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Partial Characterization of the Antimicrobial Activity of CCL28

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ABSTRACT

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This research focuses on the antimicrobial activity of the mouse chemokine CCL28. In addition to their well characterized chemotactic activity, many chemokines have been shown to be antimicrobial in vitro, including the mucosally expressed chemokine CCL28. I have investigated the primary sequence features required for antimicrobial activity, salt sensitive nature of killing/binding mechanism, and *in vivo* microbial interactions of CCL28. Through the use of protein mutation and expression techniques, I have shown that the holoprotein (108 amino acids) is necessary for full antimicrobial activity of CCL28. Furthermore, the C terminal region of CCL28 is essential for microbial killing as an almost complete loss of antimicrobial activity is seen following the removal of the C terminal 24 amino acids. The positively charged amino acids of the C-terminus directly contributed to the antimicrobial activity of CCL28. These experiments are the first to investigate the role of primary structure on the killing activity of an antimicrobial chemokine. Using flow cytometry analysis, I found that the salt-sensitive nature of CCL28 killing activity corresponds to its binding ability. Additionally, I have shown direct evidence for *in vivo* interaction between commensal bacteria and endogenously expressed CCL28 in the mouse large intestine. This interaction may directly correlate to the *in vivo* antimicrobial activity of CCL28. Lastly, I have begun to generate a CCL28 knockout mouse model to directly address the *in vivo* antimicrobial activity of CCL28. Vector construction and ES cell targeting by the vector has been completed, chimeric mouse generation remains to be done. This work represents the first systematic study of antimicrobial chemokine function. This work extends our understanding of antimicrobial proteins and their role in innate immune protection of the host and provides guidance for making better alternative antimicrobials.

Key words: antimicrobial, chemokine, CCL28

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Introduction

We are constantly exposed to microorganisms. A vast number of microorganisms inhabit the surface of our skin and mucosal tissues. Usually, these commensal microorganisms are not harmful, but in fact are often beneficial to us by competing against harmful invaders and providing necessary nutrients, such as vitamin K, to our bodies. However, these commensal microorganisms can be harmful if our mucosal barriers are damaged or our immune system is depressed. Some other microorganisms are considered pathogens because they have evolved specialized mechanisms to invade our tissues and circumvent our defense mechanisms.

Protection from pathogenic and commensal microbes is achieved through immunity. Simply put, immunity is the ability of a living organism to protect itself from foreign invaders. Our immune system consists of a wide variety of mechanisms including physical barriers, such as skin, to shield the organism, and biological or chemical components to kill the microorganisms directly. Some immune mechanisms are constitutively active, whereas others are inducible upon invasion by pathogens. Vertebrates have evolved complex immune systems which include a wide variety of molecules and cells for sensing, signal transduction, antigen processing, cell differentiation, and finally neutralizing and destroying invading microbes.

Understanding the mechanisms of immunity is very important. Our health depends on a proper functioning immune system. But the mechanisms used to destroy foreign invaders can also cause damage to our body. The efficient treatment of diseases requires understanding the infectious agents as well as the specific immune response to a given pathogen.

Vertebrates share several commonalities in immune function. All multiple celled organisms have native immunity, also called innate immunity. Vertebrates have also evolved a more complex and effective way to fight microbial invasion, which is referred to adaptive immunity. Although innate and adaptive immunity are often presented as separate entities, it is important to recognize that these two branches of the immune system must work together to achieve optimal host protection. Additionally, many cells and proteins of the immune system have roles in both innate and adaptive immunity.

Innate immunity

Innate immunity is the first line of protection that exists before an infection. It is an ancient defense mechanism shared by all life kingdoms. Cells and molecules of the innate immune system have the ability to distinguish host cells from foreign infectious agents by recognizing conserved molecular patterns which exists in microorganisms but not in host cells. These cells and molecules can be activated rapidly after encountering microorganisms. However, their efficacy is usually not improved after they encounter the same microorganism at a later date, which is the case with adaptive immunity.

The physical barriers of skin and other mucosal tissues are the front line of innate immunity. Epithelial surfaces can prevent microorganism invasion. Importantly, these "front line" tissues of the immune system can synthesize and secrete antimicrobial peptides (AMPs) to kill microorganisms directly. Three types of well-characterized antimicrobial peptides protect mucosal tissues (De Smet and Contreras 2005; Selsted and Ouellette 2005; Doss, White et al. 2010; Bevins and Salzman 2011). The first type of AMPs is defensins, which are grouped into α- and β-defensins based on their structure. The second type of AMPs expressed by epithelial cells

is cathelicidins, which have the cathelin domain (Zanetti, Gennaro et al. 1995). The third type of AMPs is the histidine-rich peptides, histatins (Edgerton, Koshlukova et al. 1998; Peters, Zhu et al. 2010).

If microorganisms penetrate epithelial barriers and enter into tissues, they are often intercepted by tissue resident immune cells: macrophages, immature dendritic cells, and mast cells. Macrophages and immature dendritic cell are professional phagocytic cells. They have phagocytic receptors to recognize microorganisms directly or to recognize opsonized microorganisms that have been coated by soluble molecules such as complement. These phagocytes contain phagolysosomes. Matured phagolysosomes contain pathogen degrading enzymes, antimicrobial peptides, and reactive oxygen and nitrogen species which kill internalized microorganism. Cells of the innate immune system also often express Toll-like receptors or the more widely expressed intracellular sensors NOD1, NOD2 and NALP3 which sense microbial invasion. Activation of these receptors results in the synthesis and secretion of proinflammatory cytokines by mast cells and macrophages to initiate an inflammatory response. These cytokines can further attract more macrophages and also activate them. Chemotactic cytokines, known as chemokines, are important players in the attraction and extravasation of leukocytes to sites of infection in both the innate and adaptive immune response. Following the rapid innate immune response, T cells and B cells are activated and contribute to adaptive immunity.

Adaptive immunity

Adaptive immunity responds slower than innate immunity. It has an almost unlimited ability to recognize different microbial antigens in a highly specific manner. Adaptive immunity

is highly efficient in clearing and preventing infection upon a re-encountering of a specific pathogen. T cells and B cells are the two main components of adaptive immunity.

Both B cells and T cells use their cell receptors to recognize antigens. The ability of B cells and T cells to recognize largely different antigens is endowed by the tremendous diversity of receptors which are generated by genetic recombination during their development.

T cells are pivotal to adaptive immunity. Naive T cells (which have matured in the thymus) recirculate through the secondary lymphoid tissues where they encounter and interact with antigen presenting cells that have ingested microbes or other debris and presented those antigens on their surfaces. Responding T cells proliferate and differentiate into different types of effector T cells. CD4 cells, one subset of T cells, regulate other immune cells of the body. Conversely, CD8 T cells function by directly killing infected host cells.

Antimicrobial peptides

Antimicrobial peptides (AMPs) are an important weapon for host defense against microbial invasion. These peptides are used by many different life forms including plants and mammals (De Smet and Contreras 2005; Selsted and Ouellette 2005). Almost all living organisms, ranging from vertebrates to bacteria, utilize AMPs to defend against microbial invasion (Giangaspero, Sandri et al. 2001; Zasloff 2002). Among the AMPs identified, defensins, cathelicidins and histatins have been the best-studied (De Smet and Contreras 2005).

Defensins are expressed by different mucosal tissues and immune cells. Paneth cells, found in the small intestines, and neutrophils express α-defensin while many mucosal epithelial cells express β-defensin (De Smet and Contreras 2005). Defensins are very effective at killing microbes *in vitro*. Knockout/knockin mouse models have confirmed the *in vivo* antimicrobial

activity of defensins (Wilson, Ouellette et al. 1999; Salzman, Ghosh et al. 2003). Crohn's disease in humans is believed to be initiated by the lack of Paneth cell differentiation or having fewer copies of the β-defensin gene (Wehkamp, Schmid et al. 2005). Defensins are also related to cancer. For example human β-defensin-1, which is produced by the epithelial cells of the genital-urinary tract, the skin, the respiratory tract, and the oral cavity, has depressed expression in most renal and prostate cancers (Valore, Park et al. 1998; Dale and Fredericks 2005). Correspondingly, over expression of this gene in bladder cancer and renal cancer has been shown to inhibit cancer cell proliferation and to result in cancer cell apoptosis (Sun, Arnold et al. 2006). Interestingly defenisins also have the ability to attract many types of immune cells (Territo, Ganz et al. 1989; Chertov, Michiel et al. 1996; Yang, Chertov et al. 1999; Oppenheim, Biragyn et al. 2003; Jin, Kawsar et al. 2010), which may partly explain their function in cancer suppression.

In the cathelicidin group of antimicrobial peptides, only one molecule, LL-37, has been described in humans (Gordon, Huang et al. 2005). Mice with the homolog of LL-37 knocked out are more susceptible to skin infection by group A *Streptococci* and urinary tract infection by *E. coli* (Chromek, Slamova et al. 2006). The expression of this molecule can be further induced in skin and lung after inflammation to more than ten-fold (Zasloff 2002). It can also bind to, and neutralize the LPS endotoxin (Bals, Weiner et al. 1999). Similar to defensins, LL-37 also acts to attract neutrophils and mast cells by binding to cellular receptors (Schiemann, Brandt et al. 2009).

The histatin group of AMPs is thought to be involved in maintaining oral health. Histantins protect oral tissues by killing *Candida albicans* (Edgerton, Koshlukova et al. 1998; Peters, Zhu et al. 2010). Multiple types of histatin exist, and the most effective one is histatin 5. They are secreted by the submandibular, sublingual, and parotid glands and are present in saliva

(De Smet and Contreras 2005). The *in vivo* antimicrobial activity of histatin 5 has been shown by an *ex vivo* murine model (Peters, Zhu et al. 2010).

There are also several other antimicrobial proteins identified in humans, including many ribonucleases (Harder and Schroder 2002; Dyer and Rosenberg 2006). For most of these ribonucleases, their antibacterial or antiviral activity is independent of their RNase activity, which can degrade microbial RNA. For example, RNase 7 is effective at nano-molar concentrations and kills *P. aeruginosa* without requiring its RNase activity (Huang, Lin et al. 2007). This kind of antimicrobial peptide is produced by eosinophils, neutrophils, and skin keratinocytes (Harder and Schroder 2002; Dyer and Rosenberg 2006). Other examples of antimicrobial proteins include Psoriasin and lysozyme from skin and air-way epithelium, dermcidin from sweat, phospholipase A2 from tears, histones from neutrophils, and platelet-derived factors from platelets (Buckland and Wilton 2000; Schittek, Hipfel et al. 2001; Kim, Cho et al. 2002; Tang, Yeaman et al. 2002; Harder, Glaser et al. 2007). Additionally, a ubiquitously expressed, multiple-functional, cellular prion-related protein (PrP^{c}) has recently been shown to have broad-spectrum antimicrobial activity (Pasupuleti, Roupe et al. 2009). These examples demonstrate that the antimicrobial activity of many AMPs is secondary or in addition to other protein functions.

Most AMPs are cationic. It has been proposed that the mechanism for the antimicrobial activity of defensins and other AMPs involves the interaction between the positively charged regions of these peptides and the negatively charged microbial cell membrane resulting in membrane lysis and cell death. Segregation of patched hydrophilic and hydrophobic residues to form an amphipathic structure is also important for AMP activity. And such a structure can be induced after the interaction with a membrane (Huang 2000; Giangaspero, Sandri et al. 2001;

Shai 2002). In bacteria, LPS and teichoic acids are negatively charged. The phosphomannan component of fungi is also negatively charged and thought to be a target of AMPs (Ballou 1990; Yeaman and Yount 2003).

Other AMPs do not lyse the microbial cell membrane but bind to the cell wall and are imported to the mitochondria where they inhibit ATP production. This has been shown to be the case for AMPs such as histatin 5 and human β-defensin 2 (Li, Sun et al. 2006; Vylkova, Nayyar et al. 2007). Additionally, some AMPs may interfere with extracellular molecule synthesis, such as in the cell wall, or intracellular molecule functions (Yeaman and Yount 2003; Schneider, Kruse et al. 2010). Still other AMPs bind to iron or sequester other trace elements from bacteria, such is thought to be the case for skin psoriasin (Glaser, Harder et al. 2005).

Through evolution some microorganisms have acquired effective strategies to evade AMPs. This resistance to AMP-mediated death can be either inherent or inducible. One mechanism commonly used by bacteria is the incorporation of positively charged components into the cell wall or cell membrane, thus making them less negatively charged and less of a target for the positively charged AMP (Peschel, Otto et al. 1999; Cao and Helmann 2004). Such mechanisms have been described in *Staphylococcus aureus* and *Bacillus subtilis*. A well characterized model of this is the bacterial PhoP/PhoQ two-component system. This two component system is used to remodel the microbial cell membrane to form a less negatively charged structure. This system is inducible following the microbe's encounter with the AMP. It has been shown in *Salmonella* and *E. coli* that this induction is accomplished through addition of aminoarbinose to the phosphate group or acylation of lipid A in the LPS (Gunn, Ryan et al. 2000; Starner, Swords et al. 2002).

The PhoP/PhoQ system can also induce the expression of outer membrane proteases, which degrade AMPs directly, in many species of bacteria including the PgtE protein expressed by *Salmonella*, the Pla protein by *Yersinia*, and the Sap protein by *C. albicans* (Guina, Yi et al. 2000; Meiller, Hube et al. 2009). *Streptococci* use M protein or SIC protein to inactivate AMPs by binding to them (Frick, Akesson et al. 2003; Frick, Schmidtchen et al. 2003; Nizet 2006). Similarly, *Pseudomonas aeruginosa* and *Enterococcus faecalis* can be induced to express proteinases which degrade proteoglycans, and then this free proteoglycan binds AMPs (Park, Pier et al. 2001). Other bacterial evasion strategies include the formation of capsules or biofilms to shield them from AMPs. Additionally, it has been demonstrated that *Yersinia* utilizes efflux pumps to expel AMPs out of the cell membrane (Bengoechea and Skurnik 2000). Finally, some bacteria have been shown to actively downregulate the expression of host AMPs (Islam, Bandholtz et al. 2001).

