Two coupled mutations abolished the binding of CEBPB to the promoter of CXCL14 that displayed an antiviral effect on PRRSV by activating IFN signaling

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is the most economically important infectious disease of pigs worldwide. Our previous study revealed that Tongcheng (TC) pigs display higher resistance to PRRS than Largewhite (LW) pigs, but the genetic mechanism remains unknown. Here, we first confirmed that CXCL14 was downregulated in lungs and porcine alveolar macrophages (PAMs) responding to PRRS virus (PRRSV) infection, but the decline in LW pigs was more obvious than that in TC pigs. Then, we found that the overexpression of CXCL14 activated type-I interferon (IFN-I) signaling by upregulating interferon beta (IFNB), which plays a major role in the antiviral effect. To further decipher the mechanism underlying its differential expression, we characterized the core promoter of CXCL14 as being located from −145 to 276 bp of the transcription start site (TSS) and identified two main haplotypes that displayed significant differential transcriptional activities. We further identified two coupled point mutations that altered the binding status of CEBPB and were responsible for the differential expression in TC and LW pigs. The regulatory effect of CEBPB on CXCL14 was further confirmed by RNA interference (RNAi) and chromatin immunoprecipitation (ChIP), providing crucial clues for deciphering the mechanism of CXCL14 downregulation in unusual conditions. The present study revealed the potential antiviral effect of CXCL14, occurring via activation of interferon signaling, and suggested that CXCL14 contributes to the PRRS resistance of TC pigs.
1 | INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a destructive disease affecting the pig industry worldwide and is caused by PRRS virus (PRRSV). As an airborne virus with a rapidly evolving single-stranded RNA genome, PRRSV is difficult to manage through vaccination and biosecurity measures. Numerous reports have shown that pigs with different genetic backgrounds display different virus clearance abilities and diverse PRRSV resistance, indicating the possibility of breeding improvement of PRRSV resistance. A major quantitative trait locus (QTL) accounting for the genetic variance of viral load at an early stage post infection was identified in sus scrofa chromosome 4 (SSC4), which further supports the possibility of breeding improvement of PRRSV resistance as an alternative and preferable strategy. Not only viral load but also immune and inflammatory responses vary greatly among different host genetic backgrounds, indicating that many loci related to PRRSV resistance remain to be identified.

Our previous study showed that Tongcheng (TC) pigs, an indigenous breed in central China, could survive well and displayed significantly less inflammatory exudation than Large white (LW) pigs. Transcriptomic studies revealed that the expression of several chemokines, such as CXCL2, CXCL9, CXCL10, CXCL12, CXCL14, CCL2, and CCL4, varied significantly in different experimental groups, among which CXCL14 was regarded as an emerging immune and inflammatory modulator. CXCL14 was initially discovered in the breast and kidney, so it is also named breast and kidney-expressed chemokine (BRAK). Unlike other chemokines induced by infection, CXCL14 is constitutively produced by epithelial cells and displays antimicrobial activity. It can also be expressed by monocyte-derived cells and macrophages and plays an immune surveillance role by chemotactically attracting those cells. In addition, it is highly expressed in many regions of the brain and regulates neurogenesis and synaptic transmission. Overall, maintenance of the normal expression of CXCL14 is important for maintaining homeostasis of the body. However, this chemokine was found to be significantly downregulated or absent in many malignant tissues or under the conditions of pathogenic infections and chemical exposure. Investigation of the mechanisms leading to its downregulation under abnormal conditions would be important for understanding the associated pathogenesis.

Given the vital role of CXCL14 in inflammatory regulation and its differential expression in response to PRRSV infection, it could be regarded as a candidate gene for investigation of the genetic basis of the highly pathogenic PRRSV (HP-PRRSV)-induced inflammatory response. In the present study, we conducted a preliminary functional examination of CXCL14 and characterized its core promoter, attempting to elucidate the molecular mechanism underlying its differential expression in pig alveolar macrophages (PAMs) of TC and LW pigs.

2 | MATERIALS AND METHODS

2.1 | Animals and sampling

The animal infection experiments were performed as described previously. Briefly, 5-week-old piglets from TC and LW populations received an intramuscular challenge of HP-PRRSV at a viral dose of $10^5$ CCID50/mL (3 mL/15 kg), and the control groups for both pig breeds were challenged with the same amount of RPMI-1640. All animals from the four groups (TC-control, TC-infection, LW-control and LW-infection) were humanely euthanized at 7 days post challenge (dpc). The spleen was collected from each pig for DNA extraction. Moreover, the left lung was collected for PAM collection via bronchoalveolar lavage, and the right lung was collected for preparation of paraffin-embedded sections.

