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Sequence analysis and expression of a CXC chemokine in resistant and susceptible catfish after infection of *Edwardsiella ictaluri*

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Abstract

Chemokines represent a superfamily of chemotactic cytokines involved in recruitment, activation and adhesion of a variety of leukocyte types to inflammatory foci. We cloned and sequenced the cDNA of a CXC chemokine that is most similar to CXCL10 from channel catfish and blue catfish. Sequence analysis of PCR amplicons from a single F1 hybrid catfish indicated that channel catfish and blue catfish may have a multigene family for the CXC chemokine. The catfish CXC chemokine was expressed in a wide range of tissues including head kidney, spleen, liver, gill, skin, stomach, and intestine, but not in the muscle. Fish challenged with intracellular bacterium *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), showed dramatically elevated levels of the CXC chemokine expression, as quantified with real time RT-PCR. Differential expression profiles were observed between resistant and susceptible channel catfish strains and blue catfish. Blue catfish were characterized by only modest induction in comparison to the drastic elevation of the CXC chemokine in channel catfish.

Keywords: CXCL10; Chemokine; Inflammatory cytokine; Gene expression; Innate immunity; Fish; Catfish

1. Introduction

The roles of the innate and acquired immune systems vary according to an organism's position in the evolutionary spectrum. Invertebrates and early chordates possess only innate immunity while higher vertebrates depend heavily on acquired immunity [1]. However, immune recognition, signaling, and gene regulatory mechanisms are remarkably conserved [2]. As immunologists learn more about the response of bony fish to prominent diseases, they have discovered that the innate immune system is often the most important factor in resistance. Diseases characterized by rapid progression through the host organisms are affected more by the fast-acting innate components than by the delayed responses of acquired immunity. Thus, research into the innate components of immunity is critical for the comprehension and eventual prevention of fish diseases.

Several innate immune systems have been identified in bony fish including chemokines, cytokines, acute phase proteins, NK cell receptors, and molecules

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upstream and downstream of the Toll signaling pathways [1]. Of these innate immune molecules, chemokines serve as pioneer passengers at inflammatory sites. Upon localized physical or chemical insult, phagocytes (macrophages and neutrophils) are activated in response to microbial antigens or compounds released from damaged cells. Among the mediators of inflammation released by the activated phagocytes are an array of chemokines and cytokines. These compounds in turn lead to increased vascular permeability, proliferation and recruitment of leukocytes, and the synthesis of acute phase proteins by the liver for fighting off the pathogens.

Chemokines represent a superfamily of chemotactic cytokines involved in recruitment, activation and adhesion of a variety of leukocyte types to inflammatory foci [3]. Based on the arrangement of the first two cysteine residues of the amino acid sequences [4,5], chemokines were divided into four groups: CXC (α), CC (β), C (γ), and CX3C (δ) subfamilies. The CXC and CC are the two major families of chemokines. Sixteen members of the CXC family, CXCL1–16, have been identified from mammalian species [6].

In mammalian species where extensive sequence analysis has been conducted, CXCL9, CXCL10, and CXCL11 share significant levels of sequence similarities. They also share the same receptor, CXCR3. Up-regulation of CXCL9, -10, and -11 has been oftentimes associated with Th1-driven immune responses against viral or intracellular bacterial infections. Up-regulation of CXCL10 expression has been reported after bacterial infection [7] while down-regulation of its expression was reported after Mumps virus infection in human leydig cells [8]. It has been suggested that CXCL10 had a role in CXCR3 mediated activation of eosinophils [9] and tissue regeneration in multiple models of liver and bile duct injury [10]. In human and mouse, all three CXC chemokine genes have been cloned. In fish, four sequences are highly similar to CXCL9–11 sequences—one from common carp [11] (Acc. No. BAB88677), and three from rainbow trout designated as VHSV-induced protein-7 and -8 (Acc. No. AAM18466 and Acc. No. AAM18467, respectively) [12], and a CXC chemokine resembling CXCL9–11 (Acc. No. AJ303075) [13]. The cDNAs for the catfish CXC chemokines were not available and their expression in early defenses after infection was not known. The objectives of this study were to characterize the cDNA highly similar to CXCL10, analyze its expression before and after infection, and compare its expression in catfish of different genetic backgrounds with different resistance to the bacterial disease enteric septicemia of catfish (ESC). Infection of the intracellular pathogen *Edwardsiella ictaluri* in channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*) causes ESC. Under artificial challenge conditions, this disease progresses rapidly causing heavy mortalities starting four days after infection when antibody-based defense is not yet in place [14]. This observation caused many to believe that innate immunity may play a crucial role for the survival of catfish from ESC disease. Under natural and artificial challenge conditions, channel catfish are highly susceptible to the infection of *E. ictaluri* while blue catfish are generally resistant [15]. Thus, the two closely-related catfish species offer an excellent research system for understanding early defense reactions after infection, especially the differential responses attributed to innate immunity. Channel catfish is a classical model within lower vertebrates for the study of comparative immunology [16–18]; and major progress has been made in understanding structure and organization of genes involved in both innate and adaptive immunity [e.g., 19–23]. We report here molecular cloning, sequencing, and expression of a CXC chemokine in catfish. The chemokine exhibits dramatic differences in expression profiles after infection in the resistant blue catfish and susceptible channel catfish.

2. Materials and methods

2.1. Fish and challenge experiments

Three strains of catfish were used: Marion Select, Kansas Random of channel catfish and D&B strain of blue catfish. Challenge experiments were conducted as previously described [24] with modifications. Briefly, the three strains of catfish were communally challenged in a rectangular tank by immersion exposure for 2 h with freshly prepared culture of ESC bacteria, *E. ictaluri*. One single colony of *E. ictaluri* was isolated from a natural outbreak in Alabama (outbreak number ALG-02-414) and inoculated into brain heart infusion

(BHI) medium and incubated in a shaker incubator at 28 °C overnight. The bacterial concentration was determined using colony forming unit (CFU) per ml by plating 10 μl of 10-fold serial dilutions onto BHI agar plates. At the time of challenge, the bacterial culture was added to the tank to a concentration of 3×10^7 CFU/ml. During challenge, an oxygen tank was used to ensure a dissolved oxygen concentration above 5 mg/ml. After 2 h of immersion exposure, 45 fish (15 fish each from the three strains) were randomly taken and placed into a rectangular trough containing pond water with constant water flow through. Eight troughs were used in the experiments including one trough for the controls for a total of 120 fish per strain. For the control fish, 15 fish of each strain were incubated in a separate rectangular tank with the same fish density as the challenge tanks. The only difference was that ESC bacteria were not added. After 2 h, these control fish were incubated in a separate trough at the same density as the challenged fish.