Due largely to the excessive use of conventional antibiotics, multiple-antibiotic resistant strains of pathogens have become an increasingly prevalent problem. Although some bacteria have evolved AMP avoidance mechanisms, due to the manner in which AMPs typically recognize and kill microbes, bacteria have generated less resistance to AMPs than to conventional antibiotics. The effectiveness and specificity of AMPs makes these molecules good candidates as alternative antimicrobials. As such, AMPs are of great interest in research and for use as clinical therapeutics.

Understanding the antimicrobial elements present in AMPs can provide guidance in engineering synthetic therapeutic drugs. To this end, primary, secondary, and tertiary structure elements important for antimicrobial activity of AMPs have been extensively explored (Pazgier, Prahl et al. 2007; Zou, de Leeuw et al. 2007; Jiang, Vasil et al. 2009). In studies of AMP

molecular structure it has been found that AMPs can adopt α-helix, β-sheet, or a mixture of these structures. Importantly, some AMPs may be unstructured in solution, however, when they are incubated with Tetrafluoroethylene, which mimics interaction with a bacterial membrane, an α-helical conformation is formed (Campagna, Saint et al. 2007). Study of α-defensins with model membranes has also shown that the presence of an organic phase is required for a conformational change in which the hydrophobic group of the peptide is exposed (Lourenzoni, Namba et al. 2007). Although appropriate structure is often important in AMP function, a fixed tertiary structure is not always required for antimicrobial activity. For example, deletion or substitution of cysteins in β-defensin-3 or in tachyplesin l does not affect their antimicrobial activity (Wu, Hoover et al. 2003; Kluver, Schulz-Maronde et al. 2005; Ramamoorthy, Thennarasu et al. 2006). On the other hand, disulfide bridges may be important in protecting the AMPs from degradation by peptidases and thus are necessary for antimicrobial activity *in vivo* (Tanabe, Ayabe et al. 2007). Dimerization can also contribute to antimicrobial activity. For example, dimerized β-defensin has a much higher activity than its monomer (Campopiano, Clarke et al. 2004). Human β-defensin-3 has the highest antimicrobial activity among all defensins, which may be explained by the fact that it can form dimers in solution even at low concentrations (Schibli, Hunter et al. 2002). Human α-defensin also mediates membrane pore formation in the form of a dimer (Zhang, Lu et al. 2010).

Chemokines

Chemokines are a group of small proteins (5-20 kDa) that are critical mediators for recruiting specific subsets of leukocytes to lymphoid tissues (constitutive homing) as well as sites of inflammation (inducible homing) via their corresponding chemokine receptor (Butcher

and Picker 1996; Lukacs 2001; Esche, Stellato et al. 2005; Allen, Crown et al. 2007). They play important roles in both innate and adaptive immunity. Based on the amino-terminal conserved cysteine arrangement, chemokines have been classified into CXC, CC, C, and CX3C subgroups (Zlotnik and Yoshie 2000). Chemokine receptors are seven-transmembrane G-protein-coupled receptors, which are named based on the chemokines they recognize. There are about 50 chemokines and 20 receptors that have been identified in humans to date (Allen, Crown et al. 2007).

Chemokines are highly variable in primary sequence but are very similar in tertiary structure, starting at the N terminus with three anti-parallel β-sheets followed by a C-terminal α-helix. Chemokines form two or three intramolecular disulfide bonds. The interactions between chemokines and chemokine receptors are usually both specific and redundant. The N-terminal amino acids are thought to be critical for specific receptor binding (Ott, Lio et al. 2004; Prado, Suetomi et al. 2007), whereas the α-helix of the C-terminus is thought to serve as a structural scaffold and the C-terminus of some chemokines can be switched between chemokines without loss of receptor specificity (Rajagopalan and Rajarathnam 2006).

Chemokines sometimes also require binding to glycosaminoglycans (GAGs) for *in vivo* function. This binding is thought to provide a platform for the interaction between chemokine and chemokine receptor and is resistant to the shearing forces of blood flow. GAGs are highly heterogeneous, extracellular molecules. Different cell types often express different types of GAGs which may be important in mediating chemokine function (Handel, Johnson et al. 2005). The exposed residues, usually positively charged arginine, lysine, and histidine in the loop regions of β-sheets and α-helix, are responsible for binding to GAG and it is thought that the binding between some chemokines and some GAGs is highly specific (Johnson, Proudfoot et al.

2005; Severin, Gaudry et al. 2010). Many chemokines are known to form dimers or other oligomers, especially when binding to GAGs (Handel, Johnson et al. 2005; Johnson, Proudfoot et al. 2005). This oligomerization has been shown to be necessary for the function of some chemokines *in vivo* (Baltus, Weber et al. 2003; Proudfoot, Handel et al. 2003).

The main function of chemokines is thought to be the chemoattraction of leukocytes. Leukocyte accumulation to tissues is a multiple-step cascade of events, in which chemokines play an important role in determining the specificity and activation of the signal required for immune cell migration. In inflammatory conditions, cytokines induce the expression of selectins, integrin receptors (addressins) on endothelial cells, and chemokines. The type of chemokine expressed will determine the type of circulating cell that will be recruited to a given tissue. After extravasation from the blood vessel into the tissue, leukocytes migrate to specific microenvironments by following chemokine gradients.

Chemokine mediated phagocytic cell and lymphatic cell extravasation is very important for the host to be able to fight infection. For example, mice deficient in CXCR2 or its receptor CCR1, are highly susceptible to *S. aureus* infection in the brain and to coronavirus infection in the central nervous system (Kielian, Barry et al. 2001; Hickey, Held et al. 2007). Constitutively expressed chemokines mediate leukocytes homing, recirculation, and development. Chemokines CCL19 and CCL21 mediate naïve B cell, naïve T cell, and mature dendritic cell migration to the secondary lymphoid organs (Ono, Nakamura et al. 2003). These chemokines are important in maintaining homeostasis in the immune system. Recently, chemokines have been recognized to be involved in many other biological process, such as lymphocyte differentiation, cell proliferation, angiogenesis, and tumor immune escape (Esche, Stellato et al. 2005; Pivarcsi,

Muller et al. 2007; Keeley, Mehrad et al. 2010; Struyf, Salogni et al. 2010). All of these functions reflect the key role of chemokines in immune processes.

In addition to their function in cell homing and migration, the majority of chemokines have been shown to exhibit direct antimicrobial activity *in vitro* (Cole, Ganz et al. 2001; Yang, Chen et al. 2003). However, it is not very clear whether these proteins play a direct antimicrobial role *in vivo*. Some chemokines have been shown to have broad antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, some fungi, and enveloped viruses (Takahata, Takada et al. 2003; Yang, Chen et al. 2003; Linge, Collin et al. 2008). However, different chemokines exhibit different antimicrobial potencies and spectra. Just like other AMPs, their antimicrobial activity is salt-sensitive. With an increase in salt concentration, the killing activity and the binding of chemokines to bacterial membranes decrease (Hieshima, Ohtani et al. 2003; Linge, Collin et al. 2008).

Like many other AMPs, antimicrobial chemokines are also cationic, suggesting that they may use the same antimicrobial mechanism as defensins. As for their structures, both human defensins and chemokines have three antiparallel β-sheets and one α-helix. It is postulated that the C-terminus of chemokines is responsible for the antimicrobial activity (Cole, Ganz et al. 2001). For example, the C-terminal helix of CCL20 is solely responsible for its full antimicrobial activity, even though the N- terminus also contains cationic residues (Hoover, Boulegue et al. 2002; Chan, Hunter et al. 2008). CXCL8 (interleukin-8) is not antimicrobial, but its C-terminus alone is antimicrobial (Bjorstad, Fu et al. 2005). The antimicrobial activity of LL-37 and CXCL chemokine platelet-derived factor thrombocidin are derived from the cleavage of their parent forms *in vivo*. However, the N-terminus of CXCL6 has higher antimicrobial activity than it C-terminus, whereas the holoprotein has the highest antimicrobial activity (Linge, Collin et al.

2008). Tertiary structure comparison between some antimicrobial chemokines and non-antimicrobial chemokines has revealed that some antimicrobial chemokines have a large, positively charged electrostatic patch on their surfaces (Yang, Chen et al. 2003). It is not known if this is true for all chemokines or other cationic AMPs.

Recently, evidence has shown that chemokines and defensins are convergent in two functions: chemotaxis and antimicrobial activity. It is a great advantage for the immune system if one molecule can kill the microorganisms directly and at the same time also attract immune cells for additional aid in microbial destruction. Both α-defensin and β-defensin can attract dendritic cells, monocytes, and/or T-cells, by using the same chemokine receptor (Territo, Ganz et al. 1989; Chertov, Michiel et al. 1996; Yang, Chertov et al. 1999; Niyonsaba, Iwabuchi et al. 2002; Yang, Biragyn et al. 2002; Rohrl, Yang et al. 2010; Rohrl, Yang et al. 2010). Defensins can also attach to GAGs as chemokines do (Seo, Blaum et al. 2010).

Chemokines and defensins kill microorganisms only at relatively high concentrations. In one way, this may be beneficial for the host. The surface of mucosal tissues is often constantly inhabited by commensal bacteria, which are vital to our health. Those bacteria may not be killed by the AMPs because of the low concentration of AMPs diffused from underlying cells. Only if the microbe has the ability to firmly attach and penetrate the cell surface will they encounter high concentrations of AMPs, which will kill them. Pathogen invasion also results in inflammation and upregulation of AMP expression (Egesten, Eliasson et al. 2007). Both chemokines and defensins may kill the microorganisms at the site of infection and diffuse to attract leukocytes. Even though the *in vivo* antimicrobial function for many chemokines is unknown, based on the expression level and location, a few of those chemokines have been proposed to play an important role in innate immunity against bacterial invasion (Egesten, Eliasson et al. 2007;

Maerki, Meuter et al. 2009; Kotarsky, Sitnik et al. 2010). For example, CCL6, is highly expressed in the small intestine and large intestine, and expression can be stimulated by LPS *in vivo*. CXCL14 is the highly expressed in epidermis and dermis and is thought to help protect human skin from microbial colonization (Yeaman, Yount et al. 2007). What's more, antibody-mediated neutralization of CXCL9, 10, and 11 was found to increase the susceptibility of mice to inhalation anthrax, suggesting an AMP role *in vivo* for this chemokine (Crawford, Burdick et al. 2010).

Antimicrobial chemokines are potential candidates for alternative antimicrobials. Discovery of effective alternative antimicrobials is a long-term task. The emergence of multiple-antibiotic resistant pathogens makes it more urgent than ever before. Studying the antimicrobial mechanisms of chemokines can be beneficial in effective drug design. However, identifying the antimicrobial functional elements and elucidating the antimicrobial mechanisms of chemokines has just begun. The next fundamental question that must be answered is which antimicrobial chemokines play an antimicrobial role *in vivo*. Finding out the antimicrobial role of chemokines will help us to understand how the immune system works. In the next few chapters, I discuss the different techniques I have used to address some of these questions.

Chapter 2: Identifying amino acids important for antimicrobial activity of chemokine CCL28

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) (Butcher and Picker 1996; Lukacs 2001; Esche, Stellato et al. 2005; Allen, Crown et al. 2007). In addition to their function in cell homing and migration, several chemokines have been shown to exhibit direct antimicrobial activity *in vitro* (Niyonsaba, Iwabuchi et al. 2002; Yang, Chen et al. 2003; Linge, Collin et al. 2008).

The chemokine CCL28 has been shown to selectively attract IgA plasma cells to various mucosal tissues through interactions with its cognate receptor, CCR10 (Wang, Soto et al. 2000; Wilson and Butcher 2004). 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 (Hieshima, Ohtani et al. 2003). Histatin 5, CCL28, and other antimicrobial peptides (AMPs), such as defensins, often require low salt (non- physiologic) solutions for maximum antimicrobial activity *in vitro*. However, mouse models in which defensin function is inhibited clearly demonstrate an essential antimicrobial role for these AMP's *in vivo* (Wilson, Ouellette et al. 1999; Salzman, Ghosh et al. 2003).

A mechanism for the antimicrobial activity of defensins and other AMPs 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 (Huang 2000; Giangaspero, Sandri et al. 2001; Shai 2002).

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 (Hoover, Wu et al. 2003; Powers 2003; Tanabe, Qu et al. 2004; Wu, Li et al. 2005; Xie, Prahl et al. 2005; Pazgier, Prahl et al. 2007; Taylor, Clarke et al. 2008). When compared with defensins and other AMPs, chemokines are larger in size and have a more complex structure (Butcher and Picker 1996). 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 (Butcher and Picker 1996; Shaw, Johnson et al. 2004). 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 (Homey, Wang et al. 2000). In contrast to CCL28, CCL27 exhibits no antimicrobial activity (Hieshima, Ohtani et al. 2003; Linge, Collin et al. 2008). 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, and the C-terminus has been hypothesized to be important for

antimicrobial activity (Hemmerich, Paavola et al. 1999; Hoover, Boulegue et al. 2002; Ott, Lio et al. 2004; Chan, Hunter et al. 2008).

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 may be important for the full antimicrobial activity of CCL28.

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). *Yersina pestis* isolates were kindly provided by Dr. David Erikson (Brigham Young University). All the bacteria were grown in tryptic soy broth at 37°C (BD Diagnostics, Sparks, MD) until they reached log phase (OD600 = 0.5~0.6).

CCL28 primary structure and tertiary structure analysis

CCL28 charge and hydrophobicity profile were analyzed by Emboss and ProtScale, respectively. CCL28 tertiary structure was predicted by the program 3Djigsaw, based on the newly published structure of human chemokine CCL27 (Kirkpatrick, et al, 2010), which among all chemokines, shares the highest homology with CCL28.