2.2 | In vitro expression analysis

To analyze the expression of CXCL14 in vivo, immunohistochemistry (IHC) analysis was conducted with the lung tissues from animals of four groups. The paraffin-embedded lung tissue was cut into 6-µm sections, deparaffinized and rehydrated, followed by treatment with 3% H2O2 to eliminate endogenous peroxidase and with 0.01 M citrate sodium for antigen retrieval. After blocking with 5% bovine serum albumin, the prepared sections were incubated with a primary antibody against CXCL14 (ab46010 from Abcam, Cambridge, United Kingdom) at 4°C overnight and a horseradish peroxidase (HRP)-conjugated secondary antibody (Beyotime, Shanghai, China) at 37°C for 30 minutes. A DAB kit (Boster, California, USA) was used for color development, and hematoxylin was used for nuclear staining. Meanwhile, immunofluorescence (IF) analysis for CXCL14 was conducted with the collected PAMs. Briefly, PAMs from one individual of the...
LW and TC control groups were cultured in a 24-well plate, receiving challenges of HP-PRRSV (multiplicity of infection (MOI) = 1) and RPMI-1640 (as control) for 24 hours. The prepared PAMs were fixed with 4% paraformaldehyde for 15 minutes and permeabilized for 10 minutes in 0.3% Triton X-100. Then, the sample was blocked in 5% bovine serum albumin for one hour at room temperature, immunostained with the primary antibody against CXCL14 (ab46010) at 4°C overnight and Alexa Fluor 555-conjugated goat anti-rabbit IgG (AS058 from Abclone, Wuhan, Hubei, China) at 37°C for 2 hours. Subsequently, DAPI (Boster, CA, USA) was used to stain nuclei. In addition, total proteins were extracted from PAMs of 12 animals (equally from four groups) and used for Western blot analysis with beta-tubulin as a control (GB11017 from Servicebio, Wuhan, Hubei, China).

2.3 Overexpression of CXCL14 and transcriptomic study

The complete coding sequence (CDS) of porcine CXCL14 was amplified (CXCL14 forward 5′-CTAGCT AGCATGAGGCTCCTGACGCC-3′, reverse 5′-CCCAAGCTTACCTGCTGAGG-3′; the reverse primer contains a FLAG tag) and inserted between the Nhel and HindIII restriction sites of the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, California, USA). The plasmid construct was confirmed by sequencing. The validated CXCL14 plasmid (pcDNA3.1-CXCL14-flag) and the empty pcDNA3.1 plasmid were transfected into CD163-transfected PK15 cells (PK15CD163) enabled for PRRSV infection. Total proteins mid were transfected into CD163-transfected PK15 cells (pcDNA3.1-CXCL14-flag) and the empty pcDNA3.1 plasmid was inserted between the restriction sites of the pcDNA3.1 (+) vector (Invitrogen, Carlsbad, California, USA) 36 hours after transfection. The effect of CXCL14 overexpression was confirmed by Western blot analysis using an anti-FLAG antibody (AE005 from Abclone, Wuhan, Hubei, China).

The RNA samples were successively checked for purity, concentration, and integrity using a Nanodrop2000, a Qubit 3.0 and an Agilent 2100 bioanalyzer, respectively. The 150-bp, paired-end, strand-specific libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB, Beijing, China). After quality control, the library preparations were sequenced on an Illumina HiSeq X platform. Clean reads were obtained by removing reads containing adapters or poly-N and low-quality reads from raw reads. The analysis of RNA-seq data was based on the Hisat-Stringtie-Ballgown pipeline.21 The criteria for differentially expressed genes (DEGs) were as follows: q-value less than 0.05 and fold change greater than 1.5. The GO and KEGG pathway enrichment analyses were performed using the online tools in DAVID (https://david.ncifcrf.gov/).

2.4 Characterization of the core promoter and variant analysis

To characterize the core promoter of porcine CXCL14, a DNA fragment from 493 bp upstream (−493 bp) to 276 bp downstream (+276 bp) of the transcription start site (TSS) was amplified with the primer pair PF1/PR (PF1: 5′-CGGGGTACCTACGTGGTGGCGC-3′; PR: 5′-CTCGAGGTCGTCGTCGTCGTCTGTCC-3′) using Phanta Max Super-Fidelity DNA Polymerase (Vazyme, Nanjing, Jiangsu, China). The obtained PCR product was inserted into the KpnI and HindIII restriction sites of the pGL3-Basic luciferase reporter vector (Promega, Madison, Wisconsin, USA), which was named PB-P1. With the PB-P1 plasmid as the template, five fragments with gradually truncated 5′ ends of the CXCL14 promoter were amplified using different forward primers PF2-PF6 (PF2: 5′-CGGGGTACCTACGTGGTGGCGC-3′; PF3: 5′-CGGGGTACCTACGTGGTGGCGC-3′; PF4: 5′-CGGGGTACCTACGTGGTGGCGC-3′; PF5: 5′-CGGGGTACCTACGTGGTGGCGC-3′; the same reverse primer (PR)). Similarly, these fragments were cloned between the KpnI and HindIII sites of the pGL3-Basic vector and named PB-P2 (−206/+276), PB-P3 (−145/+276), PB-P4 (−44/+276), PB-P5 (−12/+276) and PB-P6 (+38/+276), respectively. All these constructs were confirmed by DNA sequencing.

Genomic DNA was extracted from the spleens of 37 TC, 28 LW and 21 ET (ET, the commercial name of multigeneration hybrid pigs of LW boar and TC sows) pigs using phenol/chloroform extraction. The CXCL14 promoter was amplified from all these DNA samples with the primer pair PF1/PR. The purified PCR products were sequenced commercially and assembled for variant analysis with Seqman (DNASTAR, Madison, Wisconsin, USA). Linkage disequilibrium (LD) and haplotype analyses were performed using Haploviev 4.2 (Broad Institute, Cambridge, Massachusetts, USA) with the default parameters.

