Published in final edited form as:
*Eur J Immunol*. 2010 January ; 40(1): 186–196. doi:10.1002/eji.200939819.

# The Antimicrobial Activity of CCL28 Is Dependent on C-Terminal Positively Charged Amino Acids

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## Summary

Several chemokines have been shown to act as antimicrobial proteins, suggesting a direct contribution to innate immune protection. Based on the study of defensins and other antimicrobial peptides, it has been proposed that cationic amino acids in these proteins play a key role in their antimicrobial activity. The primary structure requirements necessary for the antimicrobial activity of chemokines however, has not yet been elucidated. Using mouse CCL28, we have identified a C terminal region of highly charged amino acids (RKDRK) that is essential to the antimicrobial activity of the murine chemokine. Additionally, other positively charged amino acids in the C-terminus of the protein contribute to the observed antimicrobial effect. Charge reversal and deletion mutations support our hypothesis that C-terminal positively charged amino acids are essential for the antimicrobial activity of CCL28. Results also demonstrate that although the C-terminal region of the chemokine is essential, it is not sufficient for full antimicrobial activity of CCL28.

### Keywords

Antimicrobial peptide; CCL28; Chemokines; Innate Immunity

## Introduction

Chemokines are a group of small proteins (5-20 kDa) that play important roles in both innate and adaptive immunity. These chemotactic proteins play a key role in recruiting leukocytes to lymphoid tissues (constitutive homing) as well as to sites of inflammation (inducible homing) [1-4]. In addition to their function in cell homing and migration, several chemokines have been shown to exhibit direct antimicrobial activity *in vitro* [5-7].

The chemokine CCL28 has been shown to selectively attract lymphocyte subsets (IgA plasma cells and skin homing T cells) through interactions with its cognate receptor, CCR10 [8,9]. This chemokine has also been shown to exhibit broad spectrum antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, and fungi. The antimicrobial activity of CCL28 was initially identified due to its homology with the antimicrobial peptide histatin 5 [10]. Histatin 5, CCL28, and other antimicrobial peptides (AMP), such as defensins, often require low salt (non physiologic) solutions for maximum antimicrobial activity *in vitro*. Through the use of mouse models, in which defensins function has been

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**Conflict of interest** The authors declare no financial or commercial conflict of interest.

inhibited, it has been shown that these AMP serve a vital immunological function *in vivo* [11,12].

A mechanism for the antimicrobial activity of defensins and other AMP has been proposed in which positively charged regions of these peptides attach or insert into the negatively charged microbial cell membrane, ultimately resulting in microbial cell death. Segregation of patched hydrophilic and hydrophobic residues and the formation of an amphipathic structure may also be important for AMP activity [13-15].

Although the antimicrobial activity of several chemokines has been described, very little is known about the structural requirements needed to facilitate the killing of bacteria by these proteins. Previous studies have explored the relationship between amino acid sequence and the antimicrobial function of defensins [16-24]. When compared with defensins and other AMP, chemokines are much larger in size and have a more complex structure [1]. No previously reported studies have explored the role of primary protein structure in chemokine mediated antimicrobial activity.

CCL28 provides an excellent model to understand the structural requirements for chemokine mediated killing. All CC chemokines are thought to have a similar structure [1,25]. The chemokines CCL28 and CCL27 share 31% identity at the amino acid level and both mediate the migration of lymphocytes via interactions with the chemokine receptor CCR10 [26]. In contrast to CCL28, CCL27 exhibits no antimicrobial activity [7,10]. Interestingly, these two proteins share high homology at the N-terminus of the protein and low homology at the C-terminus. Correspondingly, the N-terminus of chemokines has been implicated in mediating migration through chemokine receptor binding and the C-terminus has been hypothesized to be important for antimicrobial activity [27-30].

To investigate the role of primary protein structure on the activity of the antimicrobial chemokine CCL28 we used PCR based mutagenesis to generate truncation, deletion, site-specific substitution, and chimeric mutants of the protein. These mutant proteins were then assayed *in vitro* for antimicrobial activity. Results demonstrate that positively charged amino acids at the C-terminus of CCL28 significantly contribute to the antimicrobial activity of the protein. Through the generation of CCL27/CCL28 and CCL5/CCL28 chimeric proteins we also demonstrate that interactions between the antimicrobial C-terminus of CCL28 with an appropriate CC chemokine N-terminal domain is important for the full antimicrobial activity of CCL28.

## Results

### Recombinant CCL28 exhibits antimicrobial activity

In establishing our model system we first sought to demonstrate that our *in-house* protein production and purification procedures yielded CCL28 that effectively killed bacteria, as has previously been demonstrated for commercially available CCL28 [10]. To determine the antimicrobial activity of our recombinant proteins, *in-house* produced CCL28 and CCL5 proteins were used in antimicrobial assays, as described in Materials and Methods. The CCL5 chemokine was used throughout this study as a negative control, CCL5 binds the CCR1, CCR3 and CCR5 chemokine receptors and has not been shown to possess antimicrobial properties. In preliminary experiments, we found that *Staphylococcus aureus* and *Pseudomonas aeruginosa* displayed slightly different sensitivities to CCL28 mediated killing (data not shown). In all subsequent experiments a final protein concentration of 1μM for *Staphylococcus aureus* and 0.5μM for *Pseudomonas aeruginosa* was used. Antimicrobial assays confirmed that recombinant CCL5 showed no antimicrobial activity, when compared

to BSA or buffer only controls. *In-house* recombinant CCL28 demonstrated potent antimicrobial activities, similar to commercially produced CCL28 (Fig. 1).