2.2. Tissue preparation and RNA isolation

Head kidney and spleen samples were collected from 10 fish of each strain before challenge as unchallenged controls. After challenge, head kidney and spleen samples were collected at various times after infection: at 4 h, 24 h, 3 days, and 7 days. Samples were also collected from fish that were dying during a period between day 4 and day 7 after challenge. At each time point, 10 fish were sacrificed for sampling from each strain. The fish were euthanized with tricaine methanesulfonate (MS 222) at 100 mg per liter before tissues were collected. The following tissues were also collected from 10 fish of Marion Select strain of channel catfish before challenge for the analysis of tissue expression: head kidney, spleen, skin, gill, intestine, stomach, muscle, and liver. Tissues were kept in a −80 °C ultra-low freezer until preparation of RNA.

Samples of each tissue from 10 fish were pooled. The pooled tissues were rapidly frozen with liquid nitrogen. In order to obtain samples representing the average of the 10 fish, the pooled tissue samples were ground with a mortar/pestle to fine powders and were thoroughly mixed. A fraction of the mixed tissue samples was used for RNA isolation. RNA was isolated following the guanidium thiocyanate method [25] using the Trizol reagents kit from Invitrogen (Carlsbad, CA) following manufacturer's instructions. Extracted RNA was stored in a −70 °C freezer until used as template for real time reverse transcriptase PCR (real time RT-PCR).

2.3. Plasmid preparation and sequencing

Plasmid DNA was prepared by the alkaline lysis method [26] using the Qiagen Spin Column Miniplasmid kits (Qiagen, Valencia, CA). Three microliters of plasmid DNA (about 0.5–1.0 μg) were used in sequencing reactions. Chain termination sequencing [27] was performed using cycleSeq-farOUT™ polymerase (Display Systems Biotech, Vista, CA). The PCR profiles were: 95 °C for 30 s, 55 °C for 40 s, 72 °C for 45 s for 30 cycles. An initial 2 min denaturation at 96 °C and a 5 min extension at 72 °C were always used. Sequences were analyzed on an automatic LI-COR DNA Sequencer Long ReadIR 4200 or LI-COR DNA Analyzer Gene ReadIR 4200. BLAST searches [28–30] were conducted to determine gene identities. The EST analysis was conducted as we previously reported [31–33].

2.4. PCR amplified segment of the CXC chemokine gene

A blood sample from a single individual F1 hybrid catfish (channel catfish × blue catfish) was obtained for genomic DNA extraction. Genomic DNA (100 ng) was used to amplify the CXC chemokine gene segments. Specific primers (Table 1) were designed to amplify regions with single nucleotide polymorphisms (SNPs) and a 7 bp deletion to reveal sequence variation within the CXC chemokine TRP genes. The PCR profiles were: 94 °C for 30 s, 45 °C for 60 s, 72 °C for 60 s for 40 cycles. An initial 3 min denaturation at 94 °C and a 5 min extension at 72 °C were always used. PCR products were cloned into pCR 2.1-TOPO cloning vector (Invitrogen). Clones were grown, isolated and sequenced following the above protocol (Section 2.3).

2.5. RT-PCR

Total RNA isolated from uninfected fish of Marion Select was used for reverse transcription-PCR

Table 1
Primers used in analysis of sequence variation and RT-PCR or real time RT-PCR

Primer name	Primer sequences (5′ to 3′)	Nucleotide positions
CXCL10 upper for sequence variation and RT-PCR	TCTGAATTTACTAAGAAATAC	287–307
CXCL10 lower for sequence variation and RT-PCR	CTCATAAATTAAACAGT	442–458
CXCL10 upper for real time RT-PCR	CCAGTGTAAGGAGGTGTT	126–143
CXCL10 lower for real time RT-PCR	CTCTAATCCTGCCGTGATG	307–325
β-actin upper	AGAGAGAAATTGTCCGTGACATC	
β-actin lower	CTCCGATCCAGACAGAGTATTTG	

Nucleotide position coordinates are referenced from Fig. 1.

(RT-PCR) reactions. The RT-PCR reaction was conducted using SuperScript™ III One Step RT-PCR System (Invitrogen). The system contained a mixture of SuperScript™ III reverse transcriptase and the Platinum *Taq* DNA polymerase in an optimized buffer. Detailed procedures followed the instructions of the manufacturer. Briefly, the following was added to a reaction of 50μ: 25 μl 2X reaction mix, 1 μl total RNA (~100 ng), 1 μl (100 ng) each of the upper and lower primer (Table 1), 2 μl SuperScript III RT/Platinum *Taq* polymerase mix, and water to bring the reaction volume to 50 μl. The reaction also included the primers of β-actin (Table 1), serving as an internal control. The reactions were completed in a thermocycler with the following thermo-profiles: 45 °C for 15 min for one cycle (reverse transcription reaction), the samples were pre-denatured at 94 °C for 2 min, then the samples were amplified for 40 cycles with 94 °C for 15 s, 45 °C for 30 s, 68 °C for 1 min. Upon completion of PCR, the reaction was incubated at 68 °C for an additional 5 min. The RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel and documented with a Gel Documentation System (Nucleotech Corp., CA).

2.6. Real time PCR using a Lightcycler

Total RNA was used for reverse transcription real time PCR (RT-real time PCR). Concentration and quality of total RNA was determined by spectrophotometry (optical density 260/280 ratio) and electrophoresis. Primers used in real time RT-PCR are shown in Table 1. β-actin was used as an internal control for real time RT-PCR. A standard curve was constructed by using various copy numbers of a plasmid containing CXCL10 cDNA. Real time RT-PCR reactions of the standard curves were included in all runs in order to relate quantitative data from run to run. One-step real time RT-PCR was carried out in a LightCycler (Roche Applied Science, Indianapolis, IN) using a Fast Start RNA Master SYBR Green I reagents kit (Roche Applied Science) following manufacturer's instructions with modifications. Briefly, all real time RT-PCR reactions were performed in a 10 μl total reaction volume (9 μl master mix and 1 μl RNA template). A five-step experiment protocol was run on the LightCycler: (i) reverse transcription, 20 min at 61 °C; (ii) denaturation, 30 s at 95 °C; (iii) amplification repeated 50 times, 1 s at 95 °C, 1 s at 55 °C, 13 s at 72 °C; (iv) melting curve analysis, 5 s at 95 °C, 15 s at 65 °C, then up to 95 °C at a rate of 0.1 °C per second; (v) cooling, 30 s at 40 °C. Concentration of cDNA in each sample was calculated from the standard curve. Each sample was normalized to the equivalent of the reference gene, β-actin. The ratio between the CXC chemokine and β-actin was used for the purpose of comparisons.