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, Table 1), based on the DNA sequence retrieved from NCBI (NM_020279), and GeneAmp PCR Core Reagents (Applied Biosystems, Foster City, CA). All the primers used are listed in Table 1. 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, amplicons (25 µl) were separated 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 plasmids, 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

The primer sequences used to generate truncation, point mutation, and chimera are all listed in Table 1. 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 mutants were 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.

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

Table 1. Continued

C-R2	CCGAGCTCGAGCTAACGTGTTCTGTGCTTTCTCG
C-R3	CCGAGCTCGAGCTATCTCGTAGTGTGCCCTTTTCTG
C-R3	CCGAGCTCGAGCTATCTCGTAGTGTGCCCTTTTCTG
C-R4	CCGAGCTCGAGCTATTTTCTGTCCTTCCTGCTGGG
C-R5	CCGAGCTCGAGCTAGCTGGGTTGTTTTTTCCCAG
C-R6	CCGAGCTCGAGCTACCCAGAACATACGTTTTCTCTGC
C-R7	CCGAGCTCGAGCTATTCTCTGCCATTCTTCTTTACC
C-R8	CCGAGCTCGAGCTACTTTACCTCTGAGGCTCTCATCC
C-R9	CCGAGCTCGAGCTATCTCATCCACTGCTTCAAAGTACG
NF5	GCCGCTCGAGAAGAAGAATGGCAGAGAAAACG
Δ108-F	CACAGGCACGAAGCCTCTTAGCTCGAGGATCCGGCTGCT
Δ85-89-F	TCTGGGAAAAAACAACCCAGCGGGCACACTACGAGAAAGCAC
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

Site-specific mutagenesis for point and deletion mutants

To generate site-specific mutations, the circular PCR method was used. 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*, and constructs were confirmed by cycle sequencing.

Chimeric mutant preparation

To generate chimeric mutants, a two-step PCR-based method was used. 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 proteins, 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 they reached 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 0.5mM. 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 activity 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* (Hieshima, Ohtani et al. 2003). 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, pH 8.0) 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 10 µl were then added into each well. Assays using each chemokine were repeated three times. Following the incubation of recombinant proteins with bacteria, the samples were spread on to 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.

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 (Hieshima, Ohtani et al. 2003). 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. 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 this chemokine (Hieshima, Ohtani et al. 2003). In an effort to more precisely define which regions of the protein are essential for antimicrobial

Figure 1

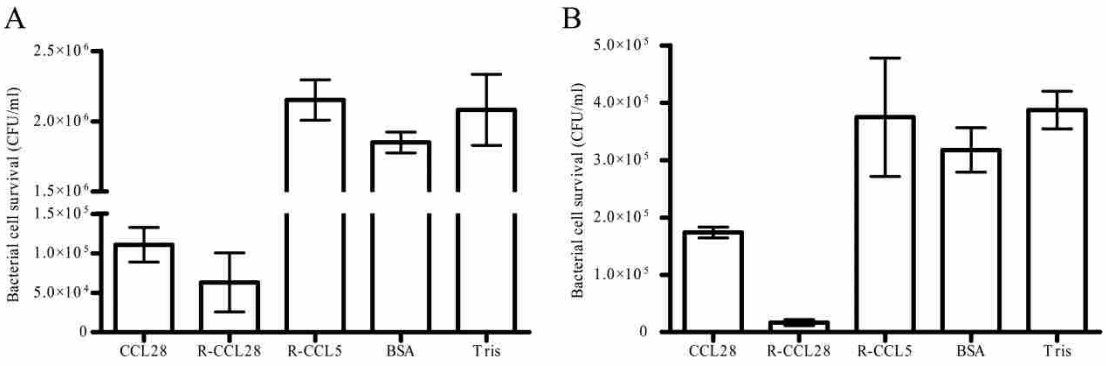


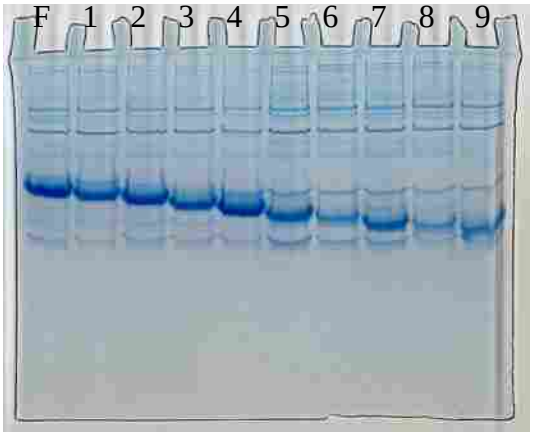
Figure 1. Recombinant mCCL28 exhibits potent antimicrobial properties, but mCCL5 does not. The antimicrobial activity of mCCL28, CCL28 (R&D), mCCL5, BSA and buffer controls demonstrate that in-house, full length CCL28 exhibits microbial killing which is comparable to commercially produced CCL28. *S. aureus* (A), and *P. aeruginosa* (B), were exposed to the substances listed in the X axis, and surviving bacteria were quantitated as CFU/ml (Y-axis). Each assay was run in triplicate. Data shown are means ± SEM.

activity, we produced several mutant versions of CCL28 through successive C-terminal truncations. The expression and purification of those mutant proteins are described in the method. The Ni-column purified mutant proteins were visualized by SDS-PAGE (16%). Both the expression and the purity of those proteins were good (Fig. 2A). These truncated recombinant proteins were then assayed for antimicrobial activity. 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 CCL28 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 all 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 decreased and the antimicrobial activity of C4 always increased when compared to C3. These results suggest that although the majority of the antimicrobial activity is highly dependent on amino acids 85-89, other regions of the C terminus may make minor contributions to the full antimicrobial effect of CCL28.

To further determine if the C4-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–

Figure 2

A

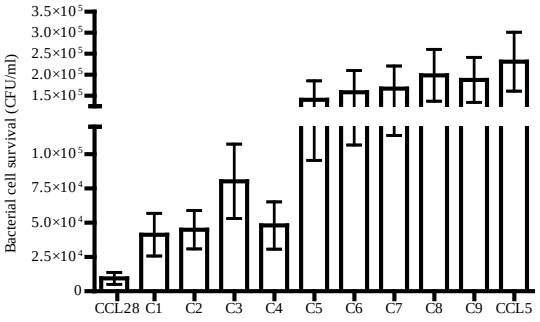


B

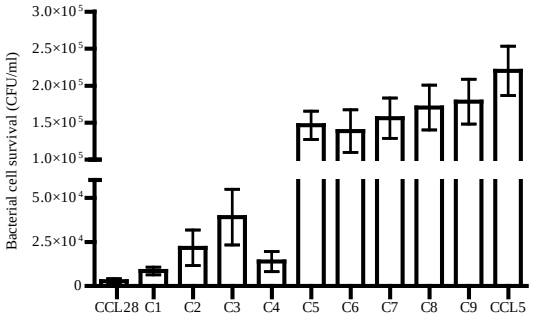
```
1         10         20         30         40         50         60

ILPMASSCCT EVSHHVSGRL LERVSSCSIQ RADGDCDLAA VILHVKRRRI CISPHNRTLK
          70         80         90         100      108
QWMRASEVKK NGRENVCSGK KQPSRKDRKG HTTRKHRTRG THRHEASR
    C9    C8    C7    C6    C5    C4    C3    C2    C1
  (1-64) (1-69) (1-74) (1-79) (1-84) (1-89) (1-94) (1-99) (1-104)
```

C



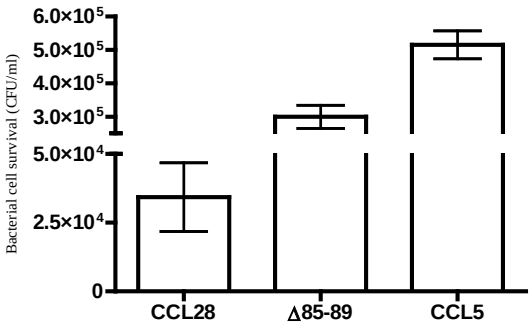
D



Continue on the next page.

Figure 2 Continued

E



F

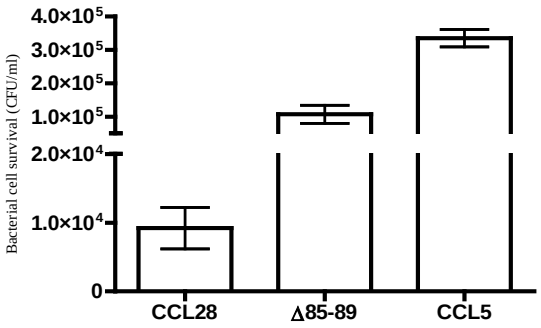


Figure 2. The C-terminus of CCL28 is indispensable for efficient antimicrobial activity of this chemokine. (A) SDS-PAGE (16%) of Ni-column purified recombinant protein. F, full length protein. 1-9, serial C-terminus truncation mutant protein, as indicated in panel B. (B) Sequential truncations (C1–C9) made to the CCL28 protein. The locations where the deletions were made are 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) was tested on *S. aureus* (C) or *P. aeruginosa* (D). Antimicrobial activity of CCL28 following the deletion of amino acids 85–89 (RKDRK) on *S. aureus* (E) and *P. aeruginosa* (F). Bacterial survival is displayed as CFU/mL. Data shown are means ± SEM (n53) and are representative of four independent experiments.

108 of the wild-type protein. In these experiments, we observed a dramatic reduction in antimicrobial activity when compared with the full-length CCL28 protein. However, although the antimicrobial activity of this mutant protein greatly decreased following the specific deletion of amino acids 85–89, antimicrobial activity was still detected in this mutant (Fig. 2D and E). 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 (Huang 2000). 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 (Figure 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 nearly 50% 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. These results suggest that the entire five amino acid region contained in

Figure 3

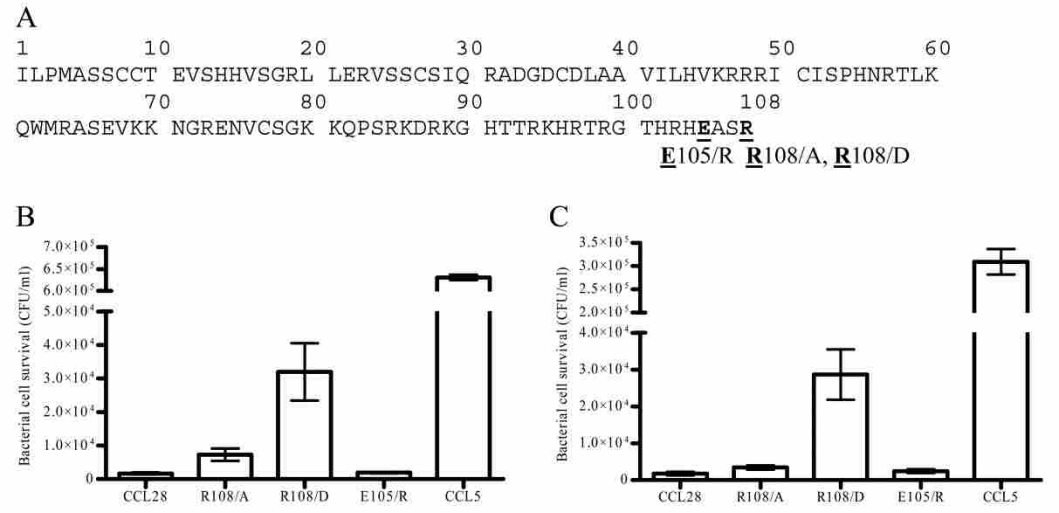


Figure 3. Antimicrobial activity of CCL28 is reduced by charge reversal of the terminal amino acid. (A) Location of amino acid substitutions in the CCL28 protein; the amino acids manipulated by substitution are in bold and underlined. The one letter amino acid abbreviation, following the numerical location, 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 means ± SEM (n=3) and are representative of three independent experiments.

truncation 4 is not highly conserved among species, but 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 human CCL28 is (Hieshima, Ohtani et al. 2003). 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 abundant 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 this chemokine and also differentiate this chemokine from the other CCR10 ligand, CCL27.

Figure 4

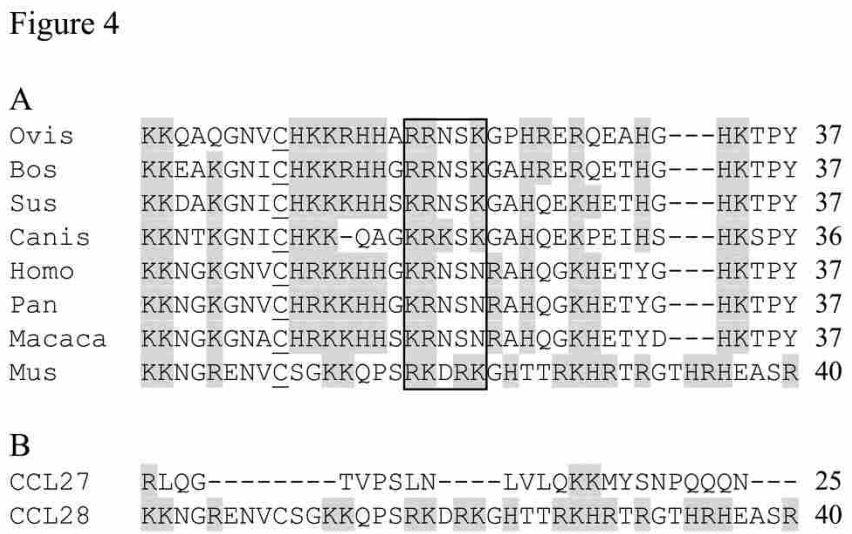


Figure 4. The highly-charged nature of the C-terminus of CCL28 is well conserved across species. The C-terminal region of CCL28 is highly positively-charged with ~45% of all amino acids in this region being arginine, histidine, or lysine. Sequence alignment was performed using the alignment program ClustalW2. Charged amino acids are shaded and conserved cysteines are underlined. The amino acids corresponding to the essential region RKDRK from each species are 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.