## The C-terminal region of CCL28 is indispensable for optimal antimicrobial activity

It has previously been suggested that the 28 C-terminal amino acids of CCL28 play a key role in the observed antimicrobial properties of the chemokine [10]. In an effort to more precisely define regions of the protein that are essential for antimicrobial activity, we produced several mutant versions of CCL28 through successive C-terminal truncations. These truncated recombinant proteins were then assayed for antimicrobial activity (Fig. 2B and 2C). The antimicrobial activity of CCL28 generally decreased as progressively larger sections of the C-terminal region were deleted. Following truncation 5 (truncation of 24 C-terminal amino acids) the antimicrobial activity of CCL28 was largely abrogated, with bacterial survival increasing 10-fold in experiments using the C5 truncation compared to the C4 truncation of the protein ($p$=0.007 and 0.039 for experiments using *S. aureus* and *P. aeruginosa* respectively). These results confirm that the C-terminal region of CCL28 is essential to the antimicrobial activity of CCL28. Furthermore, these results demonstrate that amino acids 85-89 (contained in the C4 mutant protein) are key to the antimicrobial activity of CCL28. Although differences in the antimicrobial activity of full length CCL28 and truncations C1-C4 were not strongly significant ($p$ values ranging from 0.1 to 0.04) a consistent pattern was observed in experiments using both *S. aureus* and *P. aeruginosa* in which the antimicrobial activity of mutations C1-C3 gradually decreases and the antimicrobial activity of C4 was always increased when compared to C3.

To further determine if the C5 region (amino acids 85-89) was essential to the antimicrobial activity of CCL28 we next constructed a mutation in which this region was deleted and amino acid 84 was followed by the amino acids corresponding to amino acids 90-108 of the wild type protein. In these experiments we observed a dramatic reduction in antimicrobial activity when compared to the full length CCL28 protein. However, although the antimicrobial activity of this mutant protein was greatly decreased following the specific deletion of amino acids 85-89, antimicrobial activity was still detected in this mutant (Fig. 2D and 2E). These results suggest that although the antimicrobial activity of CCL28 is highly dependent on amino acids 85-89 other regions of the C-terminus may make minor contributions to the full antimicrobial effect.

## The antimicrobial activity of CCL28 is dependent on charged amino acids at the protein's C-terminus

The C-terminus of CCL28 is rich in positively charged amino acids. It has been suggested that the recognition/killing process used by some AMP is mediated through electrostatic interactions between positively charged amino acids of the antimicrobial protein and negatively charged bacterial cell components [14]. It is clear from our results in figure 2 that a highly positively charged region of CCL28, RKDRK, which spans amino acids 85-89 is vital to the antimicrobial activity of the protein. Additionally we hypothesized that other positively charged amino acids in the C-terminal region may contribute to the antimicrobial killing observed in the full length protein. To evaluate the effect of specific, positively charged, amino acids we constructed mutant proteins which contained amino acid substitutions. The antimicrobial activity of these mutant proteins was then determined in standard antimicrobial assays. Based on our hypothesis, that positively charged amino acids outside the 85-89 region were important for full antimicrobial activity, we constructed amino acid replacement mutants in which R108 (the terminal amino acid) was changed to a neutral alanine or negatively charged aspartate. In these experiments we observed that the insertion of an alanine in place of an arginine resulted in a consistent decrease in the antimicrobial activity observed ($p$=0.056 and 0.014 for experiments using *S. aureus* and *P.*

*aeruginosa* respectively). Furthermore, conversion of R108 to a negatively charged amino acid (aspartate) resulted in a statistically significant loss of antimicrobial activity ($p$=0.038 and 0.027 for experiments using *S. aureus* and *P. aeruginosa* respectively). As an additional control we also converted a negatively charged amino acid (E105) to a positively charged amino acid. This experiment resulted in antimicrobial activity levels consistent with the full length CCL28 protein (Fig. 3). These results suggest that although the highly charged region of CCL28 included in the C4 truncation is essential to the antimicrobial activity of CCL28, other positively charged amino acids may also contribute to the overall antimicrobial effect of the protein. This is illustrated by the significant change in the antimicrobial activity of CCL28 following charge reversal of the terminal amino acid of the CCL28 protein.

## Highly charged amino acids in the C-terminal region of CCL28 are well conserved across species

The C-terminal region of CCL28 is highly positively charged with cationic amino acids constituting ~45% of the region. As shown in figure 2, truncation experiments demonstrated that a single highly charged region (amino acids 85-89) was vital in mediating antimicrobial activity. The first two amino acids in this region are well conserved with R or K being found at this position in a wide variety of species. The amino acids in the next two positions (87-88) are more variable and are generally followed by a positively charged amino acid in position 89 (Fig. 4A). These results suggest that the first two positively charged amino acids of this region are well conserved among diverse species.

Analysis of the CCL28 primary amino acid sequence of multiple species shows that the overall consensus sequence for the 85-89 amino acid region is KRNSK with the human sequence being RKNSN. It is clear that this region is essential to the activity of murine CCL28. However, it is unclear if variations in this region between different species result in differences in the antimicrobial activity of CCL28. It has previously been shown that murine CCL28 is slightly less effective as an antimicrobial chemokine than is human CCL28 [10]. The divergence of murine CCL28 from the consensus and human sequences in this essential region may contribute to the lower antimicrobial activity of mouse CCL28 compared to human CCL28.