3. Results

3.1. cDNA cloning and sequence analysis

cDNA clones for the channel catfish and blue catfish CXC chemokine genes were obtained by homology comparisons of EST sequences. During EST analysis, four clones from channel catfish and one clone from blue catfish were found to be similar to CXC chemokine CXCL9–11, with the highest similarities to CXCL10 (Table 2). All these clones harbored complete coding sequences of the chemokine gene and have been submitted to GenBank with

Table 2
Results of BLASTX searches using the channel catfish CXC chemokine as a query, listed according to the *p*-values

Species	Acc. No.	Identity	*p*-Value
Carp (*Cyprinus carpio*)	BAB88677.1	CXC most similar to CXCL10	2×10^{-21}
Rainbow trout (*Oncorhynchus mykiss*)	AF483528	VHSV-induced protein-8	4×10^{-18}
Rainbow trout	AAM18466	VHSV-induced protein-7	1×10^{-17}
Rat (*Rattus norvegicus*)	NP_620789	CXCL10	6×10^{-10}
Sheep (*Ovis aries*)	BAB63958	CXCL10	1×10^{-9}
Goat (*Capra hircus*)	BAC55183	CXCL10	1×10^{-9}
Rainbow trout	CAC83075	CXC chemokine	2×10^{-9}
Mouse (*Mus musculus*)	NP_067249	CXCL10	4×10^{-8}
Golden hamster (*Mesocricetus auratus*)	AAG16300	CXCL10	5×10^{-8}
Human (*Homo sapiens*)	P02778	CXCL10	1×10^{-7}
Pig-tailed macaque (*Macaca nemestrina*)	AAO52732	CXCL10	2×10^{-7}
Human	AAH12532	CXCL11	3×10^{-7}
Mouse	AAH25903	CXCL11	1×10^{-5}
Human	NP_002407	CXCL9	2×10^{-5}

Acc. No. BE212851, BM425159, BE468362, BE469394, and BQ097353. Sequence analysis indicated that the four cDNA clones of the channel catfish CXC chemokine represented two types of cDNAs: BE212851 and BE468362 had identical sequences, and BE469394 and BM425159 had identical sequences. The two types of the channel catfish and one type of blue catfish CXC chemokine cDNAs were completely sequenced and the sequences have been deposited to GenBank with Acc. No. AY335949, AY335950, and AY335951.

Both channel catfish CXC chemokine cDNAs harbor a 55 bp 5′ untranslated region (UTR) and a 288 bp coding sequence encoding a protein of 95 amino acids (Fig. 1), but differ in 3′-UTR. A single base change within the poly $(A)^+$ signal sequences AATAAA into AAGAAA in AY335950 led to polyadenylation at a second site of AATAAA, 75 bp downstream. The two types of channel catfish cDNAs also had 31 single nucleotide polymorphic sites within 600 bp sequences, or about 5% divergence, and one 7 bp deletion/insertion 18 bp downstream of the termination codon.

