane-active antimicrobial chemokines perturb membrane integrity. However, physiochemical similarities between the C-terminal helices of these chemokines and membrane-active AMPs suggest that these effectors may function through related modes of action. A number of models have been proposed to describe how various AMPs damage bacterial membranes. Several principal models of membrane disruption are briefly discussed below in the context of chemokine-mediated bactericidal activity.

The “carpet” model proposes that electrostatic attraction drives the accumulation of AMPs at the microbial membrane, resulting in a carpet-like coverage [61,62]. At sufficiently high concentrations, this loose arrangement of peptides causes catastrophic disruption of the membrane bilayer, micellization, and dissolution of the bacterial cell in a manner similar to detergents. Inherent, but not exclusive, to the carpet model is the notion that a certain concentration threshold must be exceeded in order for bacterial killing to occur [63]. Of interest, many antimicrobial chemokines exhibit a relatively abrupt, concentration-dependent increase in bacterial killing, possibly reflecting such a threshold effect [18,25,29,30,58]. Contrary to this model, however, immunolabeling of antimicrobial chemokines has revealed dot-like localization to the bacterial cytoplasmic membrane as opposed to widespread coverage [18,29,32,57].

Two transmembrane mechanisms describing AMP oligomerization and dynamic pore formation have been proposed: the “barrel-stave” and “toroidal-pore” models [61]. In the barrel-stave model, amphipathic peptides insert perpendicular to the membrane bilayer with the hydrophobic face of the AMP interacting with the lipid core of the membrane and the hydrophilic face lining the interior of the pore [64]. In contrast, the toroidal pore model proposes that AMP adsorption and oligomerization induces membrane thinning and positive curvature such that a continuous bend is formed that connects the inner and outer lipid

monolayers of a membrane, thus creating a hole [64,65]. Each of these pore-forming mechanisms would be expected to cause collapse of transmembrane electrochemical ion gradients, loss of osmotic regulation, and cell lysis. Depolarization/permeabilization and lysis of bacterial cells by membrane-active antimicrobial chemokines are generally consistent with these effects. Also, the ability of chemokines to dimerize and, in several known instances, form higher-order oligomers may support their ability to perturb bacterial membranes via pore formation [48,66].

In addition to the above models and their derived variants, several contemporary concepts have emerged to define the molecular basis for membrane disruption by AMPs [44,67,68]. Broadly, these descriptions focus on accounting for fundamental physiochemical alterations necessarily incurred by the membrane bilayer to accommodate AMP binding, and how these deleterious changes disrupt the multifaceted role of the bacterial membrane in organism viability. Examples of these considerations include: alterations in lipid packing that disrupt segregation between the lipid core and head-group moieties, thereby promoting permeabilization of the bilayer [68,69]; and changes in lipid clustering and domain organization that mislocalize membrane-associated protein complexes central to physiological processes important in bacterial viability [44,70].

Cumulatively, the models discussed above convey the impression that membrane-active chemokines disrupt bacterial membranes through multiple, interrelated, and overlapping mechanisms that depend on the physiochemical properties and concentration of the particular chemokine, as well as the species- and circumstance-specific composition of the target membrane. Indeed, current research appears to have just begun to elucidate the molecular mechanisms by which host-derived antimicrobial effectors compromise microbial membranes.

3.3. CXCL10-mediated disruption of peptidoglycan processing

Peptidoglycan is a highly conserved bacterial component that comprises the bacterial cell wall in both Gram-negative and Gram-positive organisms, and functions to support the structural strength necessary to withstand intrabacterial osmotic pressures [40,71]. Peptidoglycan is indispensable to bacterial viability, a feature underscored by the many antibiotics that target cell wall biogenesis (e.g. penicillin and other β-lactams, fosfomycin, daptomycin, etc.) [42,72,73]. Importantly, the cell wall requires constant remodeling and maintenance during active growth and proliferation, as well as during homeostatic periods [74,75]. These constitutive processes are administered by a number of enzymes including peptidogly-can hydrolases that are responsible for breaking old bonds within peptidoglycan structures so that new bonds maybe formed [40,76]. Hydrolase activity is tightly regulated as an imbalance between the processing of old bonds and the generation of new bonds may result in bacterial cell lysis through the loss of cell wall integrity [77–79].