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 consisted of the C-terminal 52 amino acids, and the second consisting of the 56 N-terminal amino acids of CCL28 (Fig. 4A). 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 of experiments in which the antimicrobial activity of the N-terminal peptide was compared to CCL5 resulted in P values that 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. However, the antimicrobial effect of the full length protein was clearly far superior to the C-terminal 52 amino acids peptide (Figure 5A).

Based on results from figures 2 and 5B, 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. CCL27 is the most closely related chemokine to CCL28 and both CCL27 and CCL28 bind the CCR10 chemokine receptor on lymphocytes. CCL27 has not been shown to exhibit antimicrobial properties. The second chimeric protein constructed consisted of the N-terminal region of CCL5 fused to the C-terminus of CCL28. CCL5 binds the CCR1, CCR3 and CCR5 chemokine receptors and has not been shown to possess antimicrobial properties. 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 bacteria than the CCL5/CCL28 chimera ($p=0.014$), (Fig. 4C). 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. 5B).

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 (Rajagopalan and Rajarathnam 2006; Prado, Suetomi et al. 2007). 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

Figure 5

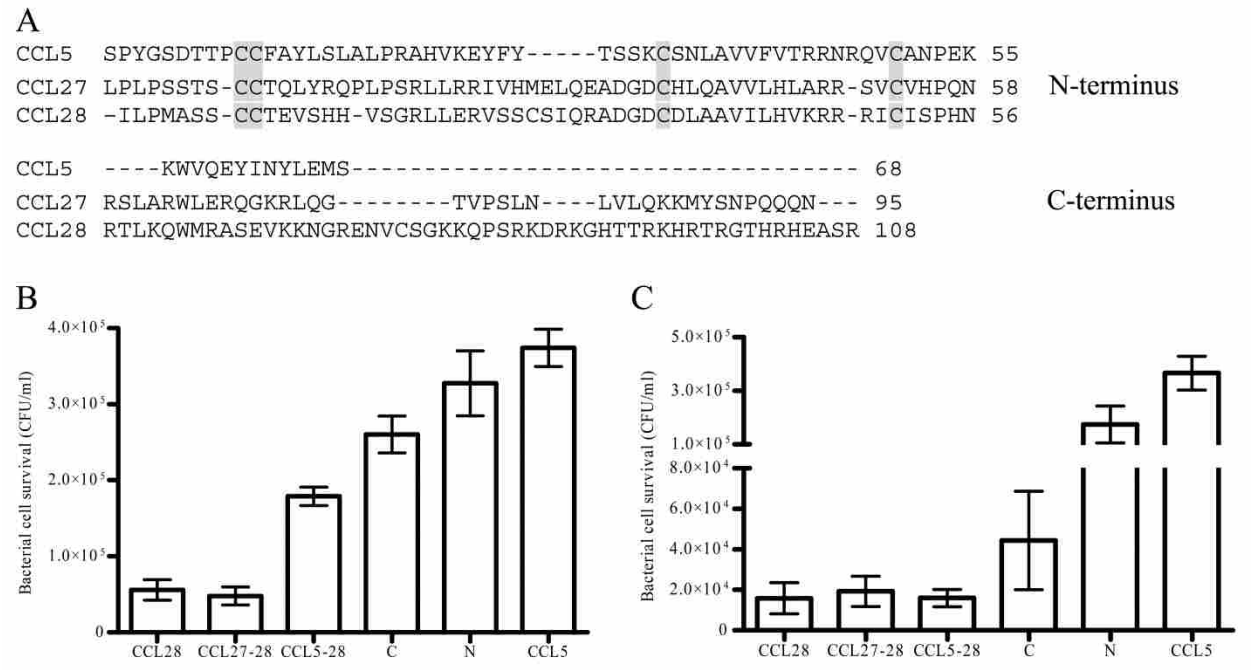


Figure 5. Antimicrobial assay of chimeric proteins. CCL27N-CCL28C, CCL5N-CCL28C, C-terminus only, and N-terminus only were used against *S. aureus* (A) and *P. aeruginosa* (B). The decision of N and C terminus are based on the alignment as shown in figure 2. Mutants are indicated (X-axis) and surviving bacterial cells were quantitated as CFU/ml (Y-axis). Their activities were compared with recombinant full length CCL28 and CCL5. Each mutant assay was triplicated. Data shown are means ± SEM.

Figure 6

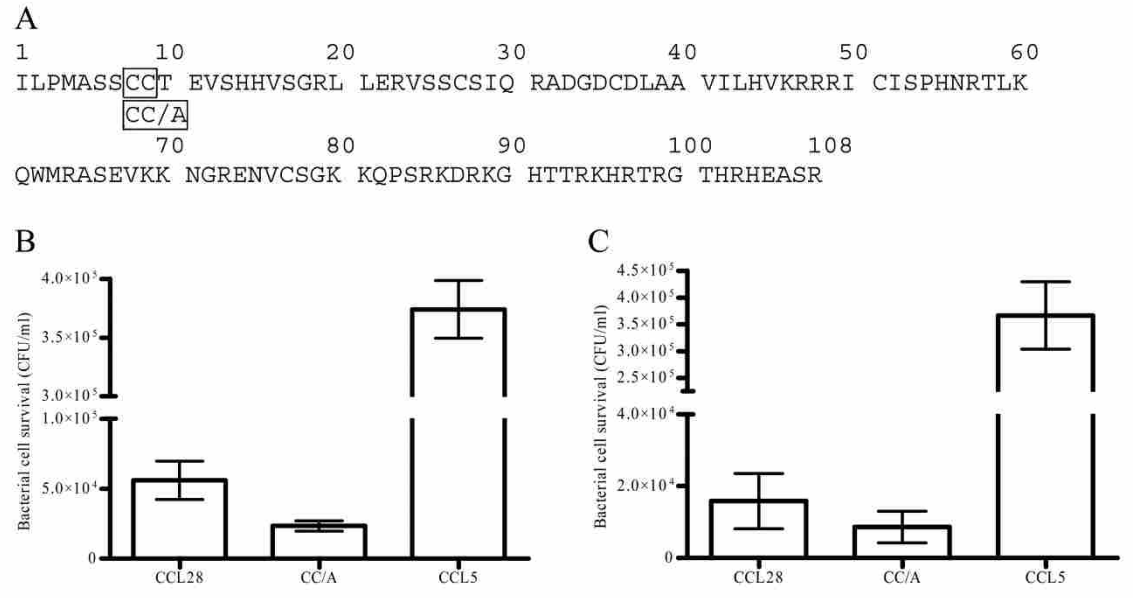


Figure 6. The first two conserved cysteines are not important for antimicrobial activity. Amino acids that have been deleted in the sequence are in boxes (A). *S. aureus* (B), *P. aeruginosa* (C), were exposed to the deletion and substitution mutant peptides, and surviving bacterial cells were quantitated as CFU/ml. Their activities were compared with recombinant full length CCL28 and CCL5. Each mutant assay was triplicated. Data shown are means ± SEM.

substituted for the two cysteines at positions 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 (Zasloff 2002). 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 (Eksteen, Miles et al. 2006; Hansson, Hermansson et al. 2008). Additionally, this chemokine has been shown to be constitutively expressed and highly concentrated in mucosal secretions such as milk and saliva (Hieshima, Ohtani et al. 2003). 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 to 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 (Chertov, Michiel et al. 1996; Yang,

Chertov et al. 1999). 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 (Lortat-Jacob, Grosdidier et al. 2002; Lau, Paavola et al. 2004; Handel, Johnson et al. 2005). The binding of chemokines to epithelial surfaces has been proposed to increase the effective concentration of CCL28 at mucosal surfaces (Hieshima, Ohtani et al. 2003).

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. Having a positively-charged amino acid in one of 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 indispensable 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 investigating or exploring the necessity of the highly positively-charged C-terminal region of CCL28, we investigated if disulfide 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 (Allen, Crown et al. 2007). 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 (Rajagopalan and Rajarathnam 2006). 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 (Ott, Lio et al. 2004; Prado, Suetomi et al. 2007). 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 disulfide 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 disulfide bonding known to be vital for chemokine receptor binding. Disulfide bonding has also been shown to be essential for the chemotactic activity of other AMP but not for the antimicrobial activity of those AMP (Rajarathnam, Sykes et al. 1999; Wu, Hoover et al. 2003; Kluver, Schulz-Maronde et al. 2005; Ramamoorthy, Thennarasu et al. 2006). Although the disulfide 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 AMPs, it has been demonstrated that essential, three-dimensional structures are formed following interaction with a bacterial membrane (Schibli, Hunter et al. 2002; Mani, Cady et al. 2006; Campagna, Saint et al. 2007; Lourenzoni, Namba et al. 2007).

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 and 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 were 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 *Staphylococcus* and *Pseudomonas* bacteria were used to compare the antimicrobial activity of in-house prepared CCL28 to the antimicrobial activity of commercially obtained CCL28. 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.

Chapter 3: CCL28 binds to bacteria *in vivo*

Abstract

Most chemokines have been found to have antimicrobial activity *in vitro*. It has been hypothesized that the decrease in killing activity at high salt concentration is a result of dissolved ions adversely affecting the charge-mediated binding between chemokine and bacteria. Here we demonstrate using the mucosal-expressed antimicrobial chemokine CCL28 and *Salmonella typhimurium* bacteria, that CCL28 binds bacteria at both high and low salt concentrations. However, more CCL28 binding per bacterium occurs in low salt conditions. This finding suggests that CCL28's inability to bind bacteria in high salt conditions does contribute its lower killing activity. We also demonstrate that endogenously-expressed CCL28 binds to commensal bacteria in the mouse large intestine. This is the first research demonstrating a direct interaction between CCL28 and bacteria *in vivo*.

Introduction

Chemokines are a group of small proteins that play essential roles in leukocyte migration and accumulation. In addition to their well-studied roles in mediating the directional migration of leukocytes via interactions with cognate receptors, these proteins also play key roles in a number of other biological processes such as angiogenesis, cancer biology, hematopoiesis, and direct antimicrobial activity against microbes (Esche, Stellato et al. 2005; Pivarcsi, Muller et al. 2007; Keeley, Mehrad et al. 2010; Struyf, Salogni et al. 2010). The pleiotropic nature of chemokines in such essential processes illustrates the importance of a better understanding of all

aspects of chemokine biology, and one such task is to understand the antimicrobial role of chemokines in innate immunity.

Antimicrobial chemokines share many traits with conventional antimicrobial proteins such as defensins, cathelicidin, and histatins. For example, they are all cationic and are thought to bind to the anionic microbial membrane, ultimately causing permeabilization or disruption of the microbial membrane (Hieshima, Ohtani et al. 2003; Brogden 2005; Lee, Hung et al. 2008; Linge, Collin et al. 2008). Like defensins, antimicrobial chemokines show reduced activity in physiological (150 mm) salt concentrations (Hieshima, Ohtani et al. 2003; Starner, Barker et al. 2003; Yang, Chen et al. 2003; Linge, Collin et al. 2008; Maerki, Meuter et al. 2009). Why these proteins function optimally in low salt solutions is unclear, but, it has been hypothesized that the large number of charged ions in high salt solutions essentially shields the negatively-charged membrane from the positively charged AMP, resulting in a failure to recognize and bind the bacterial membrane (Selsted and Ouellette 2005). However, this hypothesis has not been thoroughly tested.

The chemokine CCL28 is known to play an essential role in mediating the migration and accumulation of IgA antibody secreting B cells (IgA ASCs) to a variety of mucosal tissues (Hieshima, Kawasaki et al. 2004; Wilson and Butcher 2004). This migration is mediated by interactions with the CCR10 chemokine receptor that is predominantly found on IgA ASCs as well as some T cells (Wang, Soto et al. 2000). CCL28 is expressed by mucosal epithelial cells and is found in high concentrations in saliva and breast milk (Hieshima, Ohtani et al. 2003). CCL28 mRNA is also highly expressed in a variety of exocrine glands as well as in the large intestine (Nakayama, Hieshima et al. 2003). CCL28 has been shown to kill microbes only in low salt buffers and not in what is commonly considered “physiologically normal” conditions

(Hieshima, Ohtani et al. 2003). However, what constitutes as "physiologically normal" is clearly dependent on what part the body is being sampled. Many mucosal tissues are surrounded by low salt environments. For example milk and the intestinal lumen, both sites of high CCL28 expression, have considerably lower salinity than blood. Although, CCL28 is highly expressed at mucosal surfaces and efficiently kills bacteria in low salt solutions, it is unclear if this salt-sensitive killing is due to decreased binding of the chemokine or decreased activity of the chemokine once bound. Furthermore, although CCL28 is known to be expressed in many mucosal tissues it has not previously been demonstrated if this chemokine interacts with bacteria *in vivo*.

In this study we addressed the questions of how buffer salinity affects the interaction between CCL28 and bacteria. Furthermore we sought to determine if CCL28 protein interacts with bacteria *in vivo*. Using *in vitro* binding and killing assays we demonstrate that CCL28 kills *Salmonella typhimurium* at both 20 mM and 150 mM NaCl solutions, but much more efficiently in 20 mM conditions. Using a flow cytometry based assay to determine chemokine/bacteria binding, we demonstrated that chemokine binding occurs in both high salt (150 mM) solutions as well as in low salt (20 mM) solutions. Although the percent of bacteria bound by chemokine was identical in the two salt solutions, the intensity of chemokine binding to individual bacteria in the two salt solutions appears to vary, with more CCL28 molecules binding each bacterium in 20 mM salt solution compared to the 150 mM solution. Lastly we demonstrate that endogenously produced CCL28 binds normal flora bacteria *in vivo*. These results demonstrate that CCL28 protein is expressed in the lumen of the murine intestinal tract and that within this physiological environment this chemokine efficiently binds to bacteria.