The polymorphic rate between the two types of cDNAs of the channel catfish CXC chemokine gene was much higher than the average polymorphic rate

```
gaaaatcattaagagaatcttcagagcatcgagtgtgttcaagagcaagaggaaaatgaagtctgctgcagtttttgttg  80
                                                  M  K  S  A  A  V  F  V

tgtttgcctgtctacttattgttcatgtacaaggacaggccaggaccagtgtaaggaggtgtttgtgtcagggtcctgca 160
V  F  A  C  L  L  I  V  H  V  Q  G  Q  A  R  T  S  V  R  R  C  L  C  Q  G  P  A
gctaacgtagttcaaccacaacgtattgacaagattgaaattcatcctgcgagagcatcttgtgaaaatgtggaaatcat 240
 A  N  V  V  Q  P  Q  R  I  D  K  I  E  I  H  P  A  R  A  S  C  E  N  V  E  I  I

tgtcactctgaagaacggtgcaggaaaaaagtgcttgaatccggaatctgaatttactaagaaatacatcacggcaggat 320
  V  T  L  K  N  G  A  G  K  K  C  L  N  P  E  S  E  F  T  K  K  Y  I  T  A  G

tagagaaaaggagtgcagtgtaaagcgtgcaccgtgtcgttactgaacagaagaggaagacgagagacagcttgcttaaa 400
L  E  K  R  S  A  V  *
atattttaaatgttgtaaaaagtgtacatgtggtatgtctaactgtttaatttatgagcatcacatctgcatatgggtca 480

atgtatatagtttttgaataaacatgcaagatcttt(poly A)                                      516
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Fig. 1. Sequence of the channel catfish CXC chemokine with nucleotide sequences in small letters and deduced amino acid sequences in capital letters. The translation start codon ATG and the termination codon TAA is bold. The poly $(A)^+$ signal sequence AATAAA is bold and underlined. The locations of the RT-PCR primers are indicated by single line arrows; the locations of the real time RT-PCR primers are indicated by double line arrows.

within genes of channel catfish [34]. Even between the genes of channel catfish and blue catfish, the average SNP rate among sequenced ESTs was found to be 1.32% (1.32 SNPs/100 bp) [34]. While a 5% sequence difference existed between the two channel catfish cDNAs, one of the two channel catfish cDNAs (AY335949) was very similar to the blue catfish sequence with 2% sequence divergence. This may indicate that the two cDNAs from channel catfish are paralogous. This speculation is strengthened when the deduced amino acid sequences were analyzed (Fig. 2). There was only one amino acid difference between the channel catfish CXC chemokine (encoded by AY335949) and the blue catfish CXC chemokine, but there were seven amino acid differences between the channel catfish CXC chemokine encoded by the two different types of transcripts (Fig. 2).

Alignment of amino acid sequences of the CXC chemokine genes from catfish with those from common carp, rainbow trout, human, and mouse indicated high levels of amino acid sequence conservation (Fig. 2). The deduced amino acid sequences of catfish CXC chemokine were 51% similar to the carp sequences, 47% similar to the rainbow trout sequences, 38% similar to the mouse sequences, and 33% similar to the human sequences.

3.2. Channel catfish may have a multigene family of the CXC chemokine genes

cDNA sequence analysis indicated that the two types of CXC chemokine transcripts within channel catfish were highly divergent. They could represent transcripts of two paralogues of CXC chemokine genes from channel catfish, or could represent transcripts of alternative alleles in the population of the same gene since multiple individuals were used for the construction of the cDNA libraries. If the cDNAs represented transcripts of the same gene with alternative alleles, then only one type of PCR product should result from a single sperm or egg, considering that channel catfish and blue catfish are diploid organisms. In contrast, if the cDNAs represented transcripts from paralogous genes, then more than one type of PCR products should result. PCR primers were designed to amplify fragments spanning a region rich in SNPs and also including the 7 bp deletion/insertion (Table 1). Genomic DNA from a single F1 hybrid catfish (channel catfish × blue catfish) was amplified by PCR. The PCR products were cloned and sequenced. Among 32 clones sequenced, 14 types of cDNA sequences were found (Table 3). Obviously, in the F1 hybrid catfish, one allele each should be expressed from a given gene of channel catfish

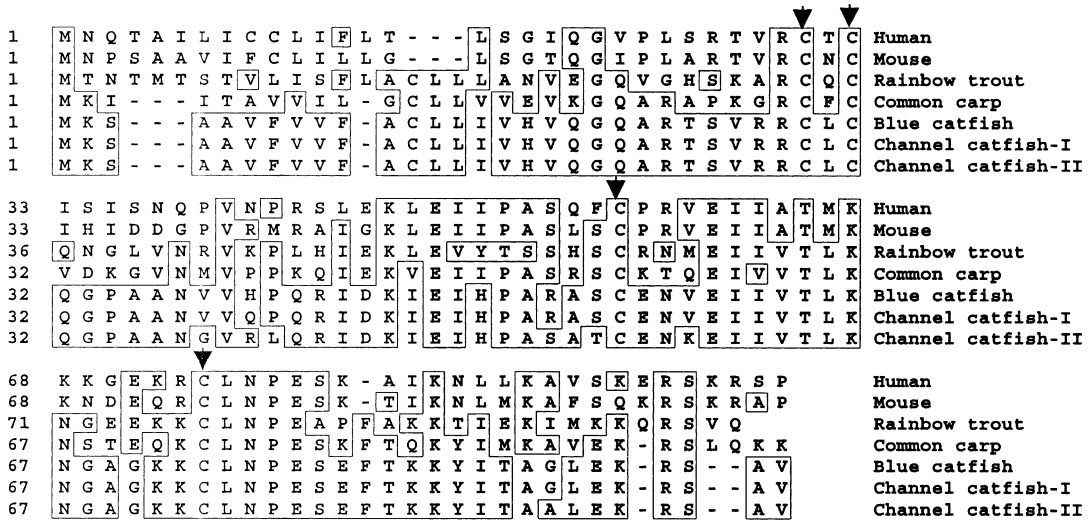

Fig. 2. Similarity comparison of the deduced amino acid sequences of the catfish CXC chemokine with the highly related CXC chemokines from human (CXCL10), mouse (CXCL10), carp, and rainbow trout. Arrows indicated the four conserved cysteine residues.

Table 3
Sequence variations as analyzed from PCR products of a single F1 hybrid catfish

STS#	Sequence variation (positions start with the PCR Primer: TCTGAATTTACTAAGAAATAC where first T was position 1)																		
	27	30	32	67	68	76	89	91	106	129	162	167	169	187	203–209	229	232	237	255	263
00043	G	A	G	C	T	T	G	C	G	T	A	C	A	G	7 bp	T	G	G	A	A
00044	G	A	G	T	T	T	G	C	G	T	G	C	G	A	7 bp	T	A	G	A	A
00045	G	A	G	T	T	T	G	C	G	C	G	C	G	A	7 bp	A	A	G	A	G
00046	A	A	C	T	T	T	A	T	G	C	G	C	G	A	7 bp	T	A	T	A	G
00047	G	A	G	T	T	T	G	C	G	T	A	C	A	A	7 bp	T	G	G	A	A
00048	A	G	C	T	T	T	G	C	G	T	G	C	G	A	7 bp	T	A	G	A	A
00049	G	A	G	T	T	T	G	C	G	C	G	C	G	A	Del	T	A	G	G	A
00050	G	A	G	T	T	T	G	C	C	C	G	T	G	A	7 bp	T	A	T	A	G
00051	G	A	G	T	T	T	G	C	C	C	G	C	G	A	7 bp	T	A	G	A	G
00052	A	A	C	T	T	T	A	T	G	C	G	C	G	A	7 bp	T	A	G	A	G
00053	G	A	G	T	T	A	G	C	G	T	G	C	G	A	7 bp	T	A	G	A	G
00054	G	A	G	C	T	T	G	T	G	C	G	C	G	A	7 bp	T	A	G	A	G
00055	G	A	G	T	T	T	G	C	G	T	G	C	G	A	7 bp	T	A	G	A	G
00056	G	A	G	C	C	T	G	C	G	T	G	C	G	A	7 bp	T	A	G	A	A

Of the 32 sequenced clones, 14 types of sequences were found with base substitutions at various positions as indicated and a 7 bp deletion (del).

and blue catfish. This data indicated that a multigene family may exist in channel and blue catfish for the chemokine resembling CXCL9, CXCL10, and CXCL11.

3.3. The catfish CXC chemokine is expressed in a wide range of tissues