The chemokines CCL28 and CCL27 both bind the CCR10 chemokine receptor and mediate migration of CCR10 expressing lymphocytes. As mentioned previously CCL27 has not been shown to possess antimicrobial properties. We next compared the C-terminal region of these two CCR10 ligands to assess if there were significant differences in the number of positively charged amino acids. Amino acid sequence comparison demonstrated that whereas positively charged amino acids were found to be highly enriched in the C-terminal region of CCL28, positively charged amino acids comprised only ~12% of the C-terminal region of the CCL27 (Fig. 4B). These results suggest that positively charged regions of CCL28 may be essential to the antimicrobial function of the chemokine and also differentiate this chemokine from the other CCR10 ligand, CCL27.

## Both N- and C-regions are necessary for full antimicrobial activity of CCL28

Based on the above observation, we sought to determine if the C-terminal region of the protein is an effective antimicrobial agent alone, or if optimal function requires the N-terminal region of the chemokine. Additionally, we designed experiments to determine if activity of the C-terminal region requires a specific CC chemokine backbone for optimal function. To address these questions we first generated two truncated proteins, the first consisting of the C-terminal 52 amino acids, and the second consisting of the 56 N-terminal amino acids of CCL28 (Fig. 5A). These proteins were then used in microbial killing assays. Results of antimicrobial assays using truncated proteins demonstrate that the C-terminal 52

amino acid peptide of CCL28 consistently killed more bacteria than the N terminal 56 amino acid peptide. Statistical analysis of the antimicrobial activity of the C-terminal peptide, compared to CCL5 killing, resulted in *p* values of 0.063 and 0.031 for *S. aureus* and *P. aeruginosa* respectively. Conversely, the *p* value for experiments in which the antimicrobial activity of the N terminal peptide was compared to CCL5 resulted in *p* values which were clearly not significant ($p = 0.185$ and 0.141). These results suggested that the C-terminus of the protein alone did exhibit some antimicrobial activity as has been reported previously [10]. However, the antimicrobial effect of the full length protein was clearly far superior to the C-terminal 52 amino acids peptide (Fig 5B and 5C).

Based on results from figures 2 and 5 which showed that amino acids in the C-terminus were essential but not sufficient for full antimicrobial activity, we hypothesized that full antimicrobial activity of CCL28 is dependent on the C-terminal region, in conjunction with an appropriate N-terminal region. We next sought to determine if the activity of CCL28 was dependent on a specific N-terminal region or if the N-terminal region of another chemokine could be substituted for the N-terminal region of CCL28 and still retain the antimicrobial properties of the full length CCL28 protein. To perform these experiments we constructed two chimeric proteins. The first consisted of the N-terminal region of CCL27 fused to the C-terminal region of CCL28. The second chimeric protein constructed consisted of the N-terminal region of CCL5 fused to the C-terminus of CCL28. In assays testing the killing activity of these chimeric proteins on *S. aureus*, full antimicrobial activity was seen in the CCL27/CCL28 chimera, when compared to full length CCL28. Additionally, full length CCL28 protein was significantly more effective in killing *S. aureus* than the CCL5/CCL28 chimera ($p=0.014$), (Fig. 5B). This was not surprising due to the high homology of CCL28 and CCL27 and low homology of CCL28 and CCL5 at the N-terminal region. In assays testing the killing activity of chimeric proteins on *P. aeruginosa*, full antimicrobial activity was seen using both CCL27/CCL28 and CCL5/CCL28 chimeras (Fig. 5C).

### The first two conserved cysteines of CCL28 are not required for antimicrobial activity

CC chemokines are so named due to the presence of a conserved CC sequence in the N-terminal region of the chemokine. Chemokine mediated chemotaxis has been shown to be dependent on these conserved cysteines and these cysteines are essential to appropriate chemokine structure [31,32]. In an effort to determine if the first two conserved cysteines of CCL28 are essential in the antimicrobial activity of CCL28, a mutant protein was constructed in which an alanine was substituted for the two cysteines at position 8 and 9 of the mature protein. In employing these mutant proteins in our antimicrobial assays, we observed no decrease in the antimicrobial activity of the mutated CCL28 protein (Fig. 6). These data suggest that these highly conserved cysteines are not required for the antimicrobial activity of CCL28.

## Discussion

Innate immunity is thought to be an ancient immune stratagem preceding the evolution of adaptive immunity. Antimicrobial peptides function as a key component of the innate immune system and have been identified in organisms as diverse as humans and plants [33]. A wide variety of antimicrobial proteins have been identified including histatins, defensins, and more recently some chemokines. Expression of the antimicrobial chemokine CCL28 has been shown to be upregulated following epithelial inflammation [34,35]. Additionally, this chemokine has been shown to be constitutively expressed and highly concentrated in mucosal secretions such as milk and saliva [10]. The localization of this antimicrobial chemokine in mucosal secretions may provide constitutive innate immune defense against a variety of bacterial pathogens.

The function of chemokines as antimicrobial proteins represents an elegant aspect of immuno-efficiency. Antimicrobial chemokines function both to recruit leukocytes to specific tissues as well as protect these surfaces through direct antimicrobial activity. Dual functionality of some defensins has also been demonstrated, in which these peptides have been shown to be chemotactic, as well as antimicrobial, suggesting evolutionary pressures have long linked these two seemingly distinct biological functions [36,37]. A potential evolutionary advantage of the inclusion of antimicrobial properties with chemokines, as opposed to histatins and defensins, is that chemokines efficiently bind heparin sulfate moieties, commonly found on the surface of epithelial cells [38-40]. The binding of chemokine to epithelial surfaces has been proposed to increase the effective concentration of CCL28 at mucosal surfaces [10].