In Gram-positive bacteria, the FtsE/X complex (comprised of the transmembrane component FtsX, together with the cytosolic ATPase FtsE), couples ATP hydrolysis to the activation of peptidoglycan hydrolases, and thereby regulates peptidoglycan processing and turnover [79–81]. As discussed in Section 3.1, CXCL10 localizes to the surface of *B. anthracis* vegetative bacilli via interaction with FtsX [55]. Recently, it was reported that this targeted interaction

disrupts FtsE/X-dependent regulation of peptidoglycan hydrolase activity, resulting in uncompensated cell wall break down and bacterial cell death [82]. Analogous bactericidal effects were observed for a CXCL10-derived peptide lacking the amphipathic C-terminal α-helix [82]. Although the C-terminus is dispensable for FtsE/X-based killing of B. *anthracis*, this region of CXCL10 directly mediates membrane depolarization [36]. Thus, CXCL10 appears capable of employing at least two disparate bactericidal mechanisms that separately target distinct, essential structures of the cell envelope (Fig. 2).

The composite bactericidal effects exhibited by CXCL10 are so far unique among antimicrobial chemokines. However, peptides derived from the N- and C-terminal regions of CXCL6 also mediate bactericidal activity [32], raising the possibility that at least several antimicrobial chemokines maintain structurally-discrete antimicrobial effector regions that function independently or, perhaps, synergistically to target and disrupt multiple elements important to bacterial viability. It is also conceivable that individual antimicrobial activities are especially tailored towards certain families of microorganisms, thereby providing an inherent, refined flexibility that may account for the broad-spectrum bactericidal effects exhibited by a number of chemokines. Future studies will be required to elucidate these emerging concepts and their biological relevance to host defense against infectious diseases.

4. Potential intracellular targets of antimicrobial chemokines

The bacterial cell envelope is generally recognized as the principal site of action for AMPs; however, a number of these peptides interact with anionic lipid bilayers without disrupting membrane integrity [83–85]. Similar observations have been reported regarding select chemokines or their derived peptides [27,59,60]. In these interesting cases, interaction with the bacterial surface is thought to facilitate effector translocation into the cytoplasm, the ultimate site of antimicrobial action [61]. Accordingly, a growing number of AMPs have been shown to target essential intrabacterial processes including protein and DNA synthesis, cell division, and central metabolism [86].

Very little is known regarding potential intrabacterial targets of antimicrobial chemokines. CXCL6 and CXCL14 have each been found to bind extracellular DNA [17,87], but these observations are perhaps not surprising as cationic antimicrobial chemokines would be expected to interact nonspecifically with polyanionic molecules such as nucleic acids. Fittingly, full-length CXCL8, which is not bactericidal, has also been shown to bind DNA [17]. Potentially more curious is that functional disruption of the cytosolic pyruvate dehydrogenase complex (PDHc), a key regulatory point of central metabolism, affords *E. coli* protection from CXCL10-mediated killing [57]. Moreover, *B. anthracis* PDHc was recently identified by our laboratory as an interacting partner of CXCL10 through co-immunoprecipitation analysis (unpublished results; M. Hughes), and genetic deletion of the E1α subunit of PDHc limits the bactericidal effects of CXCL10 against this organism (Fig. 3). It remains to be determined whether, and if so how, CXCL10 directly impacts PDHc to promote the killing of Gram-negative and Gram-positive bacteria. Nevertheless, these findings suggest bacterial components/processes contained within the cytoplasm cannot be excluded from contributing to chemokine-mediated antimicrobial activity.