Previous EST analysis indicated that the CXC chemokine gene is expressed in several tissues since it was sequenced from the cDNA libraries made from the brain, head kidney, and spleen of the four libraries that we have sequenced [31,33,35]. In order to analyze tissue expression of the CXC chemokine in channel catfish, RT-PCR was conducted using total RNA from various tissues of uninfected fish. As shown in Fig. 3, except muscle tissue from which no RT-PCR products were detected, the CXC chemokine was detected from all tested tissues including head kidney (anterior kidney), liver, spleen, intestine, stomach, skin, and gill. It appeared that the CXC chemokine was expressed at relatively low levels in all the healthy tissues tested because the RT-PCR products could not be detected at lower cycle numbers of PCR. It took 35 cycles to see a faint band from spleen, head kidney, and gill, but 40 cycles were required to visualize the band of other tissues.

3.4. Drastic induction of the CXC chemokine expression after infection of channel catfish with bacterial pathogen E. ictaluri

The production of CXCL10 has been found in several animal models of infection, especially infections where IFN-γ is known to play an important role in host defense [36,37]. In humans, several recent

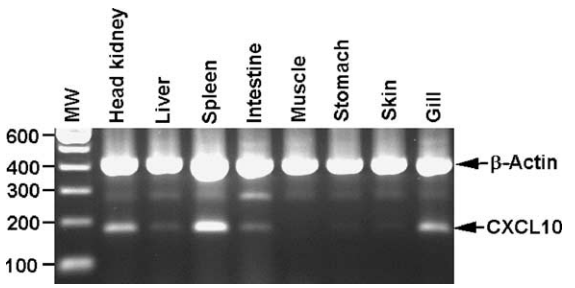

Fig. 3. Expression of the channel catfish CXC chemokine in various tissues as analyzed by RT-PCR. MW, molecular weight standards with the sizes indicated on the left margin in base pairs (bp); tissues are indicated on the top of the gel. The CXC chemokine gene-specific RT-PCR products (172 bp) are indicated on the right margin, along with β-actin RT-PCR products as an internal control. The faint bands at 273 bp represent the products amplified from the genomic DNA substrate within the total RNA preparations that were not treated with DNase, which included a 101 bp intron (Acc. No. BV079470).

studies reported elevated expression of CXCL10 after bacterial infections both in vitro [7] and in vivo [38,39], but little is known about the role of CXCL10 in bacterial infections in fish. Here we determined the levels of the CXC chemokine gene expression in channel catfish after *E. ictaluri* challenge using real time PCR. In both Marion Select and Kansas Random strains, the CXC chemokine expression was dramatically induced in head kidney after challenge (Fig. 4). As mentioned above, the background expression of the CXC chemokine before challenge was low in both strains. Upon challenge, high levels of the CXC chemokine expression were detected in Kansas Random strain as soon as 4 h after challenge. The expression reached very high levels (60 fold of the level of controls) at 24 h after challenge. The overall expression was lower three days after challenge. At seven days after challenge, the expression returned to much lower levels, only slightly higher than the level prior to challenge. In Marion Select strain, the pattern was very similar to that of Kansas Random strain except that the high level of the CXC chemokine expression was detected at 24 h after challenge, but not at the first sampling time of 4 h after challenge (Fig. 4).

The CXC chemokine expression was high in moribund fish. Mortalities started to occur four days after challenge in the experiments. In order to determine the CXC chemokine expression in moribund fish, real-time observation was made in the first week after challenge. As the infected fish became moribund, they often went through a period of 'spinning swimming'. Tissue samples were collected immediately after spinning swimming when they lost balance. These fish were regarded as moribund fish. As shown in Fig. 4, the CXC chemokine expression in the moribund fish was high as compared to the expression at either day 3 or at day 7, between which the moribund fish were collected. However, the moribund fish were collected in a period of three days, whereas the samples at day 3 and day 7 were collected at a given time point, and therefore, a direct comparison is not possible.

3.5. Differential expression profile of the CXC chemokine in channel catfish and blue catfish after infection

In spite of the differences in resistance to *E. ictaluri* among various channel catfish strains, they are generally susceptible to the disease. In contrast, blue catfish are generally resistant to the disease. To determine if the CXC chemokine expression differed between channel and blue catfish after bacterial challenge, the CXC chemokine mRNA levels were examined in head kidney and spleen at various times after challenge. As discussed above, the CXC chemokine expression was dramatically induced at 24 h after challenge in channel catfish (Fig. 5A and B). However, the CXC chemokine expression was only modestly (about 3 fold) induced in blue catfish in both the head kidney (Fig. 5A) and spleen tissues (Fig. 5B). This low induction in blue catfish is unrelated to the efficiency of PCR primers because the real-time PCR

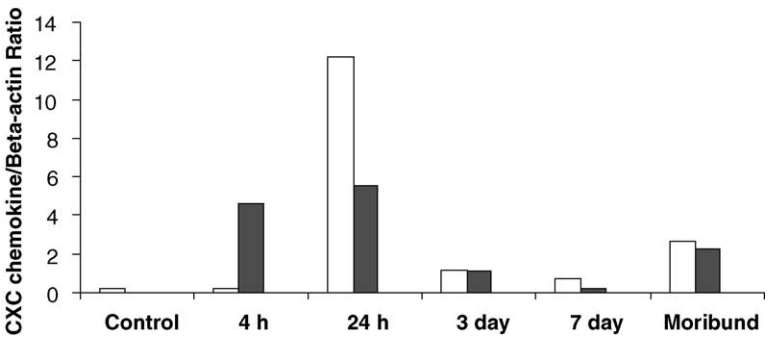

Fig. 4. Changes of the CXC chemokine mRNA levels in head kidney of channel catfish as analyzed by real time RT-PCR. Data is expressed as the ratio of the CXC chemokine RNA and the β-actin RNA at various times: control, immediately before challenge; 4 h, 24 h, 3 day, and 7 day were time points at which the head kidney tissue samples were collected. Moribund fish were head kidney samples collected from moribund fish between day 4 and day 7 after challenge. Open bar, Marion Select strain; and solid bar, Kansas Random strain of channel catfish.

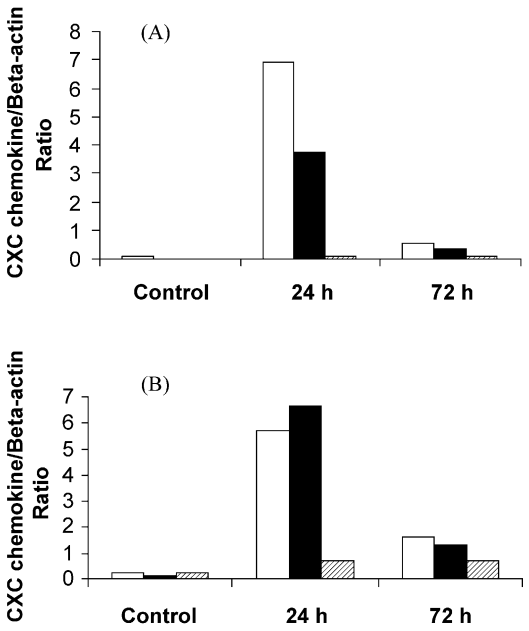

Fig. 5. Changes of the CXC chemokine mRNA levels in head kidney (A) and spleen (B) of channel catfish and blue catfish as analyzed by real time RT-PCR. Data is expressed as the ratio of the CXC chemokine RNA and the β-actin RNA at various times: control, immediately before challenge; and 24 and 72 h after challenge when the tissue samples were collected. Open bar, Marion Select; and solid bar, Kansas Random strain of channel catfish (*Ictalurus punctatus*); sketched bar, D&B strain of blue catfish (*I. furcatus*).