In this study we sought to determine which regions of the CCL28 protein are essential to antimicrobial activity and if positively charged amino acids play a role in the microbicidal properties of CCL28. Our findings strongly support a vital role for amino acids 85-89 of CCL28, as deletion of this region resulted in a dramatic reduction in the antimicrobial activity of CCL28. Positively charged amino acids in the first two positions of this sequence (K or R) is a trait strongly conserved in species ranging from rodents to primates and ruminants. In addition to this single indispensible region, charge neutralization and reversal experiments on the C-terminal amino acid of murine CCL28 suggest that other positively charged amino acids in the C-terminus contribute to the antimicrobial activity of the protein. The hypothesis that positively charged amino acids contribute to the antimicrobial properties of CCL28 is supported by sequence comparison of CCL27 and CCL28. Each of these chemokines binds the CCR10 chemokine receptor and efficiently mediates the directed migration of CCR10 expressing lymphocytes. However, the non antimicrobial CCL27 contains relatively few positively charged amino acids when compared to CCL28. The characteristic of C-terminal positively charged amino acids in CCL28 is broadly conserved across species; suggesting that evolutionary pressures have continued to select for a positively charged antimicrobial C-terminal region since the divergence of human and mouse, 65-85 million years ago.

In addition to the necessity of the highly positively charged C-terminal region of CCL28 we investigated if disulphide bonding within CCL28 was important for the observed antimicrobial function of the protein. Antimicrobial chemokines are generally larger and more structurally complex than other antimicrobial peptides. CC chemokines have a conserved structure throughout the N-terminus, with three anti-parallel β-sheets followed by a C-terminal α-helix [1]. The α-helix of CC chemokines has been postulated to serve as a structural scaffold, and this region has been switched between chemokines without loss of chemotactic specificity [32]. The N-terminus of CC chemokines and the accompanying structural complexity has been demonstrated to be important for chemokine receptor binding and activation of leukocytes [30,31]. Previous reports have not investigated the role of proper protein folding on the antimicrobial activity of CCL28. Replacement of the canonical, consecutive, cysteines is predicted to destroy disulphide bonding within the chemokine, which in turn should significantly alter the tertiary structure of the protein. Mutating these cysteines in other chemokines has resulted in a loss of receptor binding and cellular activation. In these experiments we observed no decrease in antimicrobial activity of CCL28. These results suggest that if the tertiary structure of CCL28 is important in mediating its antimicrobial activity, this structure is formed independently of the disulphide bonding known to be vital for chemokine receptor binding. Disulphide bonding has also been shown to be essential for the chemotactic activity of other AMP but not for the antimicrobial activity of those AMP [41-44]. Although the disulphide bonding of CCL28 does not appear essential for efficient antimicrobial activity, it is likely that an ordered tertiary structure of CCL28 is essential for antimicrobial function. In other AMP it has been

demonstrated that essential three dimensional structures are formed following interaction with a bacterial membrane [45-48].

Antimicrobial assays using chimeric chemokines demonstrated that the N-terminus of CCL27 fused with the C-terminus of CCL28 resulted in full antimicrobial activity against *P. aeruginosa* and *S. aureus*. However, the chimeric protein, composed of the N-terminus of CCL5 fused with the C-terminal region of CCL28, resulted in full strength antimicrobial activity against *P. aeruginosa* but only partial killing of *S. aureus*. These results suggest the possibility that there are different structural requirements for the killing of Gram-positive and Gram-negative bacteria, and these requirements may involve appropriate interactions between the N-terminus with the C-terminus of the chemokine. It appears that efficient killing of *P. aeruginosa* requires the interaction of the C-terminus of CCL28 with the N-terminus of a CC chemokine (CCL5 or CCL27). Conversely, the killing of *S. aureus* is dependent on the interaction of the C-terminus of CCL28 with the highly conserved N-terminal region of CCL27. The observed results, in the ability of these chimeric chemokines to kill *Staphylococcus* and *Pseudomonas*, are clearly not due to the N-terminal region alone, as similar antimicrobial activities are seen (compared to BSA control) in both types of bacteria when using the CCL28 N-terminal peptide in antimicrobial assays. Conversely, the C-terminal peptide alone shows higher efficacy in killing *Pseudomonas* than *Staphylococcus* bacteria. These results suggest C-terminal region interactions with the N-terminal region of the protein are less important for efficient antimicrobial activity of CCL28 against *Pseudomonas* than *Staphylococcus* bacteria. The specific requirements for efficient interaction of the N-terminal region of the CCL27 and CCL28 proteins that contribute to the killing of *Staphylococcus* remain enigmatic. However, in preliminary analyses, we have found that the hydrophobicity plots of the N-region of CCL27 and CCL28 are highly similar. Conversely, hydrophobicity analysis of the N-region of CCL5 shows that this region is distinctly different from CCL27 and CCL28 (data not shown). The distinct hydrophobicity profile seen in these proteins may contribute to an amphipathic structure necessary for CCL28 lethality against *Staphylococcus* but not *Pseudomonas* bacteria.

The potential for antimicrobial proteins interacting with Gram-positive and Gram-negative bacteria via slightly different mechanisms is also supported by experiments in which we compared *in-house* prepared CCL28 to commercial CCL28. In these experiments the antimicrobial activity of *in-house* prepared CCL28 was compared to commercially obtained CCL28 on *Staphylococcus* and *Pseudomonas* bacteria. Results indicated that when using *S. aureus* in these assays there was no difference between commercial and *in-house* preparations of CCL28. However, in assays using *P. aeruginosa*, *in-house* CCL28 consistently resulted in greater levels of CCL28 antimicrobial activity. This may be due to residual his-tag remaining on *in-house* prepared CCL28 interacting preferentially with the Gram-negative cell wall and increasing the antimicrobial activity of this recombinant protein.