2.5 Site-directed mutagenesis and dual luciferase reporter assays

To identify potential functional mutations in the promoter of porcine CXCL14, two different versions of the PB-P2 (−206/+276) reporter plasmids were constructed from individuals containing different haplotypes. Site-directed mutagenesis assays were performed directly on the promoter constructs using reverse-complemented primer pairs containing the mutant nucleotides in the middle (1-PF: 5′-GAGCGAGAGGGTAGCAGATGTGGATCGCCGAGG-3′;
add to the reaction mixture described above and incubated for 30 minutes at 4°C prior to incubation with the biotin-labeled probes. For the competition assay, a 200-fold molar excess of unlabeled double-stranded probes was added to the reaction mixture. The protein/DNA complexes were electrophoresed on a 6% (w/v) nondenaturing polyacrylamide gel in 0.5× TBE buffer prior to transfer onto nylon membranes for chemiluminescence detection.

### 2.7 | Protein pull-down and mass spectrometry

The protein pull-down assay was performed with the CC or TT probe as well as negative control (NC) (without probe). The double-stranded probe labeled with biotin at the 5′ terminus of the reverse strand was immobilized onto Dynabeads M-280 Streptavidin (Invitrogen, Carlsbad, California, USA) following the manufacturer's protocol. Nuclear extracts of the PK15 cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA) and quantified by the BCA method (Beyotime, Shanghai, China). One 200-µL reaction mixture contained 100 µL of crude nuclear extract (~500 µg), 30 µL of poly (dl:dc) (1 µg/µL), 20 µL of 10× binding buffer (100 mM Tris-HCl, 500 mM KCl, 10 mM DTT), 4 µL of protease inhibitor cocktail and 46 µL of ddH2O. The prepared mixture was preincubated on ice for 1 hour, followed by another 1-hour incubation at 4°C in a rotator after addition of 2 mg of Dynabeads (NC) or 2 mg of Dynabeads with immobilized probe. After washing the beads with 1x binding buffer three times, the proteins were eluted by adding 100 µL of elution buffer (1x binding buffer containing 500 mM NaCl). The eluted proteins were sent for mass spectrometric analysis by CapitalBio Technology (Beijing, China).

## 2.6 | Electrophoretic mobility shift assay (EMSA) and supershift assay

Nuclear extracts of the PK15 cells or PAMs were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA) and quantified by the BCA method (Beyotime, Shanghai, China). The single-stranded and reverse-complemented DNA probes were synthesized with or without a 5′-end biotin label (Sangon Biotech, Wuhan, Hubei, China) (CC probe forward strand: 5′-AGTGAGTCACCGAGTGGTT-3′; LC-TT probe forward strand: 5′-CCCCACCGCGCTGAGAAATCTCAGTGAGTCACCGAGTGGTT-3′; TT probe forward strand: 5′-CCCCACCGCGCTGAGAAATCTCAGTGAGTCACCGAGTGGTT-3′; 6-PF: 5′-GTCTCCTCCCCCCACCGCGCTGAGAAATCTCAGTGAGTCACCGAGTGGTT-3′; 7-PF: 5′-GAGAAGCAGCAGCGAGGGAGGCTACCACCGCCAGTGAGTCACCGAGTGGTT-3′; 789-PF: 5′-GAGAAGCAGCAGCGAGGGAGGCTACCACCGCCAGTGAGTCACCGAGTGGTT-3′; 23-PF: 5′-GCCAGGTGGTTGACGAGGCAG-3′; 259-PF: 5′-GCCAGGTGGTTGACGAGGCAG-3′; 76-PF: 5′-GCCAGGTGGTTGACGAGGCAG-3′; 56-PF: 5′-CGGAAGCTTATGCAACGCCTGGTGGCCTG-3′, 60-PF: 5′-CGGAAGCTTATGCAACGCCTGGTGGCCTG-3′; 50-PF: 5′-CGGAAGCTTATGCAACGCCTGGTGGCCTG-3′; 2.6-PF: 5′-GCCAGGTGGTTGACGAGGCAG-3′; 2.7-PF: 5′-GCCAGGTGGTTGACGAGGCAG-3′; 2.8-PF: 5′-GCCAGGTGGTTGACGAGGCAG-3′). The mutated fragments were cloned into the pGL3-Basic vector again using the restriction sites of KpnI and HindIII; the cloning was confirmed by DNA sequencing.

Dual luciferase reporter assays for different constructs were conducted in PK15 cells with the pGL3-Basic plasmid and the pRL-TK plasmid (Promega, Madison, Wisconsin, USA) as negative and internal controls, respectively. For each construct, the luciferase reporter assay was repeated at least three times independently, and the results from one representative experiment are shown. Statistical analysis was performed using Student's t test, and a p value smaller than 0.05 was considered significant.

