



Antimicrobial chemokines

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Chemokines are best known for their classic leukocyte chemotactic activity, which is critical for directing the immune response to sites of infection and injury. However, recent studies have suggested that at least some chemokines may also interfere with infectious agents directly. Antimicrobial chemokines tend to contain amphipathic alpha helical secondary structure, and broad-spectrum activity against both Gram-positive and Gram-negative bacteria, as well as fungi. Conversely, several bacteria have been identified that possess mechanisms for specifically blocking the antimicrobial activities of chemokines. Although the precise mechanisms by which chemokines and microbes disarm one another *in vitro* remain unknown, there is now emerging evidence *in vivo* that such interactions may be biologically significant. More research will be needed to determine whether chemokines with direct antimicrobial activity may be translated into a novel class of antibiotics.

Keywords: chemoattractant, immunology, G protein-coupled receptor, mucosa, microbiome

INTRODUCTION

Chemokines comprise a family of phylogenetically related, small proteins whose main shared function is to recruit leukocytes to sites of inflammation and infection (Murphy, 2008). In addition, some chemokines are important for tissue repair, organ development, and cancer. Many chemokines have been shown to have direct antimicrobial properties *in vitro*, however the *in vivo* significance of this has not been established.

Chemokines are classified according to the number and arrangement of conserved cysteine residues (Murphy et al., 2000). In humans, all but two chemokines contain four conserved cysteine residues with disulfide bonds linking C1–C3 and C2–C4 (Figure 1). The first two cysteines are adjacent (CC motif, $n = 24$), separated by one amino acid (CXC motif, $n = 17$), or separated by three amino acids (CX3C motif, $n = 1$). The XC chemokines ($n = 2$) have only two cysteines. Sequence identity is $<30\%$ between members of different classes, but within the same class, sequence identity ranges from ~ 30 to 99% within species. Most chemokines are cationic and ~ 7 – 12 kDa in molecular weight.

Although some chemokines are constitutively expressed, the majority are induced under inflammatory conditions, often in response to pleiotropic cytokines. Inducible chemokines are involved in host defense (both innate and adaptive immunity), and in acute and chronic inflammation. There are chemokines specialized for recruitment of neutrophils (CXCL1, CXCL2, CXCL8), monocytes (CCL2, CCL7, CCL13), Th₁ cells (CXCL9, CXCL10, CXCL11), Th₂ cells (CCL17 and CCL22), Th₁₇ cells (CCL20), and other immune cells to sites of trauma, ischemia, and infection (Olson and Ley, 2002; Singh et al., 2008). During the course of an infection, pathogens may be exposed to many chemokines.

HISTORY OF ANTIMICROBIAL CHEMOKINES

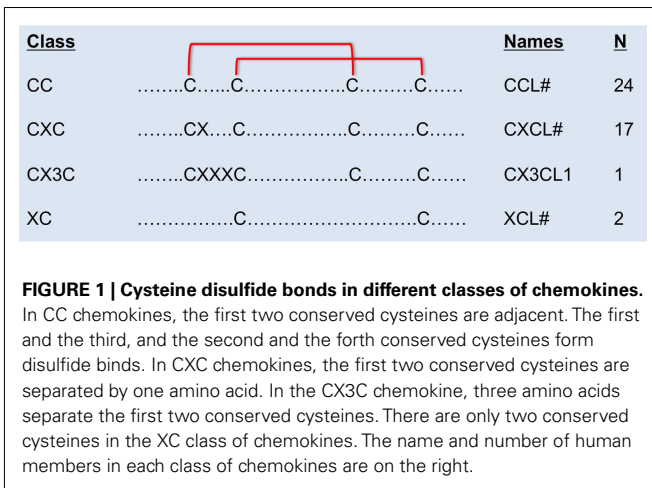
Antimicrobial activity was discovered for chemokines in the year 2000 with the identification of truncated forms of CXCL7 as

platelet microbicidal proteins (Krijgsveld et al., 2000). Blood platelets have been known to release antibacterial proteins upon thrombin activation *in vitro* (Yeaman, 1997). These antimicrobial proteins, designated as thrombocidins, had both antibacterial and antifungal properties and were identified as C-terminal deletion products of CXCL7 by protein sequencing. Within the next several years, other investigators discovered additional chemokines (CXCL9, CXCL10, CXCL11, CXCL6, CXCL14, CCL20, and CCL28) that had antimicrobial properties (Cole et al., 2001; Hieshima et al., 2003; Yang et al., 2003; Linge et al., 2008; Maerki et al., 2009). Since these chemokines can be induced during inflammatory conditions, they may act as the first line of defense against pathogens.