In conclusion, data presented here demonstrate that positively charged amino acids in the C-terminus of CCL28 play a key role in the antimicrobial activity of this dual-function chemokine. This observed antimicrobial activity is not dependent on the presence of the characteristic paired cysteines for which CC chemokines are named. Our data also demonstrate that the C-terminal region of CCL28 is necessary but not sufficient for full antimicrobial activity. Full antimicrobial activity is observed when the C-terminal region of CCL28 is combined with an appropriate N-terminal region of a non antimicrobial chemokine.

## Materials and Methods

### Reagents and bacteria

Mouse CCL28 recombinant protein was purchased from R&D systems Inc. (Minneapolis, MN). Two bacteria strains, *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 10145), were purchased from ATCC (Manassas, VA). All the bacteria were grown in tryptic soy broth at 37°C (BD Diagnostics, Sparks, MD).

### Preparation of recombinant mouse CCL28

Recombinant mouse proteins were expressed in *E. coli* as N-terminal His-tagged fusion proteins through cloning into the XhoI site of the pET19b expression vector (Novagen, Inc., Madison, WI). Briefly, total RNA was extracted from mouse large intestine using Trizol (Invitrogen, Corp., San Francisco, CA). The mouse CCL28-coding cDNA sequence, without its signal sequence, was amplified using a pair of primers (CCL28-F and CCL28-R), based on the DNA sequence retrieved from NCBI (NM_020279) using GeneAmp PCR Core Reagents (Applied Biosystems, Foster City, CA) (All the primer sequences are listed in Table I). RT-PCR reaction conditions consisted of 42 °C incubation for 15 min for reverse transcription using Oligo dT, followed by 95°C for 10 min and PCR cycles at 94 °C for 1 min, 60°C for 1 min, and 72 °C for 1 min for 30 cycles. Following PCR amplification, samples (25 μl) were gel purified using 2% agarose gels, and purified using Qiagen gel purification kit (Qiagen Inc., Valencia CA). The purified PCR products were then digested by XhoI and gel purified. The digested PCR products were ligated into XhoI digested plasmid, and transformed into DH5α *E. coli*. Transformants were selected through colony PCR. Plasmids were extracted using a Qiagen miniprep kit, and the correct CCL28 sequence was confirmed through cycle sequencing of all inserts.

### Preparation of recombinant mouse CCL5 and CCL27

Total RNA extracted from mouse spleen and ear was used to amplify CCL5 and CCL27, respectively. The primers CCL5-F and CCL5-R were used to amplify the CCL5 gene. Primers CCL27-F and CCL27-R were used to amplify the CCL27 gene. The same procedure as described above was used for the digestion, ligation, and transformation. Sequences were confirmed through cycle sequencing of the inserts.

### C-terminal and N-terminal truncation mutants

To generate successive C-terminus truncation mutants, the common forward primer, CCL28-F, was paired with the following reverse primers: C-R1, C-R2, C-R3, C-R4, C-R5, C-R6, C-R7, C-R8, and C-R9. The N-terminus mutant was generated using the common reverse primer CCL28-R with primer NF5. The same procedure was used for digestion, ligation, and transformation, as described above. All sequences were confirmed through cycle sequencing of the inserts.

### Sequence alignments

Protein sequences were retrieved from the NCBI data base for sheep, cow, pig, dog, human, chimpanzee, monkey, and mouse. Amino acid sequence alignments were then performed using the alignment program ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

### Site-specific mutagenesis for point and deletion mutants

To generate site-specific mutations, the circular PCR method was used [49]. In this method the entire plasmid containing the mouse CCL28 gene was amplified by Platinum® Pfx DNA Polymerase (Invitrogen, Corp., San Francisco, CA), and a pair of complementary primers was used to generate site-specific mutations. The following primers were used (only forward

primers are listed): R108/D-F, R108/A-F, E105/R-F, and CC/A-F. Using the above listed primers, for each mutation, two PCR reactions (25μl) were set up separately using only one of the complementary primers for each. After incubation at 95°C for 5 min, eight PCR cycles were completed using the following conditions: 94 °C for 1 min, 60°C for 1 min, and 68 °C for 6 min. The two reactions were then combined, and an additional 16 cycles were performed. DpnI was then added to the PCR reaction to digest the template plasmid. The DpnI treated PCR reaction was purified using the Qiagen PCR cleanup kit. The resulting PCR product was then transfected into DH5α *E. coli*, constructs were confirmed by cycle sequencing.

### Chimeric mutant preparation

To generate chimeric mutants, a two-step PCR-based method was used [49]. In this method two PCR reactions were set up: in the first reaction, CCL5 template and CCL5-F and CCL5-28-R primers were used, and in the second reaction, CCL28 template and CCL5-28-F and CCL28-R primers were used. For generation of CCL27 N-terminus and CCL28 C-terminus chimeric protein, CCL27 template and CCL27-F and CCL27-28-R primers were used for the first reaction and CCL28 template and CCL27-28-F and CCL28-R primers were used for the second reaction. After amplification, the two reactions were gel-purified and combined as template for PCR of a third reaction using CCL5-F and CCL28-R or CCL27-F and CCL28-R primers. After purification of the two chimeric PCR products, they were digested and ligated to the pET19b plasmid, and then transformed into DH5α *E. coli*. All constructs were confirmed through cycle sequencing.