5. Therapeutic opportunities of antimicrobial chemokines

The hastening emergence and spread of antibiotic resistance amongst human pathogens is a serious threat to global health, made worse by a steep, decades-old decline in the origination of novel antibiotics [88,89]. Countering the growing burden of multidrug-resistant (MDR) bacteria requires a multifaceted strategy that includes the development of innovative therapeutic approaches [90,91]. Towards this end, AMPs and synthetic innate defense regulator (IDR) peptides that mediate both direct antimicrobial activity and immunomodulatory effects have received considerable attention as next-generation candidate antimicrobials [92–94]. The known functions of host chemokines overlap considerably with the most promising AMPs and IDR peptides [95], suggesting that chemokines similarly merit investigation as a forward-looking therapeutic avenue for treating infections caused by MDR pathogens. In the current section, we introduce several important aspects regarding the potential therapeutic value of host chemokines.

5.1. Harnessing the functional versatility of chemokines

The importance of chemokine-dependent immune processes in host defense against infectious diseases has been well established using animal models of infection [15]. Although some redundancy exists, the precise immunomodulatory activities elicited by individual chemokines are governed by the expression of cognate receptors expressed on the surface of responsive subpopulations of host cells [3]. Thus, distinct chemokines mediate a diverse, relatively unique set of effector functions that contribute to (i) guiding immune-cell localization and infiltration to sites of infection [5]; (ii) influencing pro- and anti-inflammatory cytokine production [96–98]; (iii) regulating angiogenesis/angiostasis [99]; (iv) promoting lymphocyte priming and differentiation [100], and (v) initiating innate and adaptive immune responses [7], among others. By combining therapeutically advantageous aspects of immune control with direct bactericidal activity, antimicrobial chemokines provide an attractive paradigm for the development of stand-alone and/or adjunctive therapies to treat infections caused by pathogenic, potentially MDR, bacteria.

Perhaps the most pivotal challenge to effectively exploiting the inherent therapeutic value of chemokine-mediated bactericidal and immunomodulatory effects is successfully reconciling the disparate chemokine concentrations at which these two activities occur. Specifically, antimicrobial chemokines typically kill bacteria in vitro at concentrations 100- to 1,000-fold higher than those required to stimulate receptor-dependent, cell-based immunomodulatory effects [10,101]. While several studies suggest endogenously produced chemokines achieve bactericidal concentrations at inflammatory foci in vivo and are tolerated by the host [19,24,29,102], the therapeutic administration of intact chemokines has the potential to result in immune dysregulation and immunopathological damage [103,104]. To circumvent this potential limitation, chemokine-mediated bactericidal and immunomodulatory activities will likely need to be dissociated and/or optimized, at least to some degree.

Chemokines interact with their corresponding G-protein-coupled receptor(s) via a two-step process in which the chemokine first binds to, and then activates, the receptor [22,105,106]. Initial binding arises from interactions between the structured chemokine core domain, in particular the N-loop, and the receptor N-terminus and extracellular loops (chemokine

recognition site 1; CRS1). Subsequently, interactions between the N-terminus of the chemokine and the ligand-binding pocket of the receptor, formed by transmembrane helical bundles (chemokine recognition site 2; CRS2), triggers activation of the receptor, intracellular signaling, and effector responses. Insights resulting from a myriad of structure-activity relationships based on X-ray crystal structures, NMR structures, and site-directed mutagenesis studies provide a detailed accounting for the specific amino acid residues critical for chemokine-receptor interactions [106]. Moreover, these data inform and establish a comprehensive framework for the rational design of antimicrobial chemokine-derived peptides that display graded, finely-tuned immunomodulatory activities.