ANTIMICROBIAL ASSAY

Antimicrobial effects of chemokines were discovered *in vitro* with a gel overlay assay and quantified by microdilution comparison, or radial diffusion methods (Krijgsveld et al., 2000; Cole et al., 2001; Yang et al., 2003). In the gel overlay assay, a lysate with known antimicrobial properties is separated by SDS-PAGE. Next, the pathogen to be tested is resuspended in nutrient poor agar and poured onto a tissue culture plate (Figure 2). Then, a portion of the SDS-PAGE gel is cut and placed on top for several hours to allow transfer of proteins from gel to agar. The gel is removed and a nutrient rich agar is placed on top. After overnight incubation, band areas without pathogen growth represent the location of antimicrobial proteins.

For quantitative analysis, microdilution comparison and radial diffusion methods have been used. For microdilution comparison, a known amount of a pathogen is incubated with increasing concentration of antimicrobial chemokine in a nutrient poor broth. After several hours, the number of viable organisms is determined by serial dilution on nutrient rich agar plates. Percent inhibition is determined by the difference of viable organisms between



chemokine treated and untreated samples using the formula: $[(w/o \text{ chemokine}) - (w/\text{chemokine})] / (w/o \text{ chemokine}) \times 100$.

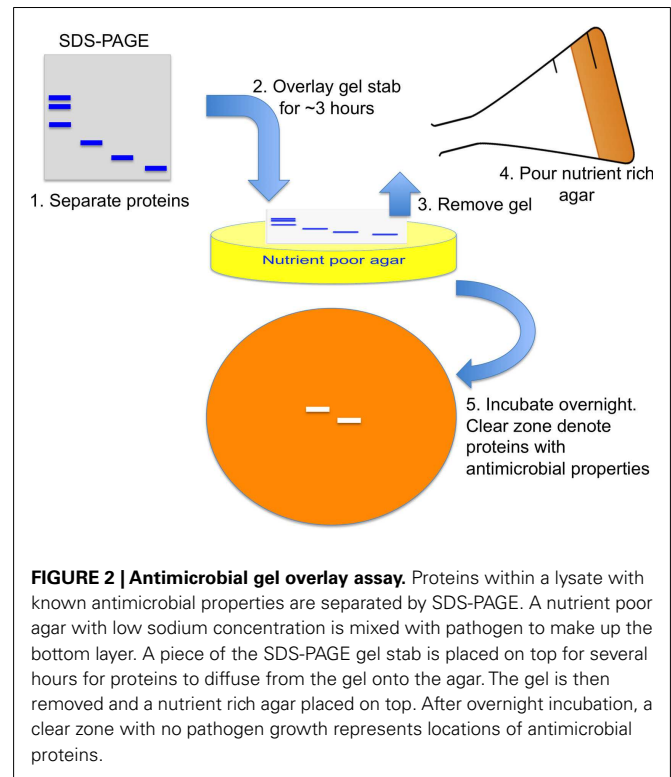
In the radial diffusion assay, organisms are mixed into nutrient poor agar. Then, 3 mm diameter wells are punched out of the agar. Next, a known concentration of chemokine is added to the wells and incubated for several hours to allow protein diffusion. Finally, a media rich agar is overlay on top. After overnight incubation, the diameters of clear zone surrounding the wells are measured and used to calculate the potency of the chemokine against the test organism.

SALT DEPENDENCE OF CHEMOKINE ANTIMICROBIAL ACTIVITIES

The nutrient poor agar and broth, described above, all contained low ionic concentrations, with most assays having 10 mM of Na^+ . As the Na^+ concentration is increased to 100 mM, the antimicrobial activity of chemokines diminishes or even disappears. **Table 1** lists the reported electrolyte concentration of different fluid compartments in humans. In particular, sweat and mucosal secretions, such as saliva, have low ionic conditions that would favor antimicrobial activities. Therefore knowing whether antimicrobial chemokines are secreted into these fluids is important. Indeed, several chemokines have been reported in sweat (CXCL8 and CCL2) and in saliva (CXCL8; Jones et al., 1995; Yang et al., 2005). Theoretically, chemokines may inhibit growth of organisms on the skin and mucosal surfaces, provided they are in high enough concentrations for antimicrobial activity.

CONCENTRATION OF CHEMOKINES NEEDED FOR ANTIMICROBIAL ACTIVITY

For most antimicrobial chemokines, micromolar concentrations are needed for pathogen killing (Krijgsveld et al., 2000; Cole et al., 2001; Yang et al., 2003; Maerki et al., 2009; Yung et al., 2011). In contrast, leukocyte chemotaxis *in vitro* typically requires 1000-fold lower concentrations of chemokine (Berkhout et al., 1997; Sivek and Hamann, 1998). A non-comprehensive list of chemokine concentrations found constitutively in different body fluids is listed in **Table 2**. The most extensive survey has been obtained by bronchiolar alveolar lavage (Pacheco-Rodriguez et al., 2009). In general,



naturally occurring chemokine concentrations are in the picomolar to nanomolar range. However, two chemokines produced by platelets are found at micromolar concentrations in serum (Brandt et al., 2000). CXCL4 (also known as platelet factor 4) was the first chemokine ever to be characterized. It binds to the receptor CXCR3B, a splice variant affecting the N-terminus. The other is CXCL7 (also known as neutrophil-activating peptide 2). Both chemokines are released during platelet activation. From the table, it is apparent that many chemokines are made constitutively and secreted into serum/plasma, sweat, tears, saliva, and breast milk.