### 2.8 | Overexpression and RNA interference (RNAi) of CEBPB

To validate the regulatory effect of CEBPB on CXCL14, overexpression and RNAi assays of CEBPB were conducted in PK15 cells. To construct the overexpression plasmid, the CDS of pig CEBPB was amplified (CEBPB forward 5′-CGGAAGCTTATGCAACGCCTGGTGGCCCTG-3′, reverse 5′-CCCTCTAGACTAGCAGTGGCCGGAGGCAG-3′) and inserted between the HindIII and XbaI restriction sites of the pCMV-N-HA vector (Beyotime, Shanghai, China). The positive plasmid (pCMV-HA-CEBPB) was confirmed by DNA sequencing. For the RNAi assay, three small interfering RNA (siRNAs) targeting pig CEBPB mRNA were synthesized by GenePharma (Shanghai, China) (siRNA-65 forward 5′-CAAGUGAAUGUGCCACAUUUTT-3′, reverse 5′-AAGUUGCCACUUCACUUGTT-3′; siRNA-770
forward 5′-CCUCGCAGGUAGAAGAGAATT-3′; reverse 5′-UUACUCUUGACCCGCGAGGT-3′; siRNA-866 forward 5′-GCCCAGCAAGGCGCAAGATT-3′; reverse 5′-AUCUUGGCUUGCGCGCTT-3′). In both assays, the expression of CEBPB and CXCL14 was first validated by qRT-PCR. The 2−ΔΔCT method was used to evaluate gene expression with the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene as an internal reference. The primers used for qRT-PCR were as follows: CEBPB forward 5′-TCGACAGGTCAAGAGTAAGACC-3′, reverse 5′-CTTGACTGCTGCTTCAGAGTT-3′; CXCL14 forward 5′-ACGGGTCAAAATGCAAGTG-3′, reverse 5′-ACCCTGCGCTTCTCATTCCA-3′; GAPDH forward 5′-ACGGGTCCAAATGCAAGTGC-3′, reverse 5′-CTTATGCTGCGTCTCCAGGTT-3′; CXCL14 forward 5′-TCGCAGGTCAAGAGTAAGACC-3′, reverse 5′-GCCTTGACTGTCGGTGAGA-3′; GAPDH forward 5′-ACCCTGCGCTTCTCATTCCA-3′; GAPDH reverse 5′-GTCCCTGAGACACGATGTG-3′, reverse 5′-GCCTTGACTGTCGGTGAGA-3′; GAPDH forward 5′-CCUCGCAGGUAGAAGAGAATT-3′, reverse 5′-GCCTTGACTGTCGGTGAGA-3′. The expression of CEBPB and CXCL14 was further validated by Western blot analysis with beta-tubulin as a control. The primary antibodies for CXCL14 were purchased from Abcam (Cambridge, United Kingdom).

### 2.9 Chromatin immunoprecipitation (ChIP) assay and qPCR

The ChIP assay was performed using a ChIP assay kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. In brief, the CEBPB overexpression plasmid (pCMV-HA-CEBPB) was transfected into PK15 cells seeded in a 10-cm plate. Cells in each plate were cross-linked and lysed at 48 hours post transfection. The cell lysate was subjected to sonication on ice with 13 sets of 40-second pulses at 35% output power and centrifuged at 13,000 g for 5 minutes at 4°C to remove insoluble material. Then, we collected 1% of the supernatant as input. Then, an equal amount of supernatant was immunoprecipitated using anti-HA antibody (ab9110 from Abcam, Cambridge, United Kingdom) and rabbit control IgG antibody (AC005 from Abelone, Wuhan, Hubei, China) separately. Protein and DNA were isolated from the immunoprecipitated products and were subjected to Western blot and qPCR analyses (qPCR primer forward 5′-AATCCACTCCTGGCCCTGCG-3′, reverse 5′-AGTGTGAGCCCGCTGGAT-3′) separately.

### 3 RESULTS

#### 3.1 CXCL14 was downregulated under PRRSV infection, and the decline varied between LW and TC pigs

Our previous transcriptomic study revealed that CXCL14 was significantly downregulated in PAMs of LW and TC pigs in response to PRRSV infection. We confirmed the expression pattern of CXCL14 by IHC. Strong signals of CXCL14 were detected in the epithelial cells in the lungs of the control groups of LW and TC pigs; however, the signals became weaker in the infection groups of both pig breeds (Figure 1A). We further compared the expression pattern of CXCL14 in PAMs between the control and PRRSV-infected individuals. The expression of CXCL14 was indeed downregulated in PAMs of both LW and TC pigs under PRRSV infection, and the decline in LW pigs was extreme at 65%, whereas a 47% decline was observed in TC pigs (Figure 1C). In summary, CXCL14 displayed varying degrees of decline in LW and TC pigs responding to PRRSV infection, indicating that the expression of this gene was regulated by genetic background.

#### 3.2 Overexpression of CXCL14 inhibited PRRSV replication by activating type-I interferon (IFN-I) signaling

As shown above, CXCL14 maintained a high expression level under normal physiological conditions but was downregulated under PRRSV infection. If CXCL14 maintains high expression, what is its effect on PRRSV? To answer this question, we conducted transient overexpression of CXCL14 in PK15CD163 cells followed by PRRSV infection (MOI = 1) at 24 hours post transfection. Western blot analysis confirmed that CXCL14 maintained a relatively high expression level within 48 hours post transfection, and the expression level decreased subsequently (Figure 2A). The qRT-PCR assay of PRRSV open reading frame 7 (ORF7) revealed that viral replication was significantly inhibited at 36 (P = .027) and 48 (P = .025) hours post infection with PRRSV (Figure 2B).