### Expression and purification of recombinant mouse CCL28 protein and its mutants

All engineered plasmids were transformed into BL21 (DE3) bacterial cells, and a single colony was selected and inoculated into a test tube containing 2ml Luria broth. These cells were then grown to an optical density ($OD_{600}$) of 0.6 at 37°C and then transferred to 50 ml of Luria broth. The cells were grown at 37°C until log phase ($OD_{600}\approx0.8$), at which time recombinant protein production was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The culture was then incubated for 6 hours at 30°C. Cells were lysed and recombinant protein was purified by nickel-nitrilotriacetic acid resin according to the manufactures protocol (His SpinTrap™, GE Healthcare, Buckinghamshire, UK). The purified His-tag fusion protein solution was then dialyzed against 1mM Tris-HCL buffer (pH 8.0) overnight in a 5L beaker with three buffer changes. The dialyzed protein solution was then centrifuged to remove any precipitated protein. The purity of the protein was confirmed by visualizing the protein following electrophoresis on a 16% SDS-PAGE gel. The concentration of the protein was determined by Bradford assay (Thermo, Sci., Rockford, IL).

### Antimicrobial peptide assays

A slight modification of the standard colony forming assay was used to test the antimicrobial activity of mutant peptides against *Staphylococcus aureus* and *Pseudomonas aeruginosa* [10]. Briefly, bacteria were grown at 37°C in tryptic soy broth to mid log phase $OD_{600}$=0.5 and then diluted to $10^6$ colony forming units/ml in sterile Tris-HCl buffer (1mM, pH8.0) supplemented with 1% (v/v) of tryptic soy broth. Bacteria were incubated with recombinant chemokines in 60 μl of buffer in a 96 well plate for 30 min at 37°C with shaking. Chemokines were 2-fold serially diluted to concentrations ranging from 2 μM to 0.125 μM in 50 μl Tris-HCl. Bacteria, in a volume of 10μl were then added into each well. Assays were preformed in triplicate and repeated three times using each protein. Following the incubation of recombinant proteins with bacteria, the samples were spread on tryptic soy agar plates. After incubation of the plates at 37°C for 12- 20 hr, surviving bacteria were counted as colony forming units/ml.

### Statistical analysis

All statistical analyses were performed using a 1 tailed, paired, Students T test. A value of $p<0.05$ was considered significant.

## Acknowledgments

We wish to acknowledge the editorial assistance of Susanne Linderman in preparing this manuscript. This work was supported by a National Institutes of Health Grant RAI072769A (to E.W.).

## Abbreviations

**(AMP)** Antimicrobial peptides

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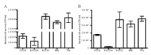

**Figure 1.**

Recombinant CCL28 exhibits potent antimicrobial activity. The antimicrobial activity of commercial CCL28 (CCL28) was compared to in-house produced recombinant CCL28 (R-CCL28), R-CCL5, BSA and buffer controls. These results demonstrate that in-house produced, full length CCL28 exhibits antimicrobial activity comparable to commercially produced CCL28. (A) CCL28 and control protein mediated killing of *S. aureus*. (B) CCL28 and control protein mediated killing of *P. aeruginosa*. Bacteria were exposed to the proteins/conditions listed in the X-axis. Bacterial survival, quantitated as CFU/ml, was determined through colony counts of bacteria surviving following 30 minute incubation with the various treatments as described. Data are shown as the mean ± SEM (*n*=3) and are representative of three independent experiments.