As discussed in the current article, antimicrobial chemokines appear to possess one or more discrete structural regions, generally comprising the N- and/or C-terminus, that mediate bactericidal activity. With few exceptions, peptides derived from these regions retain much or all of the antimicrobial activity exhibited by the intact parent molecule. Based on the mechanism of chemokine-receptor interaction, antimicrobial peptides derived from the C-terminal region of chemokines are not expected to act as receptor agonists. In contrast, antimicrobial peptides derived from the N-terminus are likely to bind and activate cognate cellular receptors, thus eliciting immunomodulatory effects in vivo. The progressive disruption of amino acid residues important for receptor engagement provides an approach to reduce or abolish immunomodulatory effects while conceivably retaining bactericidal activity. Ultimately, it may be possible to develop an armamentarium of chemokine-derived peptides that mediate direct bacterial killing and diverse, graded immunomodulatory effects. The clinical application of such peptides, either individually or in combination, would provide a timely, malleable therapeutic strategy for treating infections caused by MDR bacterial pathogens.

Future studies are required to determine how best to balance and employ the potential utility of chemokine-mediated antimicrobial and immunomodulatory activities. It is also necessary to define the ability of antimicrobial chemokines to kill MDR organisms, and demonstrate proof-of-concept for the therapeutic use of chemokine-derived peptides. Regarding the latter points, two encouraging findings have recently been reported. First, CXCL9 and CXCL10 have been shown to efficiently kill many of the most challenging MDR bacterial pathogens, including carbapenem-resistant *Enterobacteriaceae* (CRE) and Gram-negative bacteria harboring the mobile colistin resistance determinant *mcr-1* [53]. Importantly, many of these chemokine-susceptible clinical isolates maintain considerable resistance against multiple classes of antibiotics. Second, a peptide derived from the C-terminal region of CCL13 (amino acid residues 57–75) has been shown to significantly reduce bacterial burden and increase host survival in an intratracheal model of *P. aeruginosa* infection [26]. These findings highlight the potential therapeutic value of antimicrobial chemokine-derived peptides in countering infections caused by human pathogens increasingly invulnerable to currently available antibiotics.

5.2. Challenges in formulating chemokine-derived therapeutic peptides

The specific formulation(s) of chemokine-derived peptides will be dictated primarily by their intended biological activities and ultimate clinical application. However, regardless of

their potential uses, previous studies indicate that two fundamental challenges will need to be resolved: chemical instability and proteolytic degradation. In peptides, the side chains of amino acid residues are solvent exposed and, consequently, susceptible to damage caused by deamidation and oxidation [107]. These insults are dependent upon the presence of particular amino acid residues (predominately asparagine/glutamine and methionine/cysteine, respectively) and can be limited by appropriate pH, buffer composition, and/or capping strategies [108]. Peptides are also susceptible to enzymatic degradation by host and microbial proteases [56,109]. Proteolytic degradation limits bioavailability and has largely restricted first-generation peptide-based therapeutics to topical applications. Success in minimizing protein/peptide degradation has been accomplished using a variety of approaches including the use of D-amino acids, non-natural amino acid analogs, and peptide-protecting nanoparticles [110].

5.3. The potential development of bacterial resistance against chemokines

As would be reasonably expected, individual antimicrobial chemokines are not effective against every bacterial species. For example, under identical experimental conditions, CCL1 (I-309) and CCL19 (MIP-3β) are each significantly more potent against *E. coli* than *S. aureus* [10]. The fundamental basis of differential susceptibility in these cases, or others, is not known; however, intrinsic, species-specific resistance against antimicrobial chemokines clearly exists and defines an inherent range of bactericidal action. More far-reaching and impactful to the therapeutic lifetime and overall usefulness of antimicrobial chemokines or their peptide derivatives is whether existing intrinsic resistance can be mobilized and spread. Indeed, the emerging view regarding the origins of horizontally acquired antimicrobial resistance suggests that genes encoding resistance determinants become mobilized from extant reservoirs and, under selective pressure, are refined by the recipient to yield a phenotypically-resistant organism [111]. That diverse bacterial pathogens do not display comprehensive resistance to antimicrobial chemokines, despite having been exposed to these host mediators for presumably millions of years [112], indicates that existing intrinsic resistance by a certain bacterial species is unlikely to be horizontally acquired by a susceptible bacterial species.