To reveal the mechanism of CXCL14 inhibition of PRRSV replication, we conducted a transcriptomic study via RNA sequencing using samples at 36 hours post transfection. In total, we identified 51 differentially expressed genes (DEGs) (Figure S1A and Table S1), seven of which were further confirmed to be upregulated in response to CXCL14 overexpression by qRT-PCR (Figure S1B). Pathway analysis revealed that these DEGs were significantly enriched in pathways of cellular response to IFN-I and virus replication (Figure 2C), indicating that IFN-I was activated. We further analyzed the expression of β-interferon (IFNB) and γ-interferon (IFNG). The results showed that the expression level of IFNB was upregulated 4.8-fold (P = 5.04 × 10−5) (Figure 2D), and that of IFNG did not change (Figure 2E). We also detected the expression of α-interferon (IFNA)-encoding genes, but they were barely expressed.
3.3 Variant scanning in the promoter of CXCL14 identified two main haplotypes with differential transcriptional activities

To identify potential genetic differences in the regulation of CXCL14 expression between LW and TC pigs, we characterized the core promoter of CXCL14 and investigated its polymorphisms. First, we generated a series of reporter vectors with progressively truncated 5’ ends of the promoter, named PB-P1 to PB-P6, and measured their activities in PK15 using a dual luciferase reporter assay. The vector containing the longest promoter fragment (PB-P1) displayed maximal transcriptional activity, which decreased to 43.67% when the DNA fragment from −493 to −206 bp was deleted (PB-P2) (Figure 3A). When the DNA fragment continued to be shortened, the transcriptional activity increased 2.19 times (PB-P3 vs PB-P2), nearly equal to that of PB-P1 (Figure 3A). However, when the inserted promoter DNA fragment was shortened further, the activity decreased rapidly (Figure 3A), indicating that the region from 145 bp upstream of the TSS (−145 bp) to 276 bp downstream of the TSS (+276 bp) was the minimal...
FIGURE 2 Overexpression of CXCL14 inhibited PRRSV replication by activating IFN signaling. A, The overexpression of CXCL14 in CD63-transfected PK15 cells (PK15CD163) at different time points was verified by Western blot analysis. “CXCL14_OV” represents different time points post transfection of the overexpression plasmid of CXCL14. “PRRSV_IN” represents different time points post infection with HP-PRRSV. Each sample was loaded in two lanes. B, The relative expression levels of PRRSV ORF7 were analyzed by qRT-PCR at different time points post infection. The black column and gray column represent samples transfected with the pcDNA 3.1 plasmid and the CXCL14 overexpression plasmid. C, GO enrichment analysis of 51 DEGs responding to CXCL14 overexpression in PK15CD163. The DEGs are listed in Table S1. D, E, qRT-PCR analysis of IFNB and IFNG with samples used for RNA-seq. “pcDNA3.1” represents three control samples that were transfected with the pcDNA3.1 plasmid, and “CXCL14_OV” represents three samples that were transfected with the CXCL14 overexpression plasmid. *P < .05, ***P < .001.
sequence required to maintain high transcriptional activity of CXCL14.

We cloned the core promoter region of porcine CXCL14 from 28 LW pigs, 37 TC pigs and 21 ET pigs to scan potential variants, identifying nine variants (numbered from 1 to 9): G-148A (the accession number in the dbSNP database is rs708552958, similarly denoted hereafter), (AGTG)$_1$-127(AGTG)$_2$ (Novel_INDEL-1), T-121C (rs323955863), A-63G (rs1110397684), T-33C (Novel SNP-1), T-22C (rs326828426), C-96A (rs326466001),
(AGC)_6-104(AGC)_3 (Novel_INDEL-2), and C-125G (rs320041156) (Figure 3B). Linkage disequilibrium (LD) analysis revealed that the above nine variants displayed strong LD and constituted a haplotype block ($r^2 \geq 0.9$) (Figure 3C). Haplotype analysis predicted two major haplotypes that accounted for 93.7% of all potential haplotypes (Table S2). All the examined LW pigs were homozygotes of Hap1 (Hap1: G(AGTG)_2TATTC(AGC)_6C), while TC and ET pigs contained both Hap1 and Hap2 (Hap2: A(AGTG)_2CGCCA(AGC)_3G) (Table S2). A luciferase reporter assay revealed that the construct with the promoter of Hap1 had 2.03 times higher activity than that of Hap2 ($P = 5.37 \times 10^{-7}$) (Figure 3D).