Materials and Methods

Mice and bacteria

Two week old neonatal mice were used for this study. All mouse studies were performed according to Brigham Young University guidelines for animal use and care. Wide type (LT2, ATCC 15277) and phoP mutant *Salmonella typhimurium* (strain TT16826, stv(LT2) phoP::Tn10$COM rough) were kindly provided by Dr. Julianne Grose (originally from Dr. John Roth, UC Davis). All bacteria were grown in Luria broth (LB) at 37°C with vigorous shaking. *Salmonella typhimurium* containing the pAKgfp1 plasmid (kindly provided by Dr. David Erickson, originally from Addgene, 14076), which constitutively expresses green fluorescent protein were grown in LB medium supplemented with 100 µg/ml of ampicillin.

Killing assay

Subcultures of *S. typhimurium* (*wild type* and *phoP* mutant) were grown to an OD_{600} of 0.6. Bacteria were suspended in 20 mM or 150 mM NaCl supplemented with 10 mM Tris-HCl pH 8.0. Bacteria were then diluted approximately 2000 times to achieve a concentration of ~10,000 bacteria/50ul buffer. Bacteria were diluted in 20 mM or 150 mM solutions containing 0 ng/ µl, 2.5 ng/ µl, 5 ng/ µl, 10 ng/ µl, or 20 ng/ µl, final concentration of CCL28 (Peprotech). Bacteria/chemokine solutions were then incubated for 30 min at 37°C with shaking. Following incubation, the samples were spread on to tryptic soy agar plates. After incubating the plates at 37°C for 12- 20 hrs, bacterial colonies were counted. The percent killing of each solution was determined by using the number of surviving bacteria in the no chemokine control as 100% survival. The number of bacteria that grew in the CCL28 containing assays was divided by the

number of bacteria that grew in the control assays to determine percent survival in different concentrations of NaCl and CCL28.

Flow cytometry

S. typhimurium bacteria were subcultured, from an overnight culture, in TSB medium until an OD_{600} of 0.6 was achieved (~3hours). 100 µl of these this subculture was diluted in 1 ml of 20 mM or 150 mM NaCl solution and then washed by centrifuging the solution (2900Xg) for 5 minutes. The bacteria-containing pellet was then resuspended in 100 µl of the appropriate NaCl solution. Bacteria were then combined with 0.5 µg of recombinant CCL28 (Peprotech) and incubated on ice for 30 minutes. Following this incubation, the cells were washed with ice cold wash buffer consisting of PBS supplemented with 0.5% BSA (Sigma). Following washing, the bacteria were incubated with 1 µg polyclonal biotin labeled anti-CCL28 antibody (R&D Systems, Minneapolis, MN) diluted in wash buffer, for 30 minutes on ice. Cells were then washed and incubated with APC labeled streptavidin conjugate (Becton Dickinson***)*** for 30 minutes followed by a final wash. Stained bacteria were then visualized using flow cytometry. Negative controls consisted of bacteria alone and bacteria incubated with secondary antibody/APC conjugate in the absence of primary antibody.

In vivo binding of chemokine to bacteria

Purification of intestinal bacteria was performed as described previously (Kotarsky, Sitnik et al. 2010). 2~3 week old mice had their cecums and large intestines removed. Intestines were then cut longitudinally and the contents were dispensed into 2ml cold PBS. For baby mice stomach bacteria purification, the stomach was cut open and minced and dissolved in PBS. The

solution was vortexed vigorously for 1 minute and allowed to sediment for another 2 minutes on ice. 1.5 ml of supernatant was then transferred into a 1.7 ml microcentrifuge tube. The solution was spun at maximum speed for 10 seconds in a microcentrifuge tube. The supernatant was then discarded and the bacteria-containing pellet was resuspended in 1 ml cold PBS. The resuspended pellet was centrifuged at 200×g for 3 minutes and this process was repeated twice. Lastly, the clear supernatant was centrifuged at maximum speed for 10 seconds and the pellet was resuspended in 50 µl of PBS. The purified bacteria were subjected to flow cytometry analysis.

Results

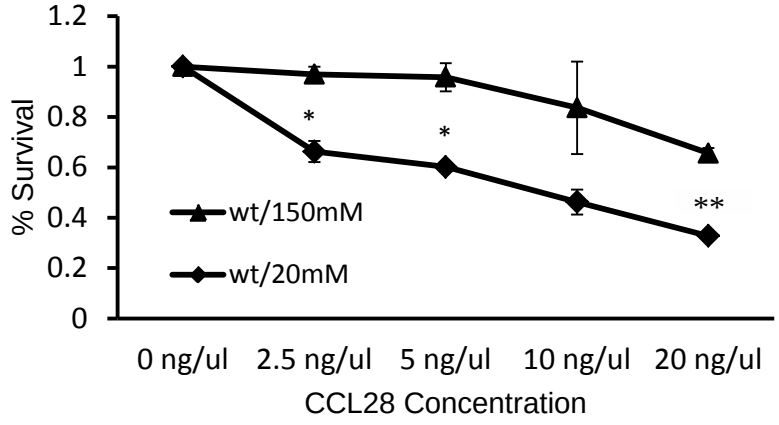
Several types of bacteria are to be susceptible to CCL28-mediated killing in low salt solutions. However, no previous research has tested susceptibility of *Salmonella* to CCL28-mediated killing. We first sought to determine if *Salmonella* was susceptible to CCL28-mediated cell death and to confirm the salt-sensitive nature of this killing. To do this, first, we incubated wild type *Salmonella* with varying concentrations of CCL28 in 20 mM NaCl or 150 mM NaCl and the percent of surviving bacteria was determined as described in Materials and Methods. We observed a statistically significant difference in bacterial death at 20 mM and 150 mM NaCl at most chemokine concentrations tested. Importantly, although bacterial killing was more efficient at 20mM concentrations, significant killing was also seen at 150mM salt concentrations (Figure 1A). *Salmonella* deficient in the phoP gene were previously shown to be highly susceptible to killing by a variety of antimicrobial peptides. However, no previous studies have tested the susceptibility of these mutant bacteria to chemokine mediated cell death, so we next tested the susceptibility of the *Salmonella* phoP mutants to CCL28-mediated cell death. As

shown in figure 1B phoP mutant *Salmonella* were highly sensitive to the chemokine CCL28 at low salt concentrations. At high salt concentrations (150mM NaCl), there were also substantial levels of bacterial cell death at chemokine concentrations of 5ng /µl and above. These studies demonstrate that the phoP mutation renders bacteria highly sensitive to CCL28-mediated killing. Furthermore, although this killing occurs optimally in low salt solutions, killing also occurs at statistically significant levels at high salt concentrations.

Recognition and binding of the bacterial membrane by antimicrobial proteins is an essential first step in effecting bacterial death. We next sought to determine if and to what extent binding of bacteria by CCL28 is influenced by the salt concentration of the solution. As phoP mutant *Salmonella* were shown to be highly sensitive to CCL28 mediated cell death we utilized this strain of bacteria in our subsequent assays. To detect the amount of CCL28 binding to individual bacteria we used a methods similar to that described by Kotarsky et al (Kotarsky, Sitnik et al. 2010). Chemokine binding to bacteria was visualized using flow cytometry as described in Materials and Methods. Flow cytometric analysis allowed the simultaneous determination of both the percentage of bacterial cells bound by chemokine, in varying salt concentrations, as well as the determination of the relative amount of chemokine bound to individual bacteria. As demonstrated in figure 2 A-C a similar percentage of bacterial cells was bound by chemokine in experiments using both 20 mM NaCl and 150 mM NaCl. However, it was also observed that the fluorescence intensity of staining was much higher for the cells stained in 20 mM NaCl than those in 150 mM NaCl. Through averaging the results from 5 independent experiments, it was observed that the intensity of staining on cells bound to chemokine in 20 mM NaCl was ~2.5 times brighter than those cells stained in 150 mM NaCl. These results combined with those in figure 1 demonstrate that the antimicrobial chemokine

CCL28 readily binds bacteria in both high and low salt solutions. However, this interaction and bacteria cell death occurs more efficiently in low salt (20 mM) conditions. These results

A



B

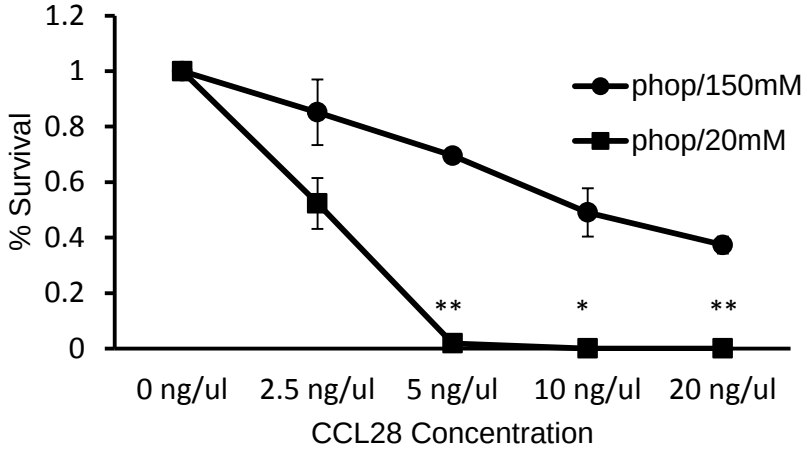


Figure 1. CCL28 kills *Salmonella typhimurium* efficiently in 20 mM NaCl concentrations. The antimicrobial activity of CCL28 in 20 mM and 150 mM NaCl solutions was determined using varying levels of the chemokine as listed on the X-axis. Bacterial survival is plotted on the Y-axis and was determined by performing plate count viability assays and comparing them to the number of bacterial colonies from assays in which no chemokine was added. (A) Results of assays using wild type *S. typhimurium* bacteria. (B) Results of assays using a phoP mutant of *S. typhimurium*. Data shown are means $\pm$ SEM. Graphs are generated from three independent experiments. Statistical differences between percent survival of bacteria in 20 mM and 150 mm NaCl are shown; * indicates $p<0.05$, ** indicates $p<0.01$.

demonstrate a direct correlation between the binding of chemokine to bacteria and the subsequent cell death of bacteria.

CCL28 mRNA is highly expressed at several mucosal sites with particularly high expression levels observed in the large intestine. The large intestine is constitutively colonized by large numbers of bacteria and preventing tissue damage caused by these bacteria is essential. Based on this fact, we next sought to determine if endogenously expressed CCL28 is expressed at high enough levels in this mucosal tissue to efficiently bind to commensal bacteria *in vivo*. In these experiments we collected intestinal contents from two-week-old mice. Using neonatal mice provided a model in which experimental animals were consuming CCL28 through the milk as well as producing it in their large intestines. In these experiments, bacteria were collected from the mice and purified as described in Materials and Methods. Bacteria were stained directly with anti-CCL28 antibodies in an effort to detect endogenously bound chemokine using flow cytometry. Controls consisted of staining cells with anti-CCL17 antibodies, since CCL17 has not been shown to be expressed in the intestinal tract. Results showed that greater than 50% of bacterial cells isolated from the large intestine were bound to CCL28 (Figure 3). These results are the first to demonstrate the direct interaction between commensal bacteria and CCL28 *in vivo*.

Discussion

The use of antimicrobial proteins to protect organisms from microbial attack is an ancient stratagem utilized by a broad array of organisms ranging from plants to humans, with over 1000 AMPs described to date (De Smet and Contreras 2005). Despite the vast numbers of organisms which utilize AMPs and the large number of AMPs identified, surprisingly little is known about