During infection and inflammation, chemokine concentrations in biological fluids may be greatly increased (Luster, 1998). For example, inflamed tonsils have nanomolar concentrations of CXCL9, CXCL10, and CXCL11 whereas in normal uninfamed tonsils these chemokines are not detectable (Egsten et al., 2007). Nanomolar concentrations of CXCL9 are sufficient to inhibit *Streptococcus pyogenes* growth *in vitro*. However, in most infection models, the concentrations of chemokines detected in biological fluids are not sufficient for antimicrobial activities *in vitro*. Therefore, the combined effect of all antimicrobial chemokines present in the infection site may be needed to achieve antimicrobial effects. However, we are not aware of any data showing that antimicrobial chemokines have additive or synergistic effects. In our own unpublished studies, we had problems obtaining consistent inhibition data at low chemokine concentrations to perform combination chemokine experiments. Another possibility is that the concentration of chemokines on mucosal surfaces may be much higher than in secretions because many chemokines can bind glycosaminoglycans on cellular surfaces.

Table 1 | Electrolytes concentration and major components in different human secretions.

Fluid	Sodium (mmol/L)	Chloride (mmol/L)	Potassium (mmol/L)	Bicarbonate (mmol/L)	Phosphate (mmol/L)	Creatinine (mmol/L)	Others (mmol/L)
Serum ¹	136–145	98–106	3.5–5.0	23–28	0.97–1.45	7–13	
Sweat ²	15–90	<40	0.002	2–40			
Saliva ³	2–50	5–40	10–36	25	1.4–39		
Tears ⁴	120–177	87–137	13–24				
Nasal ⁵	100–140	100–160	10–25				
Urine ⁶	10–200	15–200	10–0	<0.1–low		133–221 mmol/kg per 24 h	
Bile ⁷	200–260	10–60	5–8	10–60			Taurocholate 100–260
Gastric juice ⁸	100	280	15				
Pancreatic juice ⁸	150	40	5				
Intestinal fluid ⁸	150	100	5				
Stool ⁸	44–112		29–147				
Diarrh ⁸	3–139		15–115				
Milk ⁹	5–8	9–13	11–15				Lactose 193–207
PBS	137	139.7	2.7		10		

Listed are the major ions found in human secretions. Major compounds such as creatinine, taurocholate, and lactose in urine, bile, and milk are also indicated.

¹MAKSAP 14 Normal Laboratory Values. American College of Physicians, Copyright 2005. All rights reserved

²Bulmer and Forwell (1956), Kaiser et al. (1974).

³Chicharro et al. (1994).

⁴Lew et al. (2005).

⁵Cavaliere et al. (1988), Cavaliere et al. (1989).

⁶Moritz (2008).

⁷Wheeler et al. (1960).

⁸Shiau (1987).

⁹Koo and Gupta (1982), Wack et al. (1997).

RANGE OF ORGANISMS AFFECTED BY ANTIMICROBIAL CHEMOKINES

Antimicrobial chemokines have been shown to inhibit a broad-spectrum of organisms: Gram-positive bacteria, Gram negative bacteria, and dimorphic fungi. **Table 3** summarizes currently available information on this subject. In general, most antimicrobial chemokines have activity against multiple organisms. This suggests that the mechanism for antimicrobial activity is either through a conserved or non-specific pathway. All highly active antimicrobial chemokines have basic or cationic charge, but not all cationic chemokines have antimicrobial properties.

Chemokines have been surveyed most comprehensively for antimicrobial activity in the case of *Staphylococcus aureus* and *Escherichia coli*. Of 45 chemokines tested, 22 were found to have some anti-staphylococcal activity (Yung et al., 2011). For *E. coli*, 25 of 33 chemokines tested had some activity (Yang et al., 2003). A report on *S. pyogenes* showed that CXCL9, CXCL10, and CXCL11 can exert antimicrobial activities even at 150 mM sodium concentration (Egsten et al., 2007). Likewise, the antimicrobial effect of CXCL9, CXCL10, and CXCL11 on *Bacillus anthracis* may also be independent of ionic concentrations

(Crawford et al., 2010). These chemokines were able to inhibit both spore germination and bacillus viability in culture media. In addition to bacteria, several chemokines also have antifungal properties against *Cryptococcus neoformans* and *Candida albicans*.