primers were selected at a region that had 100% identity with the blue catfish CXC chemokine sequence. Similarly, the beta-actin gene primers were designed to have perfect match with both channel catfish and blue catfish and these primers have been used reproducibly in many studies [40].

4. Discussion

Innate immunity is widely believed to play a very important role in resistance against major bacterial diseases in catfish. As one of the innate immune responses, chemokines are key components in the process of leukocyte recruitment in inflammatory sites. The interactions of various chemokines with their receptors on leukocytes allow activation and chemotaxis of neutrophils, eosinophils, lymphocytes, and monocytes for migration to the sites of evolving inflammation. We have identified and characterized a CXC chemokine from both channel catfish and blue catfish. The molecular cloning of the chemokine provides necessary molecular tools for characterization of its expression during infection. To date, only very limited CXC chemokines were cloned from fish [11,13,41–43]. Therefore, it is still difficult to establish the orthologous relations for the identified CXC chemokine. However, BLASTX similarity comparisons clearly indicated that the CXC chemokine was most similar to CXCL10, followed by CXCL11, and CXCL9 (Table 2). An attempt of phylogenetic analysis failed to put the identified chemokine into the clade containing mammalian CXCL10, but it was clustered together with most other teleost chemokines. This was consistent with recent phylogenetic analysis of Laing et al. [13] where a rainbow trout CXC chemokine was found to be most similar to mammalian CXCL10, but the orthologous relation could not be established. As a matter of fact, Huising et al. [43] conducted systematic phylogenetic analysis of CXC chemokines and was able to establish unambiguous orthologues only for CXCL12 and CXCL14 in fish. In absence of any fish CXCL9 and CXCL11 chemokines, the exact identity of the cloned chemokine awaits further study. Laing et al. [13] suggested that fish may have only one representative ligand for the CXCR3, but the concrete answers for this speculation may only be answered when the full genome of fish species is sequenced.

An EST approach was used to identify the catfish CXC chemokine cDNAs. Clearly, the EST approach was effective not only in identification of the chemokine cDNAs, but also in providing detailed sequence information allowing identification of sequence variants. In this work, we have sequenced two types of the CXC chemokine cDNAs with 5% sequence divergence. Because fifteen fish were used in making the cDNA libraries, one possibility of cDNA sequence divergence is that the cDNAs represent allelic variation among the individuals used for library construction. However, this level of sequence divergence was high as compared to the observed average sequence divergence among channel catfish orthologous genes [34]. Alternatively, these cDNAs may actually represent transcripts of different genes (paralogues). Our results using PCR-sequencing indicated that channel catfish may have

a multigene family of the CXC chemokine genes resembling CXCL9–11, supporting the notion that the sequenced two types of the CXC chemokine cDNAs were transcripts of paralogous genes.

Channel catfish strains show various levels of resistance to ESC caused by infection of *E. ictaluri*. In terms of resistance within channel catfish, Kansas Random is one of the most resistant strains while Marion Select is one of the most susceptible (Dr Dunham, Auburn University, unpublished). The mechanisms of resistance are unknown at present. This work characterized the CXC chemokine expression in these two strains. Although the overall pattern of the CXC chemokine expression after bacterial challenge was very similar, the onset of elevation of the CXC chemokine expression was more rapid in Kansas Random than in Marion Select. Unfortunately, no samples were collected between 4 and 24 h after challenge so the exact time difference in the CXC chemokine induction was not revealed. It is speculated that early onset of lymphocyte recruitment to the inflammatory sites may help clear the pathogenic bacteria. CXC chemokines can be divided into two classes based on the presence of the glutamate-leucine-arginine (ELR) motif preceding the CXC sequence. While the ELR-containing CXC chemokines like interleukin-8 and growth-related oncogene alpha (Groα) preferentially attract neutrophils [44–48], the non-ELR-containing CXCL10 and monokine induced by IFN-γ (MIG) selectively attract T lymphocytes and NK cells [49–52]. If confirmed, the early onset of the CXC chemokine expression could be part of the mechanisms accounting for the greater resistance to ESC in Kansas Random. CXCL10 specifically binds to CXCR3 [51,53]. It has been demonstrated that CXCR3 is preferentially expressed on Th1-type lymphocytes [54,55]. A Th1-type immune response is associated with the release of Th1-type cytokines, such as IFN-γ and IL-2, and known to enhance cell-mediated immunity, which is important for host defense against intracellular pathogens [38]. It seems likely that CXCL10 contributes to the selective recruitment of Th1 cells in sites of inflammation with high IFN-γ production.

Mammalian CXCL9, -10, and -11 are oftentimes associated with Th1 driven responses against viral pathogens or intracellular infectious agents [37,56]. The situation after the infection of *E. ictaluri* is similar to that of a viral infection in that both infections are intracellular and up-regulating the CXC chemokine resembling CXCL9–11.