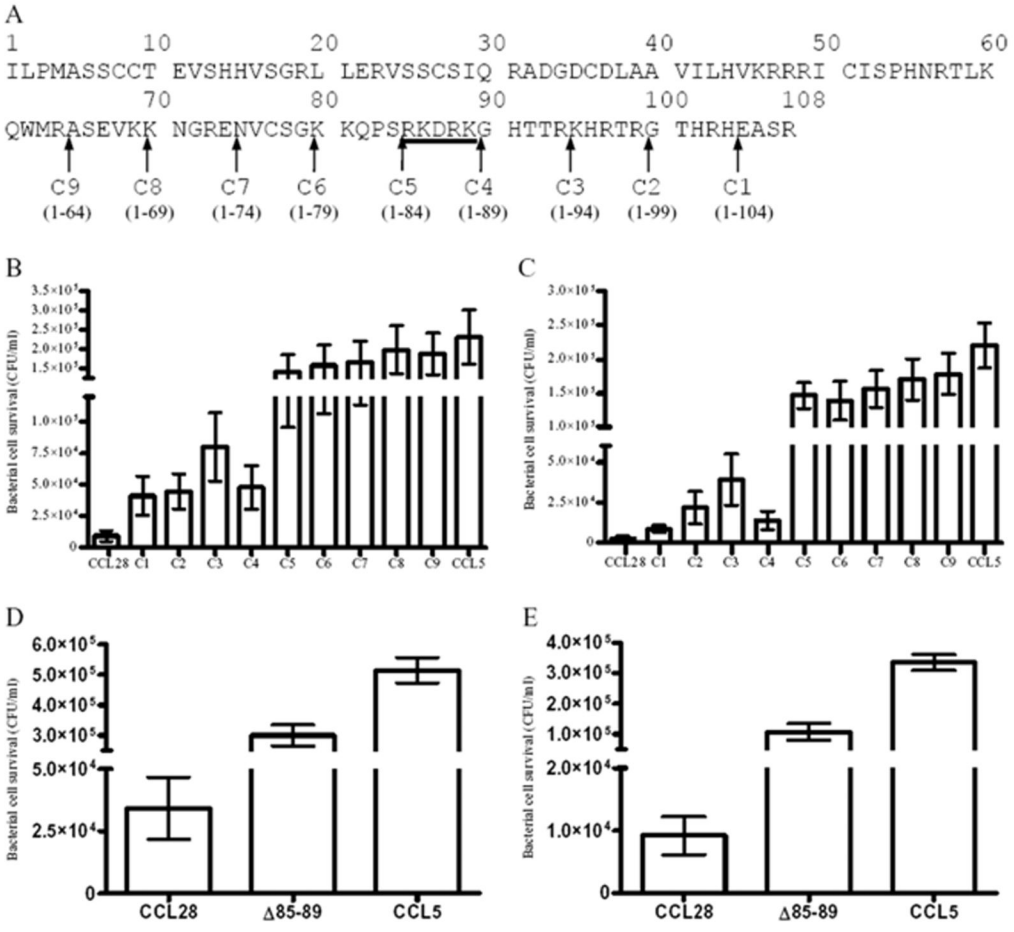


**Figure 2.**

The C-terminus of CCL28 is indispensible for efficient antimicrobial activity of the chemokine. (A) Sequential truncations (C1-C9) made to the CCL28 protein. The locations where the deletions were made are shown indicated by arrows below the protein sequence. Names (C1-C9) and amino acid residue numbers included in each protein are listed under each mutation. Antimicrobial activity of full length mCCL28, CCL5 and each CCL28 truncation mutant (C1-C9) were tested on *S. aureus* (B) or *P. aeruginosa* (C). Antimicrobial activity of CCL28 following the deletion of amino acids 85-89 (RKDRK) on *S. aureus* (D) and *P. aeruginosa* (E). Bacterial survival is displayed as CFU/ml. Data are shown as the mean ± SEM (*n*=3) and are representative of four independent experiments.

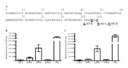

**Figure 3.**

Antimicrobial activity of CCL28 is reduced by charge reversal of the terminal amino acid. (A) Location of amino acid substitutions in the CCL28 protein; the amino acids manipulated by substitution are in bold and underlined. The one letter amino acid abbreviation, following the numerical location, indicates the amino acid present in the mutant protein. (B) Results of antimicrobial assays using mutated proteins tested on *S. aureus*. (C) Antimicrobial assay results using *P. aeruginosa*. Surviving bacterial cells were quantitated as CFU/ml. Antimicrobial activity of full length mCCL28, CCL5 and each CCL28 mutant. Data are shown as the mean ± SEM (*n*=3) and are representative of three independent experiments.


**Figure 4.**

The highly charged nature of the C-terminus of CCL28 is well conserved across species. The C-terminal region of CCL28 is highly positively charged with ~45% of all amino acids in this region being arginine, histidine, or lysine. Sequence alignment was performed using the alignment program ClustalW2. Charged amino acids are shaded and conserved cysteines are underlined. The amino acids corresponding to the essential region RKDRK from each species is boxed. (A) Sequence alignments of the C-terminus of CCL28 from sheep, cow, pig, dog, human, chimpanzee, monkey, and mouse. (B) Sequence alignments of the C-terminus of murine CCL28 and CCL27.

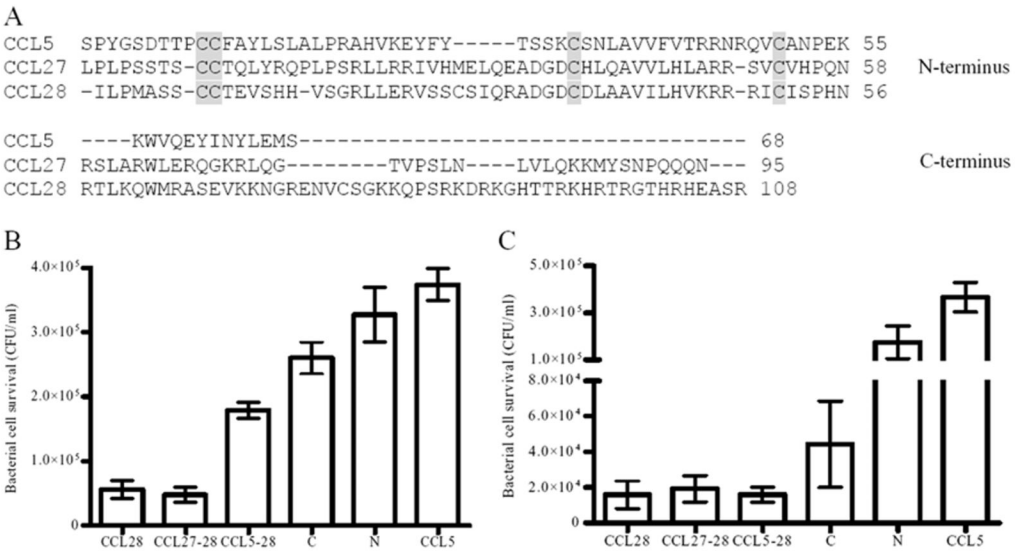


**Figure 5.**

Optimal antimicrobial activity of CCL28 requires an appropriate N-terminal region. (A) Alignment of CCL5, CCL27 and CCL28 following the signal peptide. Shaded areas indicate the conserved cysteines. Chimeric proteins were constructed by fusing the N-terminal region of selected chemokines (top panel) with the indicated C-terminal region (bottom panel). *S. aureus* (B) and *P. aeruginosa* (C) were treated with the mutant and full length proteins as indicated. Surviving bacterial cells were quantitated as CFU/ml. Data are shown as the mean ± SEM (*n*=3) and are representative of three independent experiments.