Alternatively, resistance against antimicrobial chemokines could arise adventitiously through the alteration of genomic loci. To investigate this possibility, our laboratory has previously re-challenged *B. anthracis* bacilli that survived treatment with concentrations of CXCL9 or CXCL10 that killed ~90% of assayed bacteria. After several rounds of “selection”, repeatedly-challenged bacteria remained as susceptible to subsequent chemokine treatment as the original population, indicating that neither heritable nor transient resistance was attained by these organisms (unpublished data; M. Hughes).

In contrast, transposon mutant library screens of *B. anthracis* and *E. coli* have each yielded bacterial isolates less sensitive to CXCL10-mediated antimicrobial activity [55,57]; most prominently *AftsX B. anthracis*. While resistant mutants have informed the elucidation of unique mechanistic insights into chemokine-mediated bactericidal effects, these organisms typically display morphological and/or growth defects that indicate CXCL10 resistance is associated with a considerable fitness cost [55,57,82]. Moreover, increased chemokine

concentrations effectively overcome CXCL10-resistant phenotypes [36,55]. This observation raises the intriguing possibility that CXCL10 inherently impedes the development of resistance against itself. Specifically, as discussed in Section 3, the bactericidal effects of CXCL10 are comprised of two distinct activities: (i) FtsX-dependent dysregulation of peptidoglycan hydrolysis by the N-terminal region of CXCL10, and (ii) nonspecific disruption of membrane barrier function mediated by the C-terminal region of CXCL10. Thus, bacterial resistance against such combinatorial targeting would necessitate the concurrent accumulation of multiple protective changes and, thereby, markedly reduce the development of comprehensive phenotypic resistance. That Δ*ftsX B. anthracis* resists killing by the N-terminal region of CXCL10, but remains susceptible to the C-terminal region, is consistent with this notion [36,82]. The pleiotropic antimicrobial effects mediated during a host immune response may similarly diminish the propensity of bacteria to develop specific resistance against chemokines during infection. Taken together, these observations suggest that resistance against antimicrobial chemokines is unlikely to readily occur in vivo.

6. Concluding remarks

The first descriptions of chemokine-mediated antimicrobial activity, made nearly two decades ago [24,60], originated an entirely new avenue of research in chemokine biology. Since that time, and owing to important contributions by a number of laboratories, it has become increasingly clear that no single unifying mechanism of action likely exists to describe how chemokines kill microorganisms. Rather, individual chemokines appear to employ a relatively particular blend of intersecting actions that vary according to the explicit chemokine, local concentration, proximate environment, and target organism. Curiously, overlapping diversity is also a physiological hallmark of the chemokine family, perhaps indicating that the diversity observed amongst antimicrobial mechanisms reflects a similar degree of refinement. As the mounting burden of antibiotic resistance shifts attention towards clarifying the potential therapeutic utility of antimicrobial chemokines, there is little doubt that the mechanisms by which chemokines kill bacteria will become increasingly well defined.

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Abbreviations:

AMP	antimicrobial peptide
CRS	chemokine recognition site
CRE	carbapenem-resistant *Enterobacteriaceae*

IDR	innate defense regulator
LPS	lipopolysaccharide
MDR	multidrug resistant
PDHc	pyruvate dehydrogenase complex
TFE	2,2,2-trifluoroethanol