Blue catfish is not a perfect host for *E. ictaluri*. Although blue catfish can be naturally infected by this pathogen, they are generally resistant to ESC. While it cannot be excluded that antibody-based immunity plays a role in this resistance, innate resistance appears to play a greater role because most often, heavy mortalities occur only 4–5 days after infection when specific immune response is not yet active [15]. This work demonstrated that the CXC chemokine was highly differentially expressed in channel and blue catfish. Upon infection, the CXC chemokine expression was induced drastically (60–200 fold) in channel catfish within 24 h after challenge, while its expression was only modestly induced (about 3-fold) in blue catfish. This indicates that the ESC disease progressed at drastically different stages in channel catfish and blue catfish. It is likely that the CXC chemokine would not be involved in the resistance mechanisms, however, it could serve as a molecular indicator to the disease resistance. Further analysis and comparison of expression of genes involved in early immune response between channel and blue catfish is warranted in order to gain a more complete understanding of the roles of the CXC chemokine in the resistance of channel catfish and blue catfish against ESC.

Expression analysis was conducted in pooled samples in this study. This was so conducted because of limitations of funding prohibiting analysis of a large number of individuals using real time PCR to provide meaningful expression data. However, chemokine expression in different individual fish was known to differ extensively among individuals [41]. Nonetheless, the expression analysis using pooled samples here should represent an average of the 10 fish used in the study. It should be interesting to assess variations of the chemokine expression among individuals in future studies.

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References

[1] Magor BG, Magor KE. Evolution of effectors and receptors of innate immunity. Dev Comp Immunol 2001;25:651–82.
[2] Warr GW, Chapman RW, Smith LC. Evolutionary immunobiology: new approaches, new paradigms. Dev Comp Immunol 2003;27:845–58.
[3] Neville LF, Mathiak G, Bagasra O. The immunobiology of interferon-gamma inducible protein 10 kD (CXCL10): a novel, pleiotropic member of the C-X-C chemokines superfamily. Cytokine Growth Factor Rev 1997;8:207–19.
[4] Ahuja SK, Murphy PM. The CKC chemokines growth-regulated oncogene (GRO) alpha, GRObeta, GROgamma, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. J Biol Chem 1996;217:20545–50.
[5] Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA. International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 2000;52:145–76.
[6] Bacon K, Baggiolini M, Broxmeyer H, Horuk R, Lindley I, Mantovani A, Maysushima K, Murphy P, Nomiyama H, Oppenheim J, Rot A, Schall T, Tsang M, Thorpe R, Van Damme J, Wadhwa M, Yoshie O, Zlotnik A, Zoon K. IUIS/WHO Subcommittee on Chemokine Nomenclature. Chemokine/chemokine receptor nomenclature. J Interferon Cytokine Res 2002;22:1067–8.
[7] Gasper NA, Petty CC, Schrum LW, Marriott I, Bost KL. Bacterium-induced CXCL10 secretion by osteoblasts can be mediated in part through toll-like receptor 4. Infect Immun 2002;70:4075–82.
[8] Le Goffic R, Mouchel T, Ruffault A, Patard JJ, Jegou B, Samson M. Mumps virus decreases testosterone production and gamma interferon-induced protein 10 secretion by human leydig cells. J Virol 2003;77:3297–300.
[9] Jinquan T, Jing C, Jacobi HH, Reimert CM, Millner A, Quan S, Hansen JB, Dissing S, Malling HJ, Skov PS, Poulsen LK. CXCR3 expression and activation of eosinophils: role of IFN-gamma-inducible protein-10 and monokine induced by IFN-gamma. J Immunol 2000;165:1548–56.
[10] Koniaris LG, Zimmers-Koniaris T, Hsiao EC, Chavin K, Sitzmann JV, Farber JM. Cytokine-responsive gene-2/IFN-inducible protein-10 expression in multiple models of liver and bile duct injury suggests a role in tissue regeneration. J Immunol 2001;167:399–406.
[11] Savan R, Kono T, Aman A, Sakai M. Isolation and characterization of a novel CXC chemokine in commonn carp (*Cyprinus carpio* L.). Mol Immunol 2003;39:829–34.
[12] O'Farrell C, Vaghefi N, Cantonnet M, Buteau B, Boudinot P, Benmansour A. Survey of transcript expression in rainbow trout leukocytes reveals a major contribution of interferon-responsive genes in the early response to a rhabdovirus infection. J Virol 2002;76:8040–9.
[13] Laing KJ, Bols N, Secombes CJ. A CXC chemokine sequence isolated from the rainbow trout *Oncorhynchus mykiss* resembles the closely related interferon-gamma-inducible chemokines CXCL9, CXCL10 and CXCL11. Eur Cytokine Netw 2002;13:462–73.
[14] Wolters WR, Johnson MR. Enteric septicemia resistance in blue catfish and three channel catfish strains. J Aquat Anim Health 1994;6:329–34.
[15] Wolters WR, Wise DJ, Klesius PH. Survival and antibody response of channel catfish, blue catfish and hybrids of female channel catfish and male blue catfish following exposure to *Edwardsiella ictaluri*. J Aquat Anim Health 1996;8:249–54.
[16] Clem LW, Bly JE, Ellsaesser CF, Lobb CJ, Miller NW. Channel catfish as an unconventional model for immunological studies. J Exp Zool Suppl 1990;4:123–5.
[17] Vallejo AN, Miller NW, Clem LW. Phylogeny of immune recognition: role of alloantigens in antigen presentation in channel catfish immune responses. Immunology 1991;74:165–8.
[18] Vallejo AN, Miller NW, Clem LW. Phylogeny of immune recognition: processing and presentation of structurally defined proteins in channel catfish immune responses. Dev Immunol 1991;1:137–48.
[19] Wilson M, Bengten E, Miller NW, Clem LW, Du Pasquier L, Warr GW. A novel chimeric Ig heavy chain from a teleost fish shares similarities to IgD. Proc Natl Acad Sci USA 1997;94:4593–7.
[20] Miller N, Wilson M, Bengten E, Stuge T, Warr G, Clem W. Functional and molecular characterization of teleost leukocytes. Immunol Rev 1998;166:187–97.
[21] Hawke NA, Yoder JA, Haire RN, Mueller MG, Litman RT, Miracle AL, Stuge T, Shen L, Miller N, Litman GW. Extraordinary variation in a diversified family of immune-type receptor genes. Proc Natl Acad Sci USA 2001;98:13832–7.