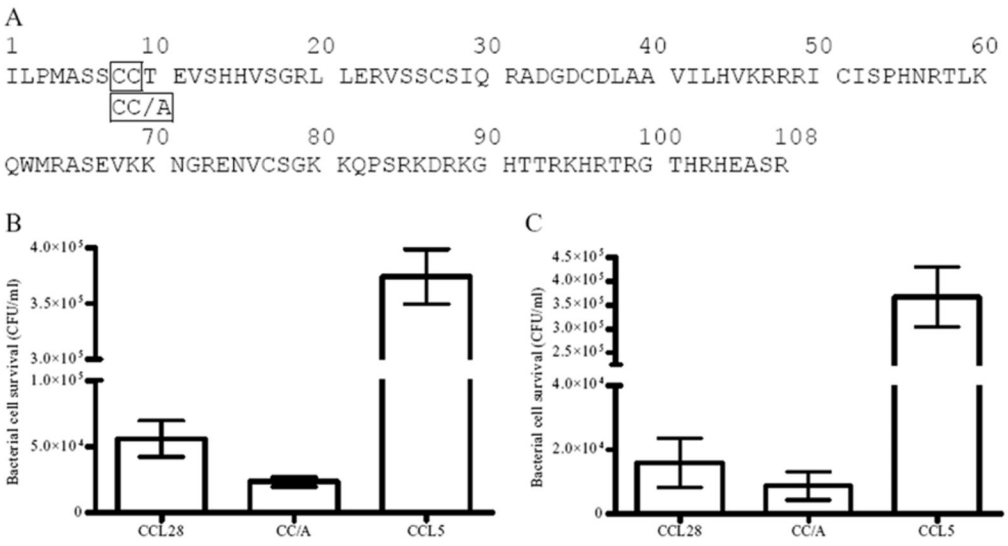


**Figure 6.**

The first two conserved cysteines in the N-terminal region of CCL28 are not essential for antimicrobial activity of the protein. (A) Amino acid sequence of CCL28, the boxed region indicates the conserved cysteines that were mutated to alanine. Bacterial killing assays using *S. aureus* (B) and *P. aeruginosa* (C). In all assays, bacteria surviving the indicated treatment were quantitated as CFU/ml as. Full length CCL28, CCL5 or the mutant protein CC/A were used. Data are shown as the mean ± SEM (*n*=3) and are representative of three independent experiments.

**Table 1**

Primer names and sequences

| Primer Name | Sequence (5′-3′) |
|---|---|
| CCL28-F | GCCGCTCGAGATACTTCCCATGGCCTCCAGCTG |
| CCL28-R | CCGAGCTCGAGCTAACGAGAGGCTTCGTGCCTGT |
| CCL5-F | GCCGCTCGAGTCACCATATGGCTCGGACACCAC |
| CCL5-R | CGAGCTCGAGCTAGCTCATCTCCAAATAGTTGATG |
| CCL27-F | GTTACTCGAGTTGCCTCTGCCCTCCAGCACT |
| CCL27-R | CGAGCTCGAGTTAGTTTTGCTGTTGGGGGTTTGAG |
| C-R1 | CCGAGCTCGAGCTAGTGCCTGTGTGTTCCACGTG |
| C-R2 | CCGAGCTCGAGCTAACGTGTTCTGTGCTTTCTCG |
| C-R3 | CCGAGCTCGAGCTATCTCGTAGTGTGCCCTTTTCTG |
| C-R4 | CCGAGCTCGAGCTATTTTCTGTCCTTCCTGCTGGG |
| C-R5 | CCGAGCTCGAGCTAGCTGGGTTGTTTTTTCCCAG |
| C-R6 | CCGAGCTCGAGCTACCCAGAACATACGTTTTCTCTGC |
| C-R7 | CCGAGCTCGAGCTATTCTCTGCCATTCTTCTTTACC |
| C-R8 | CCGAGCTCGAGCTACTTTACCTCTGAGGCTCTCATCC |
| C-R9 | CCGAGCTCGAGCTATCTCATCCACTGCTTCAAAGTACG |
| Δ85-89-F | TCTGGGAAAAAACAACCCAGCGGGCACACTACGAGAAAGCAC |
| N-F5 | GCCGCTCGAGAAGAAGAATGGCAGAGAAAACG |
| R108/D-F | ACACACAGGCACGAAGCCTCTGATTAGCTCGAGGATCCGGCTGCTAAC |
| R108/A-F | ACACACAGGCACGAAGCCTCTGCTTAGCTCGAGGATCCGGCTGCTAAC |
| E105/R-F | ACACGTGGAACACACAGGCACAGAGCCTCTCGTTAGCTCGAGGATCCG |
| CC/A-F | GAGATACTTCCCATGGCCTCCAGCGCTACTGAGGTGTCTCATCATGTTTCC |
| CCL5-28-R | TGAGGCTCTCATCCACTGCTTCAAAGTACGTGGGTTGGCACACACTTGGCGGTTCCTTCG |
| CCL5-28-F | CGAAGGAACCGCCAAGTGTGTGCCAACCCACGTACTTTGAAGCAGTGGATGAGAGCCTCA |
| CCL27-28-R | GCAGATTCTTCTACGTTTAACATGGACAAACACGACTGCAAGATTGGA |
| CCL27-28-F | TCCAATCTTGCAGTCGTGTTTGTCCATGTTAAACGTAGAAGAATCTGC |