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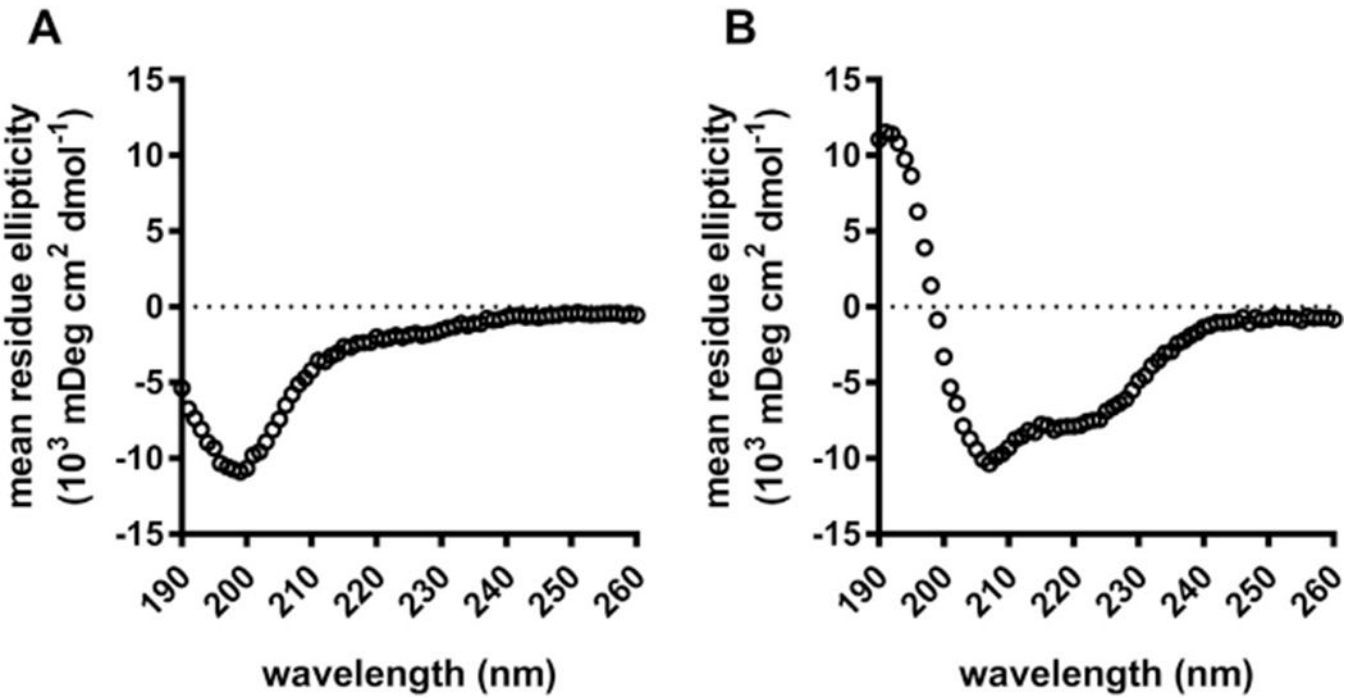

Fig. 1.

C-terminal chemokine-derived peptides require an appropriate microenvironment to form helical secondary structure. Circular dichroism spectra of a peptide derived from the C-terminus of CXCL10 (amino acid residues 53–77) in the (A) absence or (B) presence of the organic co-solvent 2,2,2-trifluoroethanol (TFE; 25% final concentration). Only in the presence of TFE is helical secondary structure observed as evidenced by peak maximum at 190 nm and peak minima at 208- and 222 nm. Mean residue ellipticity is reported; n = 3.