[22] Antao AB, Wilson M, Wang J, Bengten E, Miller NW, Clem LW, Chinchar VG. Genomic organization and differential expression of channel catfish MHC class I genes. Dev Comp Immunol 2001;25:579–95.
[23] Zou J, Secombes CJ, Long S, Miller N, Clem LW, Chinchar VG. Molecular identification and expression analysis of tumor necrosis factor in channel catfish (*Ictalurus punctatus*). Dev Comp Immunol 2003;27:845–58.
[24] Dunham RA, Brady Y, Vinitnantharat S. Response to challenge with *Edwardsiella ictaluri* by channel catfish, *Ictalurus punctatus*, selected for resistance to *E. ictaluri*. J Appl Aquacult 1993;3:211–22.

[25] Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
[26] Sambrook J, Frisch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press; 1989.
[27] Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 1977; 74:5463–7.
[28] Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
[29] Gish W, States DJ. Identification of protein coding regions by database similarity search. Nat Genet 1993;3:266–72.
[30] Zhang J, Madden TL. PowerBLAST: a new network BLAST application for interactive or automated sequence analysis and annotation. Genome Res 1997;7:649–56.
[31] Cao D, Kocabas A, Ju Z, Karsi A, Li P, Patterson A, Liu ZJ. Transcriptome of channel catfish (*Ictalurus punctatus*): initial analysis of genes and expression profiles from the head kidney. Anim Genet 2001;32:169–88.
[32] Karsi A, Cao D, Li P, Patterson A, Kocabas A, Feng J, Ju Z, Mickett K, Liu ZJ. Transcriptome analysis of channel catfish (*Ictalurus punctatus*): initial analysis of gene expression and microsatellite-containing cDNAs in the skin. Gene 2002;285: 157–68.
[33] Ju Z, Karsi A, Kocabas A, Patterson A, Li P, Cao D, Dunham R, Liu ZJ. Transcriptome analysis of channel catfish (*Ictalurus punctatus*): genes and expression profile from the brain. Gene 2000;261:373–82.
[34] He C, Chen L, Li P, Kim PS, Liu ZJ. Efficient development of type I SNP markers in interspecific hybrids of catfish by comparative EST analysis. Anim Genet 2003; 34: 445–8.
[35] Kocabas A, Li P, Cao D, Karsi A, He C, Patterson A, Ju Z, Dunham R, Liu ZJ. Expression profile of the channel catfish spleen: analysis of genes involved in immune functions. Mar Biotechnol 2002;4:526–36.
[36] Amichay D, Gazzinelli RT, Karupiah G, Moench TR, Sher A, Farber JM. Genes for chemokines MuMig and Crg-2 are induced in protozoan and viral infections in response to IFN-gamma with patterns of tissue expression that suggest nonredundant roles in vivo. J Immunol 1996;157:4511–20.
[37] Vester B, Muller K, Solbach W, Laskay T. Early gene expression of NK cell-activating chemokines in mice resistant to *Leishmania major*. Infect Immun 1999;67:3155–9.
[38] Lauw FN, Simpson AJH, Prins JM, Van Deventer SJH, Chaowakul W, White NJ, Van der Poll T. The CXC chemokines gamma interfetron (IFN-γ)-inducible protein 10 and monokine induced by IFN-γ are released during severe melioidosis. Infect Immun 2000;68:3888–93.
[39] Eck M, Schmausser B, Scheller K, Toksoy A, Kraus M, Menzel T, Muller-Hermelink HK, Gillitzer R. CXC chemokines Groα/IL-8 and CXCL10/MIG in *Helicobacter pylori* gastritis. Clin Exp Immunol 2000;122:192–9.
[40] Kocabas AM, Kucuktas H, Dunham RA, Liu Z. Molecular characterization and differential expression of the myostatin gene in channel catfish (*Ictalurus punctatus*). Biochim Biophys Acta 2002;1575:99–107.
[41] Laing KJ, Zou JJ, Wang T, Bols N, Hirono I, Aoki T, Secombes CJ. Identification and analysis of an interleukin 8-like molecule in rainbow trout *Oncorhynchus mykiss*. Dev Comp Immunol 2002;26:433–44.
[42] Huising MO, Stolte E, Flik G, Savelkoul HF, Verburg-van Kemenade BM. CXC chemokines and leukocyte chemotaxis in common carp (*Cyprinus carpio* L.). Dev Comp Immunol 2003;27:875–88.
[43] Huising MO, Stet RJ, Kruiswijk CP, Savelkoul HF, Lidy Verburg-van Kemenade BM. Molecular evolution of CXC chemokines: extant CXC chemokines originate from the CNS. Trends Immunol 2003;24:307–13.
[44] Oppenheim JJ, Zachariae CO, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergenes intercrine cytokine family. Annu Rev Immunol 1991;9:617–48.
[45] Miller KD, Krangel MS. Biology and biochemistry of the chemokine: a family of chemotactic and inflammatory cytokine. Crit Rev Immunol 1992;12:17–46.
[46] Baggiolini M, Dewald B, Moser B. Interleukin-8 and related chemotactic cytokines-CXC and CC chemokines. Adv Immunol 1994;55:97–179.
[47] Schall TJ, Bacon KB. Chemokines, leukocyte trafficking, and inflammation. Curr Opin Immunol 1994;6:865–73.
[48] Mukaida N, Harad A, Yasumoto K, Matsushima K. Properties of proinflammatory cell type-specific leukocyte chemotactic cytokines interleukin 8 (IL-8) and monocyte chemotactic and activating factor (MCAF). Microbiol Immunol 1992;36: 773–89.
[49] Luster AD, Ravetch JV. Biochemical characterization of a γ-interferon-inducible cytokine (CXCL10). J Exp Med 1987; 166:1084–97.
[50] Liao BF, Rabin RL, Yannelli JR, Koniaris LG, Vanguri P, Farber JM, Human MIG. chemokine: biochemical and functional characterization. J Exp Med 1995;182:1301–14.
[51] Loetscher M, Gerber B, Loetscher P, Jones SA, Piali L, Clark-Lewis I, Baggiolini M, Moser B. Chemokine receptoe specific for IP10 and MIG: structure, function and expression in activated T-lymphocytes. J Exp Med 1996;184:963–9.
[52] Farber JM. MIG and CXCL10: CXC chemokines that target lymphocytes. J Leuk Biol 1997;61:246–57.
[53] Baggiolini M, Dewald B, Moser B. Human chemokines: an update. Annu Rev Immunol 1997;15:675–705.
[54] Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Investig 1998;101:746–54.
[55] Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J Exp Med 1998; 187:875–83.
[56] Hamerman JA, Aderem A. Functional transitions in macrophages during in vivo infection with *Mycobacterium bovis* Bacillus Calmette-Guerin. J Immunol 2001;167:2227–33.