### 3.4 Site-directed mutagenesis and dual luciferase reporter assays identified two functional point mutations in the core promoter of CXCL14

To identify functional variants accounting for the differential promoter activity between Hap1 and Hap2, we created a series of mutant reporter vectors from the alleles of Hap1 to those of Hap2. First, we generated mutations for each of the three nearby sites, such as Hap1-M (1+2+3), representing the mutant Hap1 vector containing mutations in the first three sites, and, similarly, Hap1-M (4+5+6) and Hap1-M (7+8+9). The promoter activity of Hap1 was slightly higher than that of Hap1-M (1+2+3) and Hap1-M (7+8+9) (Figure 4A-C), while it was 2.07 times higher than that of Hap1-M (4+5+6) ($P = 1.81 \times 10^{-7}$) (Figure 4B). Then, the allele from the fourth to sixth site of Hap1 was mutated to the corresponding allele of Hap2 one by one. When the allele in the fourth site of Hap1 was mutated (Hap1-M4), the promoter activity did not show a significant change ($P = .15$) (Figure S2), whereas when the alleles in the fifth (Hap1-M5) and sixth sites (Hap1-M6) of Hap1 were mutated, the promoter activity decreased to 46.08% ($P = 9.8 \times 10^{-6}$) and 54.05% ($P = .000211$), respectively, of that of Hap1 (Figure 4D,E). Furthermore, the promoter activity of Hap1 was 2.00 times ($P = 1.45 \times 10^{-5}$) higher than the activity of the vector containing the corresponding Hap2 alleles in both the fifth and sixth sites (Hap1-M (5+6)) (Figure 4F). Therefore, we concluded that both the fifth (T-33C) and sixth (T-22C) sites respond to the differential promoter activities of Hap1 and Hap2.

### 3.5 EMSA and supershift assays verified that CEBPB can interact with the promoter of porcine CXCL14

Multiple sequence alignment revealed that the DNA element containing the fifth (T-33C) and sixth (T-22C) mutation sites in the porcine CXCL14 promoter was conserved among mammals (Figure 5A). To verify the potential interactions of this DNA element and unknown trans-acting factors, an EMSA was carried out using nuclear proteins extracted from PK15 cells. We synthesized two 41-bp probes containing C alleles (a CC probe) or T alleles (a TT probe) in both sites, as well as shorter competition probes, an RC probe, an LC-CC probe and an LC-TT probe (Figure 5B). Both the CC probe and TT probe detected a common band (Figure 5C, arrow 1 in lanes 2 and 5), while the TT probe detected an additional shifted band (Figure 5C, arrow 2 in lane 5). The specificity of the observed shifted bands was confirmed using the competition assay with excess unlabeled CC (U-CC) or TT (U-TT) probe (Figure 5C, lanes 3 and 6). The common band could be prevented by excess unlabeled RC probe (U-RC) (Figure 5D,E, lane 5) but not unlabeled LC-CC (U-LC-CC) or LC-TT probe (U-LC-TT) (Figure 5D,E, lane 4), indicating that the common band arose due to the conserved DNA element in the right part of the probe. The specific band for the TT probe could be eliminated only by excess unlabeled LC-TT probe (Figure 5E, lane 4), not by unlabeled RC probe (Figure 5E, lane 5), indicating that the specific band was due to the DNA element in the left part of the probe. Thus, the specific band indicated that some unknown trans-acting factors could uniquely bind to the TT probe and probably respond to the differential promoter activities of Hap1 and Hap2.

We predicted potential transcriptional factors (TFs) that could interact with the 41-bp DNA element, identifying the binding sites of several TT probe-specific TFs, such as CEBPA, CEBPB, and Gfi1b (Table S3). To experimentally identify possible TFs, we conducted protein pull-down assays and mass spectrometry. Mass spectrometry analysis characterized a total of 2,163 proteins captured in the CC or TT group but not in the NC group, of which CEBPB, CEBPD, and CEBPG were identified in the TT group (Table S4). However, only one nonspecific peptide of CEBPB was detected in the CC group (Table S4). Thus, we selected CEBPBA, CEBPB, and CEBPD as candidate TFs for further supershift EMSA verification. The results revealed that the antibody of CEBPB could visibly weaken the signal of the TT probe-specific band (Figure 5F, arrow 2 in lane 5 vs that in lane 2/4) and yield a supershifted band (Figure 5F, arrow 3 in lane 5), whereas the antibodies of CEBPA and CEBPB did not produce any supershifted band for either the CC or TT probe (Figure S3). Meanwhile, the CEBPB antibody had no effect on the common band of both probes (Figure 5F, G, arrow 1 in lane 5) and did not produce a supershifted band for the CC probe (Figure 5G), confirming that CEBPB could specifically bind with the TT probe. The specific binding capacity of CEBPB was further confirmed by the supershift EMSA using nuclear extracts from PAMs, in which the TT probe could form a very strong shifted band with CEBPB (Figure 5H), whereas the
CC probe formed a very weak shifted band (Figure 5I). Thus far, we have identified CEBPB as a transcription factor that can uniquely bind to the TT probe in vitro.

3.6 | CEBPB can regulate the expression of CXCL14 in vivo

To evaluate the potential interaction of CEBPB and the promoter of CXCL14 in vivo, transient overexpression of HA-tagged CEBPB (pCMV-HA-CEBPB) was performed in PK15 cells. A ChIP assay was performed at 48 hours post transfection, which was confirmed by Western blot analysis using an HA tag antibody (Figure 6A). Then, the DNA levels were measured in the precipitates by qPCR, which showed 11.1 times enrichment of the DNA fragments from the CXCL14 promoter in the precipitate of HA-CEBPB vs that of IgG (P = .0066) (Figure 6B).