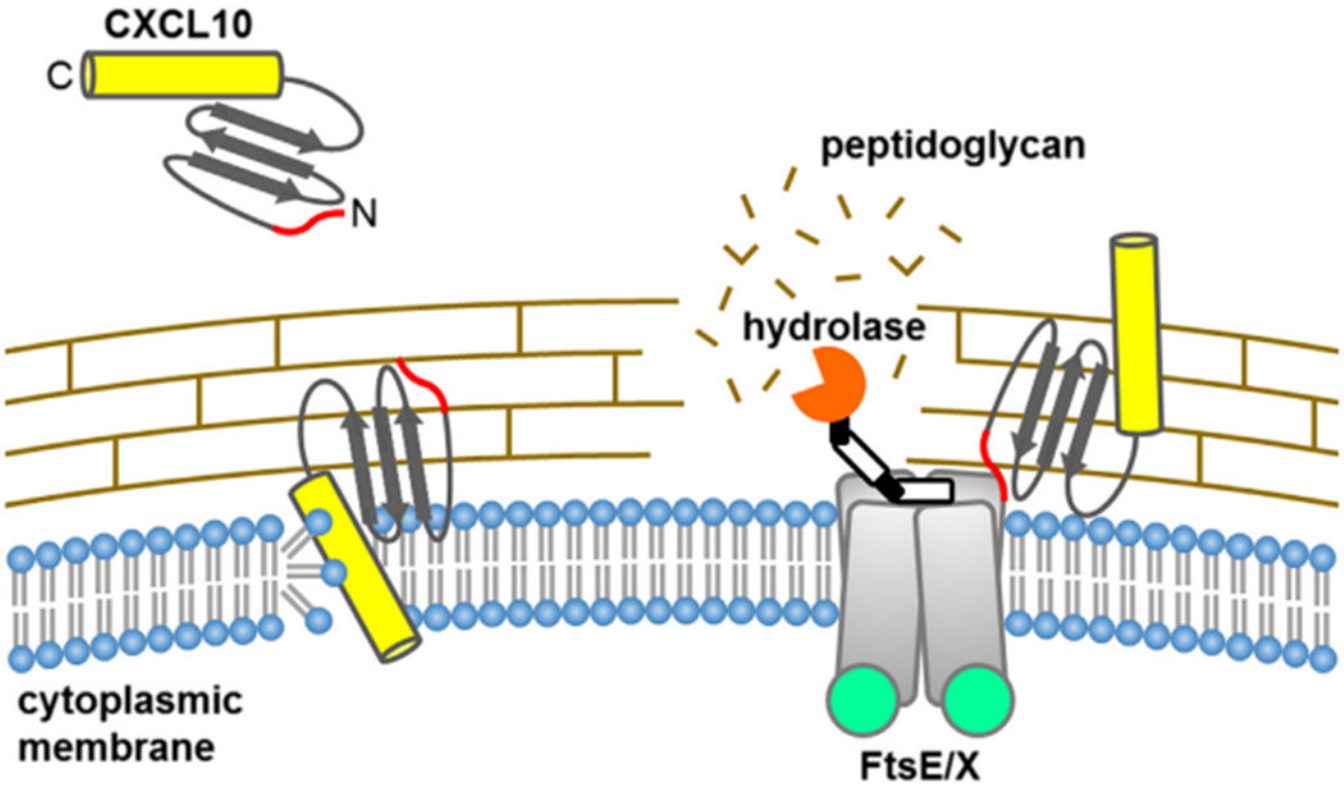

Fig. 2.

CXCL10 targets multiple essential components of the bacterial cell envelope. A schematic illustration of CXCL10-mediated antimicrobial effects against *B. anthracis* bacilli. The C-terminal α-helix of CXCL10 interacts nonspecifically with the cytoplasmic membrane, thereby disrupting barrier function and causing membrane depolarization/permeabilization (left). In contrast, the N-terminal region of CXCL10 interacts with FtsX, thus stimulating the activation of peptidoglycan hydrolases by the FtsE/X complex and, consequently, causing uncompensated destruction of the cell wall (right). Each mechanism promotes the loss of cellular integrity and bacterial lysis.