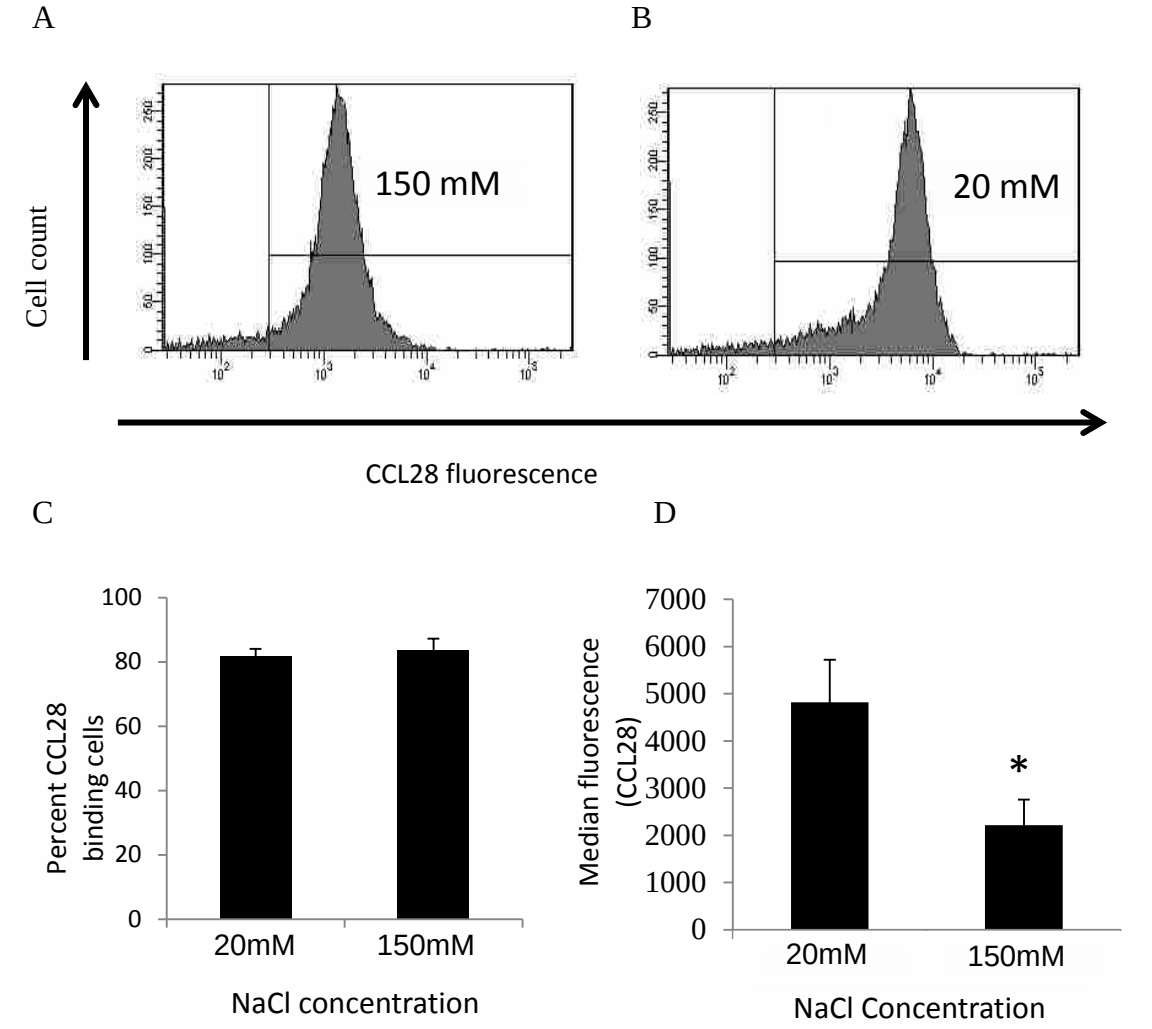


Figure 2. CCL28 efficiently binds *S. typhimurium in vitro*. The ability of CCL28 to interact with and bind to bacteria in high and low salt concentrations was determined using flow cytometry. (A & B) Representative histograms of bacterial cells bound by CCL28 in 150 mM and 20 mM NaCl concentrations respectively. Markers indicating positive and negative staining cells were set using negative controls. The Y-axis represents the cell count of *S. typhimurium*. The X-axis represents the intensity of CCL28 binding to individual bacteria. (C). Percent of bacteria bound by CCL28. Data shown are means $\pm$ SEM. (D) Median fluorescence intensity of bacteria following staining with CCL28 (Y-axis) in 20 mM and 150 mM NaCl solutions (X-axis). * Indicates that differences between median fluorescence staining in 20 mM and 150 mM are statistically significant $p<0.05$. Panel C and panel D represent the combined results of 5 independent experiments.

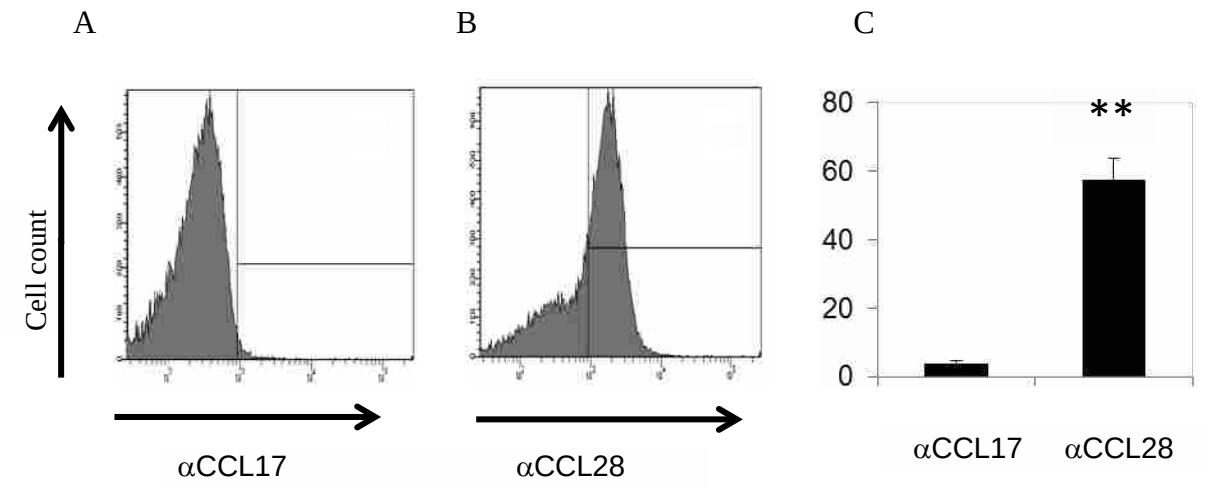


Figure 3. Endogenous CCL28 binds to commensal bacteria *in vivo*. (A & B) Representative histograms showing the percent of bacteria binding to CCL28 *in vivo* was measured through the addition of anti-CCL28 antibody to bacteria collected from the mouse large intestines. Negative controls consisted of treating bacteria with anti-CCL17 antibody. Y-axis indicates the number of bacteria. X-axis indicates the fluorescence intensity of individual bacteria. (C) Combined results from 5 mice. Y-axis represents percent of bacteria stained by the antibody indicated in the X-axis. Data shown are means $\pm$ SEM. ** indicates statistically significant differences $p<0.01$.

the actual antimicrobial role of these proteins *in vivo*. Antimicrobial chemokines constitute a recently described subgroup of dual function AMPs, which means they have antimicrobial characteristics and are also involved in cell chemotaxis and migration (Esche, Stellato et al. 2005). Cationic AMPs, including antimicrobial chemokines, appear to kill microbes in a salt-sensitive manner (Hieshima, Ohtani et al. 2003; Yang, Chen et al. 2003; Linge, Collin et al. 2008). The *in vivo* antimicrobial role of antimicrobial chemokines remains severely understudied.

Previous research has clearly shown that CCL28-mediated bacterial cell death occurs optimally at low salt concentrations (Hieshima, Ohtani et al. 2003). It has been proposed that cationic AMPs are more able to bind bacteria through electrostatic interactions in the absence of excess free ions (Zasloff 2002). However, previous research has not quantified how the binding of chemokines to bacteria is influenced by environmental salt concentrations. Here, we demonstrate by flow cytometry that CCL28 efficiently binds bacteria in both 20mM and 150mM salt concentrations. Although equivalent percentages of bacteria were found to bind CCL28 in both salt concentrations, CCL28 binding per bacterium was significantly enhanced in low ionic strength conditions. Previous research by Linge et al has shown that human CXCL6 binding to *S. aureus* is totally abrogated in 150 mM NaCl (Linge, Collin et al. 2008). This discrepancy could be a result of the bacteria used in our assays or the binding of CXCL6 could be fundamentally different than that of CCL28. The Gram-negative bacteria *S. typhimurium* phoP strain used in our assays is efficiently killed by CCL28 even at 150 mM NaCl solutions. Our results clearly demonstrate that CCL28 binds bacteria more efficiently at low salt concentrations.

Enhanced CCL28 binding in low salt solutions may be a result of the bacteria swelling under the osmotic stress of the low salt solution which may then expose more chemokine binding sites on each bacterium. The swelling of bacteria under low osmotic conditions may also

accelerate bacterial membrane rupture and cell death. Alternatively, it is possible that increased numbers of positively and negatively charged ions in high salt buffers interfere with the electrostatic attraction of chemokines to their targets as previously hypothesized. This data suggests that although CCL28 binding to bacteria occurs over a broad range of salt concentrations efficient bacterial cell death occurs preferentially once a threshold of CCL28 binding has been achieved.

It has been hypothesized that physiologically normal salt concentrations (150mM) will decrease efficient chemokine killing in the blood stream because chemokines will not be able to bind to bacteria efficiently. This is supported by our results and the results from Linge et al (Linge, Collin et al. 2008). Such findings may cast doubt on the physiological relevance of such salt sensitive proteins in the blood stream. However, the use of gene deficient animals has shown *in vitro* that the killing of some defensins is salt-sensitive using *in vivo* assays. It is clear that these proteins play an essential role in protecting the host from microbial challenge (Wilson, Ouellette et al. 1999; Morrison, Kilanowski et al. 2002; Salzman, Ghosh et al. 2003; Chromek, Slamova et al. 2006). These observations could be a result of these proteins being active only in low salt microenvironments within the body. Conversely, these results could reflect low levels of binding under *in vivo* conditions which, when combined with other components of the innate immune system, may play a significant role in the clearance of invading microbes. It is clear from the high levels of CCL28 binding observed in the murine large intestine that appropriate conditions exist in this tissue to support high levels CCL28 binding to a large population of commensal bacteria. Similar results have been observed for small intestine commensal bacteria (data not shown). These results are the first evidence that endogenously produced CCL28 binds to bacteria *in vivo*. However, it is still unclear how much chemokine must bind bacteria to effect

CCL28-mediated bacterial cell death *in vivo*, and whether this threshold is reached under normal *in vivo* conditions in the murine colon. It is likely that the *in vivo* activity of CCL28, and many other antimicrobial peptides, does not solely depend not on a single protein but rather on the combined effects of various components of the innate immune system working in concert.

Chapter 4: Exploring CCL28 in vivo antimicrobial activity through the generating of a CCL28 knockout mouse model

Introduction

Since identification of their antimicrobial activity, it has been proposed that some mucosally expressed antimicrobial chemokines play a role in direct innate immune defense. This proposal was made based on indirect research experiments. For example, some antimicrobial chemokines are highly expressed on mucosal tissues and this expression can be further increased after contact with bacteria or their components, such as CCL6, CXCL9, CCL20 (Hieshima, Ohtani et al. 2003; Starner, Barker et al. 2003; Egesten, Eliasson et al. 2007; Maerki, Meuter et al. 2009; Kotarsky, Sitnik et al. 2010). More supporting evidences came from studies in which neutralizing antibody was administered to pulmonary expressed chemokines, such as CXCL9, CXCL10, and CXCL11 (Crawford, Burdick et al. 2010). However, the *in vivo* antimicrobial function of most of the antimicrobial chemokines in innate immune protection still remains unclear.

Definitive evidence is necessary to understand the *in vivo* antimicrobial role played by chemokines. The direct *in vivo* antimicrobial function of mouse α-defensin, β-defensin-1, human β-defensin-5, and LL-37 has been demonstrated using knockout/in mouse models (Wilson, Ouellette et al. 1999; Morrison, Kilanowski et al. 2002; Salzman, Ghosh et al. 2003; Chromek, Slamova et al. 2006). Since mouse gene knockout techniques are not as formidable as before, establishing a CCL28 knock-out mouse model may be a good way to demonstrate its antimicrobial role *in vivo*.

CCL28 is a good antimicrobial chemokine to use for demonstrating chemokine antimicrobial function *in vivo* by a knockout mouse model. Namely, CCL28 is highly expressed in a variety of mucosal tissues. Mucosal tissues are constantly challenged by different microbes. If CCL28 plays a role in defending against microbial invasion *in vivo*, it may be effectively demonstrated through a knockout mouse model. However, like most other chemokines, CCL28 is bi-functional. A simple CCL28knockout mouse model would be both deficient in CCL28 chemotactic and antimicrobial function. The ability of CCL28 to attract B cells and T cells is important in the host's ability to defend against microbial invasion. A simple knockout will not work in this case. However, there is another chemokine, CCL27 that uses the same chemokine receptor as CCL28 (CCR10), and according to previous research is not antimicrobial. Therefore driving CCL27 expression under the control of the CCL28 promoter should provide efficient recruitment of CCR10 bearing cells yet not provide any direct antimicrobial activity at mucosal sites.

If successfully generated, a mouse model could be used to evaluate the antimicrobial activity of CCL28 on the bacteria burden and mouse mortality following challenge with pathogenic or on commensal bacterial. I have worked extensively to generate such a mouse model. Although successful generation of transgenic mice has not yet been achieved, I have successfully constructed targeting vectors and ES cell lines. The following section will document my approach and methods.

Material and Method

Targeting vector construction

A CCL28 targeting vector was constructed using a recombineering (recombination-mediated genetic engineering) method according to the paper published by Wu et al (2008). In this method, a low copy number plasmid (pKD46) encoding lambda phage Red-recombination proteins alpha, beta, and gamma mediates the homologous recombination in *E. coli*. In this system, a short homologous arm (~50bp) is enough for high efficiency recombination.

The BAC clone (WI1-1632B20) containing the whole genomic DNA sequence for CCL28 was ordered from BACPAC Resource Center. The CCL28 genomic sequence has 3 exons; the first one encodes for the signal peptide. The strategy utilized in this work was to replace the second exon of CCL28 with the coding sequence of CCL27, as shown in the following figure.

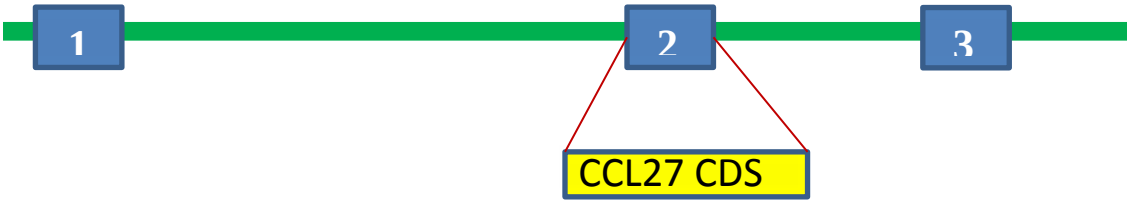


Figure 1. Strategy to make a gene swap between exon 2 of the CCL28 gene and the CCL27 coding sequencing without the signal sequence but with a stop code.

Vector construction was performed as described in detail by Wu et al (2008). A brief description of the methods used is described below.

Capturing the CCL28 gene from its BAC vector

A homologous arm was added to each end of the linear plasmid pSTART-K by PCR and the linear plasmid was then used to capture the ccl28 gene by homologous recombination.