COMPARISON WITH OTHER ANTIMICROBIAL PROTEINS

Antimicrobial chemokines represent a fraction of known antimicrobial proteins.

In general, antimicrobial proteins can be divided into three different classes. The largest class consists of β -stranded proteins with four to six conserved cysteines linked by disulfide bonds. Defensins and chemokines are members of this class. Whereas chemokines typically have four conserved cysteines that form two disulfide bonds, defensins have six conserved cysteines that form three disulfide bonds to stabilize β -strands. Both β -defensins and chemokines have overall cationic charge.

The antimicrobial effect of defensins occurs at micromolar concentration and in low sodium conditions just as antimicrobial chemokines. For example, micromolar concentrations of human beta defensin two are required for activity against

Table 2 | Chemokine concentrations in different body fluids.

Chemokine		Size MW (kD)	Serum or plasma nM	BAL (nM)	Sweat (nM)	Tears (nM)	Saliva (nM)	Parotid Secr (nM)	Breast milk (nM)
Family	Member								
CXC ELR+	CXCL1	7.8	0.01 <i>D</i>	3.07 <i>T</i>					0.4/ 5.96/ 0.62/
	CXCL2	7.9	0.03 <i>X</i>						
	CXCL3	7.9	0.09 <i>P</i>						
CXC ELR–	CXCL4	7.8	1100 <i>B</i>						
CXC ELR+	CXCL5	8.0	0.12 <i>D</i>	0.56 <i>T</i>					0.66/ 0.001/ 0.02/ 0.01/ 0.01 <i>K</i> 0.06 <i>K</i>
	CXCL6	7.9	0.04 <i>R</i>						
	CXCL7	7.6	3200 <i>B</i>						
CXC ELR–	CXCL8	8.4	0.003 <i>L</i>	0.22 <i>T</i>	0.02 <i>A</i>	0.038 <i>V</i>	0.03 <i>O</i>		
	CXCL9	11.7	0.012 <i>M</i>	0.32 <i>T</i>					
	CXCL10	8.5	0.009 <i>K</i>	0.22 <i>T</i>		0.358 <i>V</i>	0.008 <i>Y</i>		
	CXCL11	8.3	0.005 <i>S</i>	0.08 <i>T</i>					
	CXCL12	8.0	0.3 <i>S</i>	1.74 <i>T</i>			0.005 <i>Y</i>		
	CXCL13	10.3	0.003 <i>U</i>				0.000 <i>Y</i>		
	CXCL14	9.4	0.11 <i>W</i>						
	CXCL16	10.1	0.26 <i>Q</i>	0.02 <i>T</i>					
	CXCL17	11.5		<0.005 <i>z</i>					
CX3C	CX3CL1	8.5	0.1 <i>P</i>			0.005 <i>V</i>			
C	XCL1	10.0	0.08 <i>P</i>	0.23 <i>T</i>					
CC	CCL1	8.5	0.003 <i>P</i>	0.04 <i>T</i>					
	CCL2	8.6	0.02 <i>E</i>	0.55 <i>T</i>	0.01 <i>A</i>	0.019 <i>V</i>	0.044 <i>Y</i>		
	CCL3	7.8	0.003 <i>E</i>	0.19 <i>T</i>			0.004 <i>Y</i>		
	CCL4	7.6	0.001 <i>P</i>	0.17 <i>T</i>		0.001 <i>V</i>			
	CCL5	7.8	1.79 <i>E</i>	0.05 <i>T</i>		0.009 <i>V</i>	0.000 <i>Y</i>		0.003 <i>F</i>
	CCL7	9.0	0.003 <i>P</i>	0.03 <i>T</i>					
	CCL8	8.9	0.002 <i>P</i>	0.05 <i>T</i>					
	CCL11	8.3	0.01 <i>P</i>	0.13 <i>T</i>		0.007 <i>V</i>			0.006 <i>F</i>
	CCL13	8.6	0.026 <i>P</i>	0.03 <i>T</i>					
	CCL14	8.4	1.31 <i>C</i>						
	CCL15	10.1	0.1 <i>P</i>						
	CCL16	11.2	0.42 <i>P</i>						
	CCL17	8.0	0.02 <i>S</i>	0.04 <i>T</i>					
	CCL18	7.8	4.23 <i>J</i>						
	CCL19	8.8	0.009 <i>P</i>	0.02 <i>T</i>					
	CCL20	8.0	0.001 <i>S</i>	0.11 <i>T</i>					
CCL21	12.2	0.01 <i>S</i>	0.12 <i>T</i>						
CCL22	8.1	0.07 <i>S</i>	0.07 <i>T</i>						
CCL23	11.3	0.036 <i>P</i>							
CCL24	8.8	0.025 <i>H</i>							
CCL25	14.2								
CCL26	8.4	0.004 <i>H</i>							
CCL27	10.2	0.038 <i>S</i>							
CCL28	12.3	0.004 <i>N</i>					47 <i>G</i>	149 <i>G</i>	24 <i>G</i>