To further investigate the regulatory effect of CEBPB on CXCL14, the overexpression vector and siRNAs targeting CEBPB were transfected into PK15 cells. When CEBPB was overexpressed, the expression of CXCL14 did
FIGURE 5  In vitro analysis of the interaction between CEBPB and CXCL14. A, Multiple sequence alignment among mammals of the DNA elements surrounding the fifth and sixth mutation sites. The functional mutation sites in the porcine CXCL14 promoter are labeled with arrows, and their positions are relative to the TSS of porcine CXCL14. B, Schematic of EMSA probes. The red bases represent the two functional mutations. The “LC-CC(TT) probe” represents the sequence of the left competition probe of the CC type or TT type, and the “RC probe” represents the sequence of the right competition probe. C-E, The results of EMSAs. Arrow 1 represents the common band and arrow 2 represents the specific band for the TT probe. U-CC represents unlabeled CC probe, similar to U-TT, U-LC-CC, U-LC-TT and U-RC, and B-CC represents biotin-labeled CC probe, similar to B-TT. “protein” represents the PK15 nuclear extracts. F-I, The results of supershift EMSAs. F and G are the results of assays with nuclear extracts of PK15 cells, and H and I are the results obtained for PAMs. “IgG” represents the IgG control antibody, and “CEBPB” represents the antibody of CEBPB. Other labels have similar meanings as those in C-E. Arrow 1 represents the common band and arrow 2 represents the specific band for the TT probe, arrow 3 represents the supershifted band in PK15, arrow 4 represents the specific band for the TT probe in PAMs, and arrow 5 shows the supershifted band in PAMs
not change significantly at the mRNA and protein levels (Figure S4A-C). The interference effect of siRNAs targeting CEBPB was evaluated by qRT-PCR, in which two siRNAs (siRNA-770 and siRNA-866) displayed very strong inhibitory effects (Figure S4D). When two validated siRNAs were transfected into PK15 cells, the expression of CXCL14 decreased significantly at the mRNA and protein levels (Figure 6C,D). Similar to CXCL14, CEBPB was also downregulated in PAMs from PRRSV-infected TC and LW pigs vs control pigs (Figure 6E). Thus, these results confirmed that CEBPB could regulate the expression of CXCL14 in vivo.

4 | DISCUSSION

4.1 | Function of CXCL14 in PRRSV infection

As described above, CXCL14 plays antimicrobial\textsuperscript{9,10,23} and immune surveillance\textsuperscript{11,12} roles and regulates neuronal activity.\textsuperscript{13,14} Although its expression is altered significantly during infection by viruses, such as murine West Nile virus,\textsuperscript{24} human papillomavirus\textsuperscript{25} and hepatitis B virus,\textsuperscript{26} its function during viral infection is less well understood. In the present study, we observed that transient overexpression of CXCL14 inhibited PRRSV replication (Figure 2A,B). To our knowledge, this is the first direct evidence of the antiviral effect of CXCL14, which should be further confirmed by other experiments, such as the detection of PRRSV itself via Western blot analysis or IF and cytopathic effect analysis.

The antiviral effects of chemokines are consistent with their regulation of immunity and inflammation via cooperation with tumor necrosis factor (TNF).\textsuperscript{27} It seems that the antiviral effect of CXCL14 is mediated by the IFN-I signaling pathway, which is highly consistent with the up-regulation of IFNB (Figure 2D) and many interferon-stimulated genes (ISGs) responding to CXCL14 overexpression (Figure S1A,B). Until now, there has been no direct evidence of the relationship between CXCL14 expression and interferon, but a mechanistic relationship between the expression level of CXCL4, another CXC chemokine, and the IFN-α signature in systemic sclerosis was reported recently.\textsuperscript{28} Therefore, a similar mechanistic relationship between CXCL14 expression and IFN-β signaling could be proposed; this relationship requires further investigation.

It is well known that IFN-I (IFN-α/β) are major antiviral cytokines, displaying a strong inhibitory effect on PRRSV.\textsuperscript{29,30} However, both IFN-α and IFN-β are generally inhibited in PRRSV-infected pigs and PAMs.\textsuperscript{30,31} Here, we showed that CXCL14 probably exerted an antiviral effect by activating IFN-I signaling, but its expression was also
inhibited by PRRSV. Therefore, the restriction of PRRSV to CXCL14 probably represents a joint inhibition mechanism for immune evasion triggered by PRRSV infection.

### 4.2 Regulation of CXCL14

Under PRRSV infection, CXCL14 displayed different degrees of downregulation in PAMs of TC and LW pigs, indicating the involvement of breed genetic variants. Accordingly, we constructed a series of reporter vectors to characterize the core promoter of porcine CXCL14 and potential functional variants. We noticed that the PB-P2 construct with an extended sequence displayed lower transcriptional activity than PB-P3 (Figure 3A). Bioinformatic prediction revealed that the extended sequence contains multiple binding sites for transcriptional repressors, such as SNAI2 and ZEB1 (data not shown). The PB-P2 construct probably provided the chance for transcriptional repression, leading to decreased transcriptional activity; this, however, remains to be validated. The characterized core promoter (from −145 to +276 bp of TSS) is perfectly aligned to that of human CXCL14 (from −250 to +193 bp of TSS), which contains four essential cis-elements: an AP1 binding site, a TATTAA sequence and two tandem GC boxes as the binding sites of SP1. We detected one common shifted band (Figure 5C, arrow 1 in lines 2 and 5) by EMSA using both CC and TT probes, which was probably due to the predicted binding site of AP1 in the right part of the probe. Therefore, EMSA in the present study confirmed the existence of the AP1 binding site, but the exact transcription factor of the API family remains to be identified.