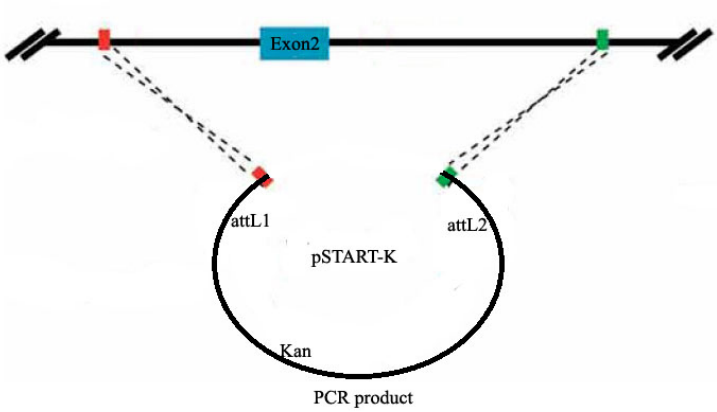


Figure 2. The homologous recombination between the ccl28 gene and the plasmid. This will result in the insertion of the ccl28 gene into the plasmid. This picture was modified from Wu, et al., 2008.

The upstream and downstream primers are,

Bac-F forward:

GGTGAAAATGAATGGTTTAGAGTGGATGAGGGCAGTGTCAAGATTCTATTCGACTG

AATTGGTTCCTTTAAAGC

Bac-R reverse:

TGCACTGTGAGGGTGAACTGGTTTTGTTGGATGAAGTTTAAACATGGAAGGCCGCAC

TCGAGATATCTAGACCCA

Capture of the gene was confirmed by sequencing the recombination junctions through the use of the following sequencing primers,

Pst-B-R5 reverse: CCAAGCAACTCTCCATCATGACACG

Pst-B-F5 forward: GTTAGTTCCCACTATGCATAAGGGC

PCR conditions were set as follows: 1µl of 10µM of each of forward and reverse primer, 1 µl of 50 ng/µl pStart-K plasmid, 2 µl of dNTP (2.5µM each), 2.5 µl PLATINUM pfx50 DNA polymerase buffer and 0.5 µl polymerase (Invitrogen) in a total volume of 25 µl. PCR product

was then gel purified. Competent BAC strain was transformed with plasmid pKD46, which encodes for the recombinase. The pKD46 positive BAC strain was electroporated with 500 ng of the above purified PCR product. Colonies grown on kanamycin selection plates were picked up and then were grown in LB medium for plasmid extraction. Plasmid DNA samples were run on agarose gel and those of the right size were chosen for sequencing to further confirm that they were from the desired homologous recombination.

Generation of CCL27-chloramphenicol fusion gene for replacement into the CCL28 exon 2 by homologous recombination

First, the chloramphenicol gene was amplified by PCR. An AscI restriction site was added to each end and a homologous arm was added to the C-terminus of the chloramphenicol gene through use of the following primers.

Cat F:

CTCAAACCCCCAACAGCAAAACTAATAGGGCGCGCCCACTTAACGGCTGACATGGGAATTA

Cat R:

GGGGATAATCCGCATTCGGTTTCCCATGTCCTTGCTGAGGGACACTCACATGGCGCGCCAGCATTACACGTCTTGAGCGATTGT

Second, the ccl27 gene was amplified by PCR. A homologous arm was added to the N-terminus of ccl27 through the use of following primers:

CCL27 F:

AGGCTGGTACTCTGCGGACGGTAATGCAAGCTCTCCCCTTTCTTGTAGCCTTGCCTCTGCCCTCCAGCACTAGCTGCTGT

CCL27 R:

TAATTCCCATGTCAGCCGTTAAGTGGGCGCGCCCTATTAGTTTTGCTGTTGGGGGTTT

GAG

Third, CCL27 F and Cat R primers were used to make the fusion gene of ccl27 - chloramphenicol.

Finally, the fusion gene was then used to replace exon 2 of the ccl28 gene by homologous recombination. Successful recombination colonies were selected on chloramphenicol selection plates. Four colonies were picked and grown. Plasmids were extracted from those colonies and the junctions were sequenced to confirm that the right recombination events had occurred.

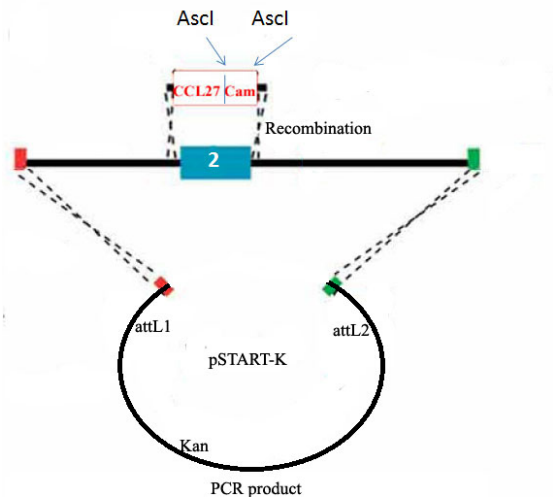


Figure 3. The homologous recombination for replacement of exon 2 by ccl27- chloramphenicol fusion gene. The plasmid pStart-k carrying ccl28 gene was generated from a previous step. The chloramphenicol gene has an AscI site on each end, which will be used for the next step. This picture was modified from Wu, et al., 2008.

Insertion of positive selection marker---Ligate GFP-LoxP-Cre-Neo cassette with CCL27-pStart-K plasmid

A positive selection marker is required to screen out the ES cells which have the target vector recombined. Neomycin is used as the positive selection marker. For convenience, the plasmid pEGFPpA-ACN containing EGFP-loxp-Ace-Cre-Neo-loxP was used. The plasmid was digested by AscI and ligated into AscI digested pStart-K plasmid from the previous step. Ligated plasmid was transformed into *E. coli* and grown on kanamycin selection plates. Plasmids from 8 colonies were extracted and the junctions were sequenced to confirm that the right insertion of the Neo-cassette was present.

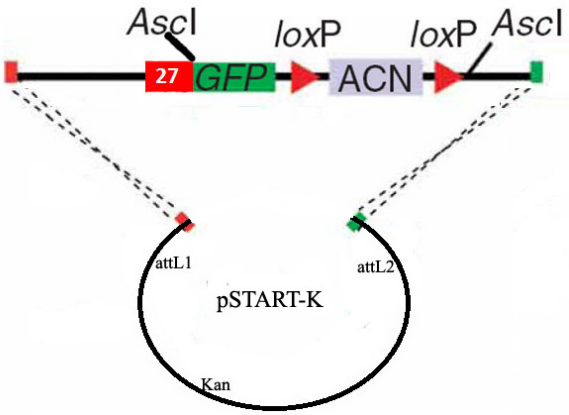


Figure 4. The insertion of a Neo-cassette after the ccl27 gene into the pStart-k plasmid by cut and paste using the AscI restriction sites. This picture was modified from Wu, et al., 2008.

Insertion of a negative selective marker by gate-way recombination

Negative selection can greatly increase the efficiency of the screening process by eliminating those colonies resulting from random insertion. Herpes simplex virus thymidine kinase was used as a negative selection marker. Plasmid pWS-TK6, which contains the negative selection marker, was kindly provided by the Capecchi lab (University of Utah). The gateway

recombination kit was purchased from Invitrogen. Following the kit instructions, incubation of the pStart-K plasmid with pWS-TK6 resulted in a gene swap between attL1/attL2 and attR1/attR2. Successful recombination colonies were selected on ampicillin-containing plates. Colonies were then retrieved and a large quantity of plasmid (~500 µg) was prepared by using a QIAGEN plasmid Midi kit.

Gene targeting in mouse ES cells by electroporation

In conjunction with the laboratory of Dr. Jeffery Barrow, two tubes of prepared ES cells at a concentration of 1.3E7/0.8 ml were freshly grown. An aliquot of 30 µg of FseI digested targeting vector and 0.8 ml of cells were added to a 4mm cuvette and mixed well. Electroporation was performed at 220 v, 500 µF. A control cell without DNA was also performed. Electroporated cells were grown and screened by using the positive and negative selection drugs as described in the protocol (Wu, 2008). 96 colonies were picked up and grown on two 48-well plates. Replicate plates were made. One was frozen at -80°C and the other was used for genomic DNA extraction to find out which cell lines came from true recombination. ES cell genomic DNA was then extracted as previously described (Wu, 2008).

Screening the ES cell clones by Southern blot and PCR

As described in the Wu protocol, southern blotting was performed to screen the knock-out ES cells. Non-radioactive Digoxigenin-labeled probes were used. The template for the left and right probes was PCR amplified and then labeled using a Roche Dig-DNA labeling and detection kit (Roche Applied Science). The templates are about 1kb. The primers used for left probe is: Forward-GGCTTTGCCTCCTTAAAGTTCTA, Reverse-

CCACTCCACTGCTTGCTTTGATTTCAGTC. Primers for right probes are: Forward-GACTTTACCTGGGTCTGTTACTGG, Reverse-CTCCTCTGTAGAGCTGGAAAACTTG. Genomic DNA was digested with BsrG1. Successfully targeted loci should contain an additional BsrG1 site. Wild type DNA produces a 12 kb band with either the left or right probe. Mutants with the homologous recombination produce three bands: a wild-type genotype band of 12 kb is seen and 7.2 kb band for the left probe and 10.5 kb band for the right probe will exist if homologous recombination has occurred in the correct place.

At the same time, screening for the correct insertion was also done through PCR. A Roche long template PCR kit was used. The PCR reaction was set up and the Mg^{2+} concentration varied from 0.8 mM ~1.6 mM as described in the manual (Roche applied science). Two pairs of primers were used for each arm. Primers for the left arm were: Forward 1-CAACTCCCACAAAACAGCTCCACCC, Forward 2-GGGTACCAAGTGTCCAAATCCCCG, Reverse 1- CTGGGTGCTCAGGTAGTGGTTGTCG, Reverse 2-CGATGGGGGTGTTCTGCTGGTAGTG. Primers for the right arm were: Forward 1-GACGTAAACGGCCACAAGTTCAGCG, Forward 2-CTACGTCCAGGAGCGCACCATCTTC, Reverse 1- GCGGGCTGATGCAGATTCTTCTACG, Reverse 2- GTGCCCTTTTCTGTCCTTCCTGCTG.

Morulae aggregation and embryo implantation

Following confirmation of appropriate recombination events via southern blot or PCR, cell lines were thawed and cultured in ES medium until they were ready for aggregation. Superovulation female mice were mated with male mice and morulaes were collected 2.5 days thereafter by cutting and flushing the oviducts. Each morula was placed into a divot in a petri

dish. Clusters of 4-12 ES cells were added to each divot. A total of 50-100 good morulaes were used. After a few hours of incubation, the merged morulae (checked for aggregation by dissecting microscope), were surgically implanted into the uterine horns of the pseudo-impregnated female, which was induced by mating with vasectomized males.

Chimeric, heterozygous, and homozygous mice generation

According to the protocol we followed, after the chimeric mice are born and reach sexual maturity, the males mate with females to produce germline heterozygous pups. The heterozygous mice would then be crossed to produce homozygous knockout mice.

Genotyping

PCR assays were to be used to genotype the knockout mice. Primers that used to identify WT mice are, F-Wild, ATTCTAGAAGGTGGAGACACGTCC. R-Wild, ACTCCAGGTAGCCTGGTGCTCAAAGG. Primers for knockout mice identification are, F-Wild (the same as wild type). R-KO, TGCGCTCCTGGACGTAGCCTTC. Wild type mice show amplification of a 450 bp size band. For knockouts, a 760 bp size band is amplified.

Results

Targeting vector construction

Retrieving ccl28 gene

Part of ccl28 gene including exon 2, was pulled down from BAC Fosmid by recombination as described in the Methods. The left homologous arm is 5.2 kb and the right

homologous arm is 4.5 kb. The plasmid pStart-K used for homologous recombination is 2.7 kb. A plasmid with a size of 12.4 kb is expected after extraction of the plasmids from the colonies grown on kanamycin selective plates. Two plasmids were chosen to run in the gel (Figure 5). The junction regions of the plasmids were also sequenced for further confirmation that the correct sequence was obtained.

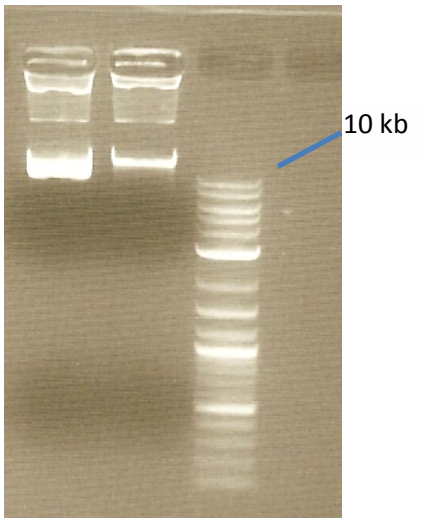


Figure 5. Gel electrophoresis of plasmids which captured ccl28 through homologous recombination. The left two lanes are plasmids extracted from two different colonies. The third lane is the DNA ladder (BioLabs 2-log DNA ladder).

Replacing ccl28 exon 2 with ccl27-chloramphenicol

CCL27-chloramphenicol fusion gene was made by PCR as described in the Methods. Homologous arms (50bp) identical to the border regions of CCL28 exon 2 were added to the ends of the fusion gene. Colonies grown on chloramphenicol selective plates were picked and grown and their plasmids were extracted and run on a gel (figure 6). Plasmids with the right

recombination are 13kb. Recombination junction regions were also sequenced to confirm that the correct sequence was obtained.