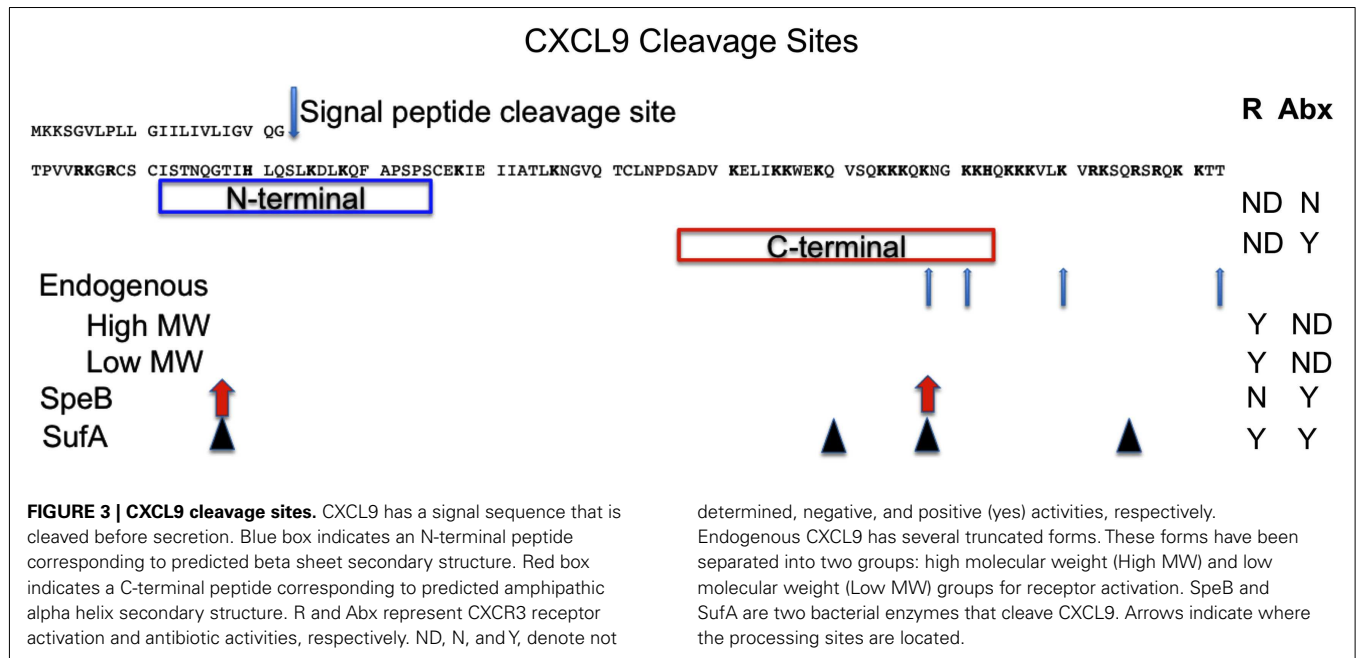
Jones et al. (1995)^A, Brandt et al. (2000)^B, Nomiyama et al. (2001)^C, Holven et al. (2002)^D, Parissis et al. (2002)^E, Bottcher et al. (2003)^F, Hieshima et al. (2003)^G, Kagami et al. (2003)^H, Maheshwari et al. (2003)^I, Struyf et al. (2003)^J, Takahata et al. (2003)^K, Antonelli et al. (2005)^L, Chuang et al. (2005)^M, Kagami et al. (2005)^N, Yang et al. (2005)^O, Bauer et al. (2006)^P, Sheikine et al. (2006)^Q, Alzogaibi et al. (2008)^R, Narbutt et al. (2009)^S, Pacheco-Rodriguez et al. (2009)^T, Schiffer et al. (2009)^U, Carreno et al. (2010)^V, Izukuri et al. (2010)^W, Dong et al., 2011)^X, Hernandez-Molina et al. (2011)^Y, Burkhardt et al. (2012)^Z.

Family and members of chemokines are listed on the left. The predicted molecular weights (MW) of mature chemokine forms are denoted in kilodaltons (kD). Reported serum or plasma concentrations of chemokines are grouped together in a single column. Italicized letter next to value designates reference source. BAL, bronchoalveolar lavage. All concentrations are reported as nanomolar (nM). Blanks indicate not determined.

Table 3 | Antibacterial and antifungal properties of chemokines.