In the present study, we identified two closely located functional variants that altered the binding status of CEBPB. CEBPB belongs to the C/EBP family of basic region-leucine zipper (bZIP) proteins and regulates a variety of important biological processes, such as monocytic cell differentiation and mammary luminal cell fate determination. Importantly, CEBPB plays an essential role in the generation, activation and polarization of macrophages, and CEBPB knockout mice lack alveolar macrophages. Interestingly, CXCL14 is also highly expressed in the CEBPB-positive cells mentioned above, especially epithelial cells and macrophages, which was further confirmed in the present study (Figure 1). The consistent tissue expression pattern of CEBPB and CXCL14 suggests that the regulation by CEBPB of CXCL14 is probably a conserved mechanism among different types of cells. This assumption is partly supported by the EMSAs in PK15 cells and PAMs, but much more evidence is needed from other tissues or cells.

CXCL14 was downregulated in both PAMs and epithelial cells of the lung in response to PRRSV infection (Figure 1). Meanwhile, CEBPB displayed a similar downregulation trend in PAMs under PRRSV infection (Figure 6E), contributing at least in part (if not completely) to CXCL14 downregulation under PRRSV infection. Unlike other infection-triggered chemokines, CXCL14 maintained constitutive expression in a normal physiological state but was downregulated in unusual conditions such as malignant tissue, pathogenic infections and chemical exposure. Abnormal hypermethylation of the CXCL14 promoter and regulation via both the MAPK/ERK and JAK3/STAT6 pathways were proposed to be the mechanisms leading to CXCL14 downregulation. However, to our knowledge, no transcriptional factor that directly regulates CXCL14 expression has been identified to be responsible for its downregulation. Therefore, the direct regulatory effect of CEBPB on CXCL14 could provide valuable clues for deciphering the mechanism of CXCL14 downregulation under the unusual conditions mentioned above.

### 4.3 Two coupled mutation sites in the promoter of CXCL14

In the promoter region of porcine CXCL14, we identified two coupled mutation sites, which formed two major haplotypes representing the distinct binding status of CEBPB and the transcriptional activity. It seems that Hap1 with the T allele at both sites is the major haplotype in both LW and TC pigs, and Hap2 with the C allele at both sites is a TC pig-specific haplotype. No Hap2 was detected in LW pigs, but the population size of the present study was relatively small; further analysis is needed in other populations with large sizes.

PRRSV infection leads to downregulation of both CEBPB and CXCL14. Interestingly, the Hap2 haplotype could not bind with CEBPB and may evade the inhibitory effect of PRRSV mediated by CEBPB. Therefore, the individuals with the Hap2 haplotype could maintain a relatively high level of CXCL14 expression under PRRSV infection, which is probably one of the reasons that TC pigs displayed relatively high expression levels of CXCL14 under infection. Given the functionality of the two characterized mutation sites and the potential antiviral effect of CXCL14 via IFN-I, we speculate that the two mutation sites probably contribute to the PRRSV resistance (or tolerance) of TC pigs. Therefore, it would be interesting to analyze the association of their polymorphisms and interferon levels with PRRSV resistance.

### 4.4 PRRSV resistance in TC pigs

We found that CEBPB could regulate the expression of CXCL14. A previous study showed that CEBPB is necessary for the expression of interleukin 10 (IL-10) in macrophages. IL-10 is the key Th2 anti-inflammatory cytokine
inducing PRRSV-triggered immune suppression,\(^4\),\(^5\) which is highly upregulated in LW pigs but mildly upregulated in TC pigs within 7 dpc with PRRSV.\(^5\) The balance between IL-10 and interferon gamma (IFN-\(\gamma\)), the major Th1 pro-inflammatory cytokine, plays an essential role in determining the outcome of PRRSV infection,\(^4\)\(^6\)\(^7\) which may be the key to dissecting the genetic difference between the two pig breeds. Therefore, one interesting question is whether and how the CEBPB-CXCL14 signaling axis affects the balance of IL-10 and IFN-\(\gamma\).

Given the obvious difference in PRRSV resistance between TC and LW pigs, we will build a segregation population with these two breeds, in which the phenotype will be measured using more accurate indicators, such as the percentages of specific lymphocytes and the levels of IL-10 and IFN-\(\gamma\). Related work on the segregation population is ongoing, and QTLs and related functional variants for PRRSV resistance in TC pigs are expected to be identified. Regarding this point, the present study may provide a good reference.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
X. Xu and B. Liu designed the research; L. Niu, Q. Xue, Y. Liu, and X. Hu conducted the experiments; Z. Zheng analyzed the transcriptome data; X. Xu and L. Niu wrote the manuscript; H. Cheng, A. Zhang, and H. Wang participated in discussion, data analysis, and manuscript revision. All authors have read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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