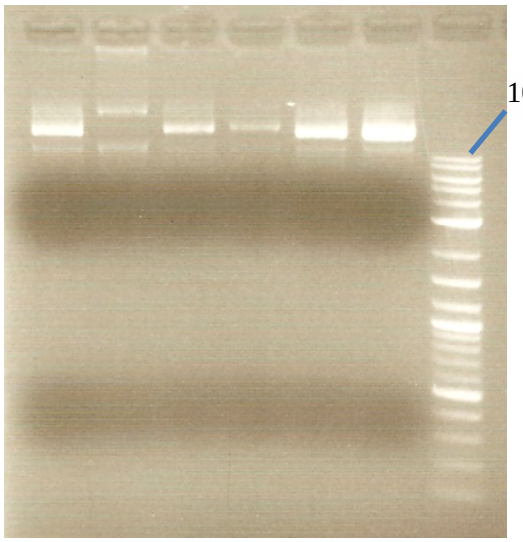


Figure 6. Gel electrophoresis of plasmid DNA after replacement of ccl28 exon 2 with ccl27 through homologous recombination. The right-most lane is the DNA ladder (BioLabs 2-log DNA ladder), and all the other lanes are plasmids extracted from different colonies.

Insertion of the positive selection marker

Insertion of the positive the selection marker into the constructor was done by the cut-paste method. Plasmid pEGPpA-ACN and pStart-K with ccl27 were cut with AscI. EGFP fragment of the plasmid pEGFPpA-ACN was inserted into plasmid pStart-K by ligation. The plasmid was then transformed into *E. coli* and grown on kanamycin selection plates. Colony PCR was done to select for the plasmids that had the insertion in the right orientation. As shown in the following figure, the samples showing a band have the insertion with the right orientation. These plasmids were also confirmed by sequencing of the ligation junctions.

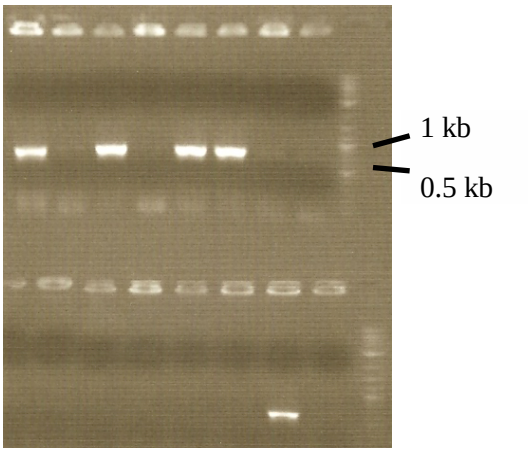


Figure 7. Gel electrophoresis of the colony PCR products to confirm the insertion of the positive selection marker. The right-most lanes are DNA ladders (BioLabs 2-log DNA ladder), and all the other lanes show PCR amplification results using plasmid templates extracted from different colonies.

Insertion of the negative selection marker

The negative selection marker was inserted by gateway recombination. Successful recombination colonies were selected on ampicillin LB plates. Plasmids were extracted. Four colonies were picked up and plasmids were extracted. As shown in figure 8, all picked colonies had the correct recombination. The junctions were confirmed by sequencing.

Screening out the ES cells with the homologous recombination

Genomic DNA extracted from the ES cell colonies was subjected to southern blot and PCR to identify the ES cells with the correct homologous recombination event. Non-radioactive probes for southern blot did not work well. However, the PCR screening method consistently worked. Figure 9A shows the PCR amplification products of the wild type ES cell DNA from two different PCR conditions. The first condition was chosen as the PCR condition to be used for

all the other samples. 18 ES cell colonies were confirmed by PCR to have the right homologous recombination event, as shown in Figure 9 B and C, represented by those lanes having the amplification band.

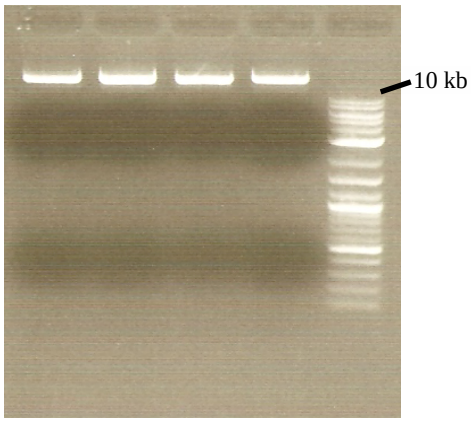


Figure 8. Gel electrophoresis of plasmid DNA after gateway recombination to detect the insertion of the negative selection marker. The right-most lane is the DNA ladder (BioLabs 2-log DNA ladder), and the other lanes are plasmids extracted from different colonies.

Morulae aggregation and chimera production

This project was aimed at developing a mouse line that would express no endogenous CCL28 but rather express the non-antimicrobial CCL27 under control of the CCL28 promoter. This would theoretically have resulted in a mouse in which the CCR10 receptor-bearing lymphocytes would traffic normally to all tissues but have none of the CCL28-mediated antimicrobial activity. Such a mouse would be a powerful tool in understanding the role of CCL28 as a component of the innate immune system. Unfortunately, although the construction of the constructs and ES cell knockout worked perfectly, morulae implanted pseudo-impregnated

females have not produced any chimeric pups. We feel it is beyond our current capabilities to complete this project without extensive corporation with another lab.

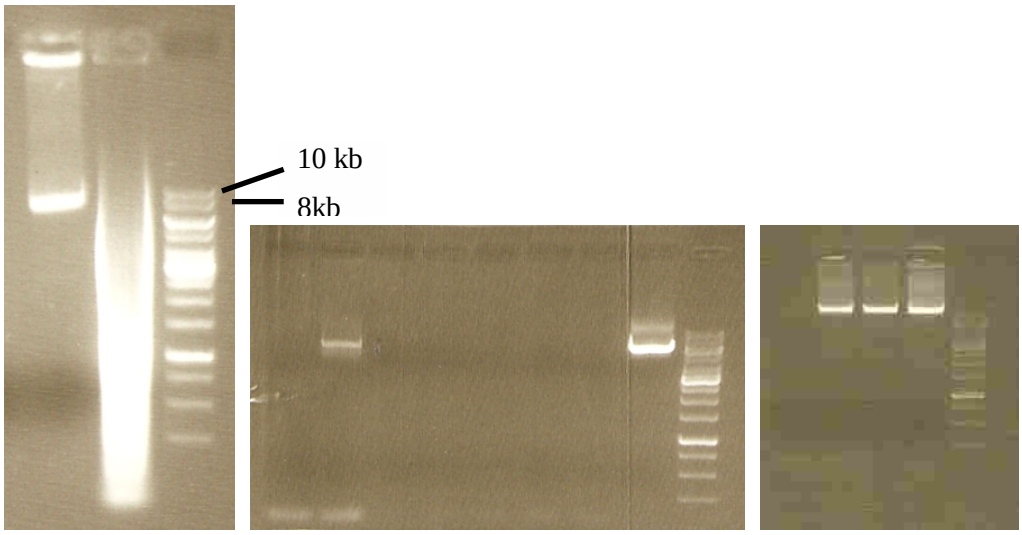


Figure 9. Gel electrophoresis of the PCR products for confirmation of the right homologous recombination ES cells. The right-most lanes of all the panels are loaded with DNA ladder (BioLabs 2-log DNA ladder). A, WT ES cell DNA was used to for long PCR amplification. The left-most lane represents the condition (1.6 mM Mg^{2+} concentration) that amplified the long target fragment successfully, while the middle lane did not (0.8 mM Mg^{2+} concentration). Panels B and C are representatives of PCR amplification results through the use of left border primer or the right border primer, respectively.

Chapter 5: Discussion and conclusions

This research project focused on the study of the antimicrobial activity of the chemokine CCL28. The structure-function relationship, killing mechanism and *in vivo* binding of the chemokine have been all explored in this study. This work has improved our understanding about the antimicrobial mechanism of chemokines and their contribution to direct innate immune protection.

The first goal of this project was to explore the antimicrobial determinants of CCL28 at the amino acid sequence level. Using protein mutation and recombination techniques, we have compared the antimicrobial activity of different mutants of CCL28 protein. Our findings indicate that C-terminal positively-charged amino acids are the major functional elements for the antimicrobial activity of CCL28, although full length protein is optimal for the maximum antimicrobial activity. Based on the result that full activity is only achieved when the full length sequence is used, it would be interesting to further investigate the role of the N-terminus in the antimicrobial activity of the protein. Hydrophobic amino acids have been considered important players in helping AMPs insert into the bacterial membrane. In the future, the significance of those hydrophobic amino acids should also be examined.

Although we have shown that disruption of the disulfide bonds does not affect the antimicrobial activity of CCL28, obtaining its tertiary structure might be required to elucidate the structure-function relationship in CCL28 and further provide guidance to generate specific mutants for study. As CCL28 is a relatively small protein, it should not be difficult to determine its tertiary structure. This could be an important research direction in the future. Importantly, some AMPs that are found to be unstructured in aqueous state, are found to be structured only

after interaction with an organic state (Mani, Cady et al. 2006; Campagna, Saint et al. 2007; Lourenzoni, Namba et al. 2007). Determining CCL28 structure, as well as its predicted structure when interacting with a bacterial membrane, could be another important avenue for future research.

The structure-activity analysis in this study contributes to the understanding of the antimicrobial mechanism of CCL28. The antimicrobial mechanism for other conventional cationic AMPs has been proposed to be charge-mediated, in which the positively charged AMPs and the negatively charged microbial membranes interact with each other. We have provided evidences that the chemokine CCL28 likely uses a similar mechanism for its antimicrobial activity. This conclusion is based on several independent observations. First, by using confocal fluorescent microscopy, I have observed that CCL28 binds to *S. typhimurium* phoP bacteria, but the highly similar, non-antimicrobial, chemokine CCL27 does not bind bacteria (data not shown). The CCL27 chemokine has been shown to lack the extended C-terminus of CCL28, which is highly positively charged. Second, mutant bacterial strains, such as phoP *S. typhimurium* and *Y. pestis* strains, which don't have the ability to reduce the negative charges on their membranes, are more sensitive than their wild type parent strains to CCL28-mediated cell death. Third, CCL28 binds and kills phoP *S. typhimurium* better in low salt concentrations than in high salt conditions. These three observations all support the conclusion that CCL28 shares many similarities with other better studied conventional AMPs.

It has generally been accepted that the antimicrobial activity of AMPs is receptor-independent; however, there is evidence suggesting that specific microbial proteins, including antimicrobial chemokines, could be actively involved in the process of AMP bacteria recognition (Li, Sun et al. 2006; Crawford, Lowe et al. 2011). It is quite possible that in addition to simply

recognizing the negatively charged phospholipids of the bacterial membrane, other bacterial components may be involved in AMP recognition of microbes. Our results provide evidence to support this proposition. For example, we have shown, by counting the number of bacteria bound by CCL28 and the level of CCL28 binding on each bacterium, that high salt concentrations decreased the intensity of CCL28 binding to bacteria but not the percentage of cells bound. The charge could mediate the initial binding of chemokine to bacteria, but when bacterial cells have swelled in the low salt solution, it may result in increased CCL28 binding to the membrane. Swelling of bacterial cells may also expose their other membrane proteins which may interact with CCL28. This interaction between CCL28 and bacteria could be specific for the antimicrobial chemokine CCL28, but not for the non-antimicrobial chemokine CCL27, since we have observed that CCL27 does not bind to bacteria (data not shown). Additionally, bacteria killing by AMPs in low salt conditions may be augmented by osmosis-assisted rupture, since we have observed that there was more GFP leakage when bacteria were treated with CCL28 under low salt concentration (data not shown).

To definitively understand the antimicrobial role of CCL28 *in vivo* it may be necessary to generate a transgenic mouse model. In our attempts to do this we have successfully constructed an appropriate vector and knockout ES cells have been successfully generated. However, the subsequent generation of chimeric mice didn't work for our collaborators. In the near future, the construct will be sent to commercial knockout mouse center to obtain chimeric mice. It is anticipated that in the future the use of such a model, where CCL27 is substituted for CCL28, could provide definitive answers to the in *vivo* role of CCL28 in modulating bacterial numbers or communities. In an effort to circumvent the challenges of constructing a transgenic mouse line we also explored the idea of using antibody-mediated chemokine inactivation to examine the role

of CCL28 *in vivo*. These studies confirmed that through the addition of exogenous CCL28 it was possible to increase binding of CCL28 on commensal bacteria as well as S*almonella* bacteria. We then confirmed that endogenously produced CCL28 efficiently bound to commensal bacteria. Finally, in an effort to determine if these levels of binding resulted in *in vivo* bacterial cell death we tried to block chemokine activity by feeding polyclonal antibody to mice and then tracking *Salmonella* numbers in the animal. Although these experiments resulted in findings which were suggestive of an *in vivo* role for the chemokine in protecting the stomach from *Salmonella* colonization there was a high degree of variability resulting in no statistical differences in these experiments.

In summary, in the course of this work I have conducted experiments in which I have constructed a large panel of mutant versions of the chemokine CCL28. Using these mutants I have demonstrated that positively-charged amino acids in the C-terminal end of the chemokine play an indispensable role in mediating microbial cell death in both Gram-positive and Gram-negative bacteria. This study, published in the *European Journal of Immunology*, represents the first molecular dissection of the antimicrobial activity of any chemokine. In addition, my work has shown that CCL28 binds bacteria in both high and low salt concentrations *in vitro* with most efficient binding occurring at 20 mM NaCl concentrations. Furthermore, I demonstrated that CCL28 produced within the murine gastrointestinal tract is at high enough concentrations to efficiently bind commensal bacteria within the murine large intestine. All of these studies suggest that CCL28 may play an important *in vivo* role in controlling bacterial numbers at mucosal surfaces. In an effort to definitively determine if CCL28 serves an important role *in vivo*, I have designed and constructed a molecular construct to make a transgenic mouse which would express the non-antimicrobial CCL27 under the promoter of the antimicrobial CCL28

gene. Such a transgenic animal would theoretically traffic CCL10 bearing lymphocytes effectively to mucosal surfaces but not play a direct role in innate immune protection of the animal, making it an ideal model to study the antimicrobial properties of CCL28 *in vivo*. Although this project is not yet completed I have generated ES cells which can be used to generate chimeric mice in the future.

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