Chemokine	MRSA	<i>S. coeag</i> neg	<i>S. pyogenes</i>	<i>E. faecium</i>	<i>B. subtilis</i>	<i>L. monocytogenes</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. neoformans</i>	<i>C. glabrata</i>	<i>C. albicans</i>
Family	Member	pl	[Na+] 10mM	[Na+ or K+] 10mM	[K+] 10mM	[Na+] 10mM	[Na+ or K+] 10mM	[Na+ or K+] 10mM	[Na+ or K+] 10mM	[Na+] 10mM	[Na+ or K+] 10mM
CXG ELR+	CXCL1	10.09	-	-	-	-	+++D	-	-	-	-
	CXCL2	10.45	-	-	-	-	+++D	-	-	-	-
CXG ELR-	CXCL3	10.15	+	-	-	-	+++D	-	-	-	-
	CXCL4	8.71	-	-	-	-A	-A	-	-	-	-
CXG ELR+	CXCL5	11.4	-	-G	-	-	-B	-	-	-	-
	CXCL6	10.44	++	+G	-	-	+,++++D,G	+G	-	-	-
CXG ELR-	CXCL7	8.73	-	-G	-	-	+(cleaved)A	-	+(cleaved)A	-(cleaved)A	+
	CXCL8	9.85	+	-H	-	-	-,+D,B,H	-	-	-	+
CXG ELR-	CXCL9	11.1	++++	+E	+E	+B	+++D	-B	-	-	-
	CXCL10	10.77	++++	+E	+E	+B	+++D	-B	-	-	-
CXG ELR-	CXCL11	11.1	++++	+E	+E	+B	+++D	-B	-	-	-
	CXCL12	10.48	++++	+E	+E	+B	+++D	-B	-	-	-
CXG ELR-	CXCL13	10.92	++++	-	-	-	+++D	+++H	-	-	-
	CXCL14	10.47	-	+	-	-	+++D	+++H	-	-	+
CX3C	CXCL16	8.75	+	-	-	-	+++D	+++H	-	-	+
	CXCL17	10.95	-	-	-	-	+++D	+++H	-	-	+
XC	CX3CL1	10.24	-	-	-	-	+++H	+	-	-	-
	XCL1	11.28	-	-	-	-	-D,B	-	-	-	-
CC	CCL1	10.48	++	-	-	-	+++D	-D,B	-	-	-
	CCL2	9.74	-	-	-	-	-D,B	-	-	-	-
CCL3L1	CCL3	4.48	-	-	-	-	-	-	-	-	-
	CCL4	4.47	-	-	-	-	-	-	-	-	-
CCL5	CCL5	8.61	-	-	-	-	-	-	-	-	-
	CCL7	10.33	-	-	-	-	+	-	-	-	-
CCL11	CCL8	9.88	-	-	-	-	+	-	-	-	-
	CCL11	10.74	-	-	-	-	+++D	-	-	-	-
CCL13	CCL13	10.62	+++	+	-	-	+++D	-	-	-	-
	CCL14	8.25	+	-	-	-	+	-	-	-	-
CCL15	CCL15	8.01	-	-	-	-	-D	-	-	-	-
	CCL16	9.86	-	-	-	-	+++D	-	-	-	-
CCL17	CCL17	9.81	-	-	-	-	+++D	-	-	-	-
	CCL18	9.47	+	-	-	-	+++D	-	-	-	-
CCL19	CCL19	10.31	+++	+	-	-	+++D	-	-	-	-
	CCL20	10.26	+++	+	-	-	+++D	-	-	-	-
CCL21	CCL21	10.71	+	+	+	+	+++D	+	+	+	+
	CCL22	8.98	+	-	-	-	+++D	-	-	-	-
CCL23	CCL23	9.09	+	-	-	-	+++D	-	-	-	-
	CCL24	10.76	-	-	-	-	+++D	-	-	-	-
CCL25	CCL25	10.92	+++	+	-	-	+++D	-	-	-	-

(Continued)



for chemotactic and antimicrobial properties, and whether these regions overlap.

MECHANISM OF ACTIVITY

By electron microscopy criteria, antimicrobial chemokines appear to induce lysis of the bacterial membrane (Hieshima et al., 2003; Egesten et al., 2007; Linge et al., 2008). Since the outer membrane of bacteria are highly anionic, charge interaction is thought to mediate initial binding (Brogden, 2005). In eukaryotic cell membranes, negatively charged phospholipids are sequestered in the inner leaflet of the lipid bilayer, and the outer leaflet is composed mostly of zwitterionic and uncharged lipids. Therefore, the lipid content of the outer membranes of eukaryotic cells are devoid of electrostatic charge (Wiesner and Vilcinskas, 2010). In contrast, both leaflets of bacterial cell membranes are enriched with acidic phospholipids such as phosphatidylglycerol and cardiolipin, making these membranes negatively charged.