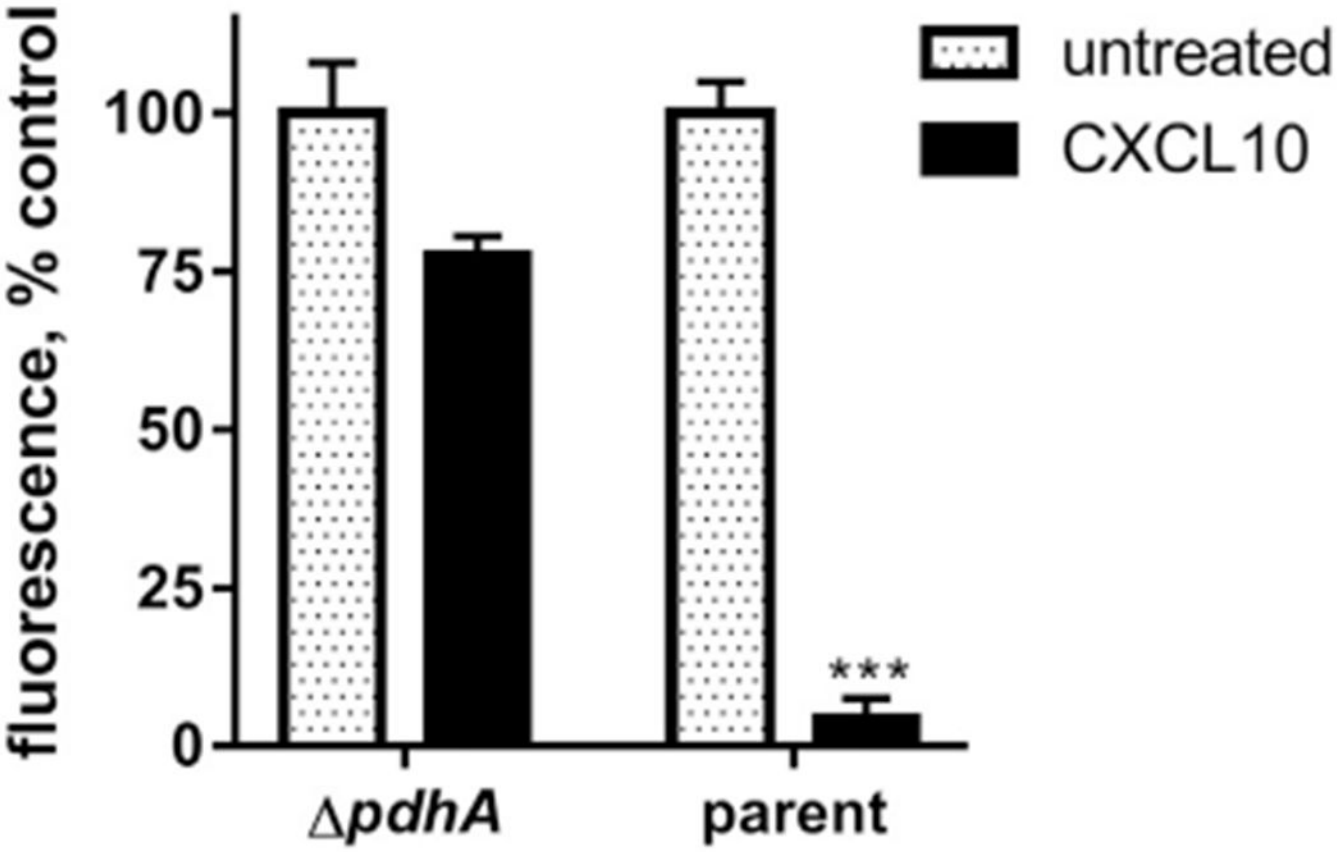

Fig. 3.

Functional inactivation of PDHc protects *B. anthracis* from CXCL10-mediated bactericidal activity. A *pdhA* deletion mutant (lacking the E1α subunit of PDHc) was derived from *B. anthracis* Sterne strain 7702 (parent). Vegetative bacilli from each strain were treated ±24 μg/ml human recombinant CXCL10 for 6 h. Relative cell viability was determined using the fluorometric indicator AlamarBlue as previously described [18]. Genetic complementation of *pdhA* using the inducible vector pUTE973 [55] restored CXCL10 susceptibility to Δ*pdhA* organisms (unpublished data; M. Hughes). Data are expressed as a percentage of the strain-specific control and represent the mean ± SEM; n = 3, ***$p < 0.001$ as compared to CXCL10-treated Δ*pdhA B. anthracis*.