The exact mechanism for antimicrobial killing is not known nor is it known whether all chemokines kill microbial targets by a common mechanism. Defensins, which have been studied in greater detail, are absorbed onto the bacterial membrane by electrostatic attraction and subsequently aggregate there to cause local membrane thinning and ion channel formation (Ganz, 2003). Rupture of bacterial membranes has been demonstrated in *S. pyogenes* for CXCL9, in *E. coli* for CXCL6, and in *P. aeruginosa* for CCL28 (Hieshima et al., 2003; Egesten et al., 2007; Linge et al., 2008).

For the bacterium responsible for anthrax, *B. anthracis*, the membrane protein FtsX has been identified as a unique target for CXCL10 (Crawford et al., 2011). The authors used a transposon-based genetic screen to identify *ftsX*, and two other genes (*BAS0651* and the cell wall autolysin *lytE*) to be critical for resistance against CXCL10. *B. anthracis* genetically deficient in *ftsX* could not bind CXCL10 as assessed by transmission electron microscopy. FtsX is a cell division ABC transporter that is conserved among

Gram-positive and Gram negative bacteria. Comparative protein analysis of FtsX with CXCR3, the receptor for CXCL10, showed a 27-residue region of FtsX that has 45% similarity with the N-terminal chemokine-binding domain of CXCR3. This is low, and how FtsX actually functions in antimicrobial chemokine resistance remains to be determined.

If charge–charge interaction were the sole means of chemokine-binding and antimicrobial action, chemokines would not be predicted to have antifungal properties since fungi have eukaryotic membranes. Histatin, a known antifungal peptide, inhibits *C. albicans* by binding to fungal mitochondria and inhibiting respiration by inducing reactive oxygen species (ROS) formation (Helmerhorst et al., 2001). The level of induced ROS correlated with fungicidal activity. However, it is not known whether antimicrobial chemokines act through similar pathways. The chemokine CCL28 has been shown to cause membrane disruption in *C. albicans* by scanning electron microscopy (Hieshima et al., 2003). How it binds to the fungal membrane and the domain responsible for antifungal activities has not been determined.

By whatever mechanisms chemokines kill pathogens, they do so rapidly. CXCL6 can disrupt liposome membranes within minutes, and pathogens treated with CCL28 show numerous surface blebs within 1 h (Hieshima et al., 2003; Linge et al., 2008). It is unclear whether gene activation or signal transduction needs to occur for antimicrobial chemokines to kill targets. Unlike traditional antibiotics, which work best on dividing organisms, antimicrobial proteins typically can exert effects on non-dividing organisms (Wiesner and Vilcinskas, 2010).

The salt dependence seen with antimicrobial chemokines is typical of most antimicrobial proteins. With the exception of human beta defensin 3, all human defensins exert antimicrobial properties at ~10 mM Na⁺. Killing of pathogens is thought to occur *in vivo* even in host environments with >10 mM Na⁺ because defensins

are packed in high concentration within granules. Activation and degranulation of leukocytes by pathogens would produce very high local concentrations of defensins for optimal antimicrobial effects. In addition, two recent reports have shown that the antimicrobial activity of chemokines is not dependent on sodium concentration for certain bacteria. In particular, CXCL9, CXCL10, and CXCL11 are able to inhibit growth of *S. pyogenes* even in 150 mM sodium chloride solutions. The same chemokines could also inhibit *B. anthracis* germination and growth in culture media with serum.

INHIBITION OF ANTIMICROBIAL CHEMOKINES BY BACTERIA

In addition to degrading chemokines with SpeB, *S. pyogenes* produces additional factors that inhibit antimicrobial chemokines. Streptococcal interleukin-8 inactivating cell envelope protease (SpyCEP) cleaves CXCL1, 2, 6, and 8 (Zinkernagel et al., 2008), and Streptococcal inhibitor of complement (SIC) binds CXCL9 to inhibit its antimicrobial properties (Eggesten et al., 2007). As mentioned previously, the bacterium *F. magna* expresses a subtilisin-like serine-proteinase (SufA) that partially degrades CXCL9, abolishing its antimicrobial activity (Karlsson et al., 2009). Therefore, several bacteria have developed means to degrade antimicrobial chemokines and evade host defense.

IN VIVO EVIDENCE FOR ANTIMICROBIAL PROPERTIES OF CHEMOKINES

Many inflammatory chemokines with antimicrobial properties are induced during inflammatory or infectious processes. It is unclear to what extent control of pathogens depends on direct antimicrobial activities of chemokines versus recruitment of leukocytes. One study demonstrated that neutralization of either CXCL9 or CXCL10 but not CXCR3 (the receptor for CXCL9 and CXCL10) increased susceptibility of C57BL/6 mice to *B. anthracis* (Crawford et al., 2011). Increased mortality was shown to arise from extrapulmonary dissemination of bacteria to kidneys, spleen, and liver and from toxemia with lethal factor. However, the bacterial load at the site of inoculation, the lung, was similar between treated and untreated mice.

We are not aware of genetic mutations in chemokine genes leading to increased susceptibility to infectious diseases in humans. However, there is persuasive evidence that the chemokine system mediates control of pathogens in mammals, including humans. For example, a form of congenital neutropenia, WHIM syndrome, arises from gain-of-function mutations truncating the C-tail of CXCR4 (Hernandez et al., 2003). Patients with WHIM syndrome have recurrent bacterial infections, especially in the sino-pulmonary tract, apparently caused by neutropenia and hypogammaglobulinemia (Kawai and Malech, 2009). However, this susceptibility appears to be due to leukocyte trafficking defects, not direct antimicrobial effects of chemokines.

FUTURE DIRECTIONS

There is no doubt that many chemokines have antimicrobial properties *in vitro*. However, the *in vivo* significance of chemokines as antimicrobial agents is still unclear. Direct antimicrobial activity will depend on the chemokine concentration and the environmental context between host and pathogen. In theory, chemokines

in mucosal surfaces might inhibit colonization of certain bacteria in the host since antibiotic usage has clearly been shown to alter normal flora. New studies will be needed to determine whether lack of constitutive chemokines affect skin and mucosal flora.

Studying the domains important for antimicrobial activities might lead to more potent antimicrobial peptides. Cationic charge and amphipathic alpha helix secondary structure appears to be important for antimicrobial properties. However, with the great diversity of antimicrobial peptides there has been neither consensus primary sequence nor a predominant tertiary structure. This makes molecular modeling to generate more potent synthetic antimicrobial proteins difficult. There is also the issue of unwanted side effects. Several potent antimicrobial peptides have systemic toxicity because they lyse red blood cells as well as pathogen membranes (Wiesner and Vilcinskas, 2010). In addition, we have found that many chemokines can induce the release of a virulence factor, protein A, in *S. aureus* (Yung et al., 2011). This potentially would harm the host if bacteria deploy virulence factors in response to chemokines. There is also evidence that subinhibitory concentrations of aminoglycoside antibiotics induce biofilm formation (Hoffman et al., 2005). Antibiotics trigger a protective response by bacteria to form biofilms. Bacteria in biofilms are very difficult to eradicate and are generally thousands of times more resistant to antimicrobial therapy than planktonic bacteria. In the same manner, antimicrobial proteins might trigger a protective response by bacteria to form biofilms.

Currently, there are no antimicrobial proteins in clinical use. The antimicrobial peptide MSI-78 or pexiganan completed a phase III clinical trial for the treatment of diabetic foot ulcers. MSI-78 was originally derived from magainin, an antimicrobial peptide found on the skin of frogs (Zasloff, 1987). However, it failed to receive approval from the FDA on the grounds that efficacy was not sufficiently demonstrated. Few companies are actively developing antimicrobial proteins as therapeutics. Both MBI-226 (a 12 amino acid peptide developed for the prevention of catheter-related bloodstream infections) and Neuprex (a recombinant fragment of bactericidal/permeability increasing protein developed for meningococcal meningitis) finished phase III clinical trials and never made it to market. It is unclear how antimicrobial proteins will be used in the clinics. Topical formulations to treat skin infections may be most applicable but effective topical antiseptics such as chlorhexidine already exist. To treat invasive infections, an injectable or intravenous form would be needed since proteins would most likely be degraded in the gastrointestinal tract. If developed, such potential broad-spectrum antimicrobial peptides might be useful in the setting of life-threatening infections caused by multi-drug resistant organisms.

Even though antimicrobial chemokines have been shown to generally disrupt bacterial membranes, unique targets on pathogens may actually mediate killing. As mentioned previously, the cell membrane protein FtsX in *B. anthracis* binds CXCL10 specifically and mediates antimicrobial chemokine resistance. Understanding this pathway and determining whether the same or related pathways exist in other organisms may lead to new antibiotics.

Genome-wide association studies have not linked increased infection susceptibility to single nucleotide polymorphism in chemokine genes. This could imply either no association, a weak association that has not been analyzed, or redundancy of the antimicrobial protein system. Further genetic studies in mice will be able to address whether genetic deficiencies in chemokines correlate with pathogen control, especially when compared with their respective chemokine receptor deficient controls.

CONCLUSION

In conclusion, the utility of chemokines likely extends beyond leukocyte recruitment. Understanding domains essential for

antimicrobial activities in antimicrobial chemokines could lead to more potent antimicrobial peptides. In addition, specific pathways may be involved in pathogen recognition of antimicrobial chemokines. Much work is needed to determine whether antimicrobial chemokines affect susceptibility to infection in humans, how chemokines kill microbes, and whether antimicrobial chemokines can be used to treat infections.

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