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Exacerbated Type II Interferon Response Drives Hypervirulence and Toxic Shock by an Emergent Epidemic Strain of *Streptococcus suis*

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***Streptococcus suis*, a major porcine pathogen, can be transmitted to humans and cause severe symptoms. A large human outbreak associated with an unusual streptococcal toxic shock-like syndrome (STSLS) was described in China. Albeit an early burst of proinflammatory cytokines following Chinese *S. suis* infection was suggested to be responsible for STSLS case severity, the mechanisms involved are still poorly understood. Using a mouse model, the host response to *S. suis* infection with a North American intermediately pathogenic strain, a European highly pathogenic strain, and the Chinese epidemic strain was investigated by a whole-genome microarray approach. Proinflammatory genes were expressed at higher levels in mice infected with the Chinese strain than those infected with the European strain. The Chinese strain induced a fast and strong gamma interferon (IFN-γ) response by natural killer (NK) cells. In fact, IFN-γ-knockout mice infected with the Chinese strain showed significantly better survival than wild-type mice. Conversely, infection with the less virulent North American strain resulted in an IFN-β-subjugated, low inflammatory response that might be beneficial for the host to clear the infection. Overall, our data suggest that a highly virulent epidemic strain has evolved to massively activate IFN-γ production, mainly by NK cells, leading to a rapid and lethal STSLS.**

Streptococcus suis, an encapsulated Gram-positive bacterium, is a major porcine pathogen endemic worldwide. It is responsible for a wide range of clinical symptoms in swine, such as meningitis, arthritis, endocarditis, pneumonia, and septicemia with sudden death, and causes vast economic losses in the swine industry. To date, 35 serotypes or capsular types of *S. suis* based on the capsular polysaccharide (CPS) composition have been described. *S. suis* serotype 2 is the type most commonly isolated from diseased animals and humans in most countries (1). Besides, *S. suis* serotype 2 is considered an emerging zoonotic pathogen, raising considerable international concerns among public health professionals. At the outset, infections in humans had usually been considered sporadic in people working with pigs or pork-derived products (1). After being an old neglected zoonotic infection for many decades, *S. suis* is now considered one of the most important emerging infectious diseases in Asian countries, where most people have regular contact with raw pork meat. In fact, *S. suis* has become the leading cause of adult meningitis in Vietnam, the second leading cause in Thailand, and the third leading cause in Hong Kong (2–4). In humans, *S. suis* usually produces a purulent meningitis, albeit other pathologies have been described (5, 6). The clinical characteristics typical of acute meningitis in humans caused by *S. suis* changed after the 2005 outbreak in the Chinese province of Sichuan (7). The most important feature of this outbreak was a high incidence of systemic disease with a high fatality rate and a relatively low number of meningitis cases (7). The clinical presentation was characterized as streptococcal toxic shock-like syndrome (STSLS), similar to that usually associated with *Streptococcus pyogenes* (7, 8) (Fig. 1A). Although the 2005 disease outbreak in China is the largest recorded outbreak of *S. suis* infection in humans, another smaller Chinese outbreak with the same clinical characteristics took place in Jiangsu Province in 1998 (9). The Chinese human outbreaks are the only ones reported with many patients presenting acute symptoms related to STSLS.

Interestingly, and differing from what has been observed in Europe and Asia, the prevalence of serotype 2 strains recovered from diseased pigs in North America remains relatively low compared to that of strains of other serotypes (10, 11). In addition, most zoonotic cases of *S. suis* in Western countries have taken place in Europe, and only three cases of human disease with *S. suis* serotype 2 have been reported on the mainland of the United States and Canada, with all of them being nonfatal (12). These observations led to the hypothesis that Asian, European, and North American *S. suis* serotype 2 strains possess different potentials for virulence.

Multilocus sequence typing (MLST) has shown that many serotype 2 strains from North America belong to sequence type 25 (ST25), which differs from the sequence types of European and Asian isolates, which are mainly ST1 (13, 14). The clonal strain responsible for both Chinese outbreaks was classified as ST7, a new sequence type included within the ST1 complex (Fig. 1B) (15). Although responsible for cases of STSLS, to date no typical superantigens could be found in this strain (9). It has been proposed that the ST7 strain recently evolved from a pathogenic ST1 strain, which in turn evolved from the intermediately virulent ST25 strain that is usually found in North America (16). After genome-wide comparisons (17–19), the contribution of diverse

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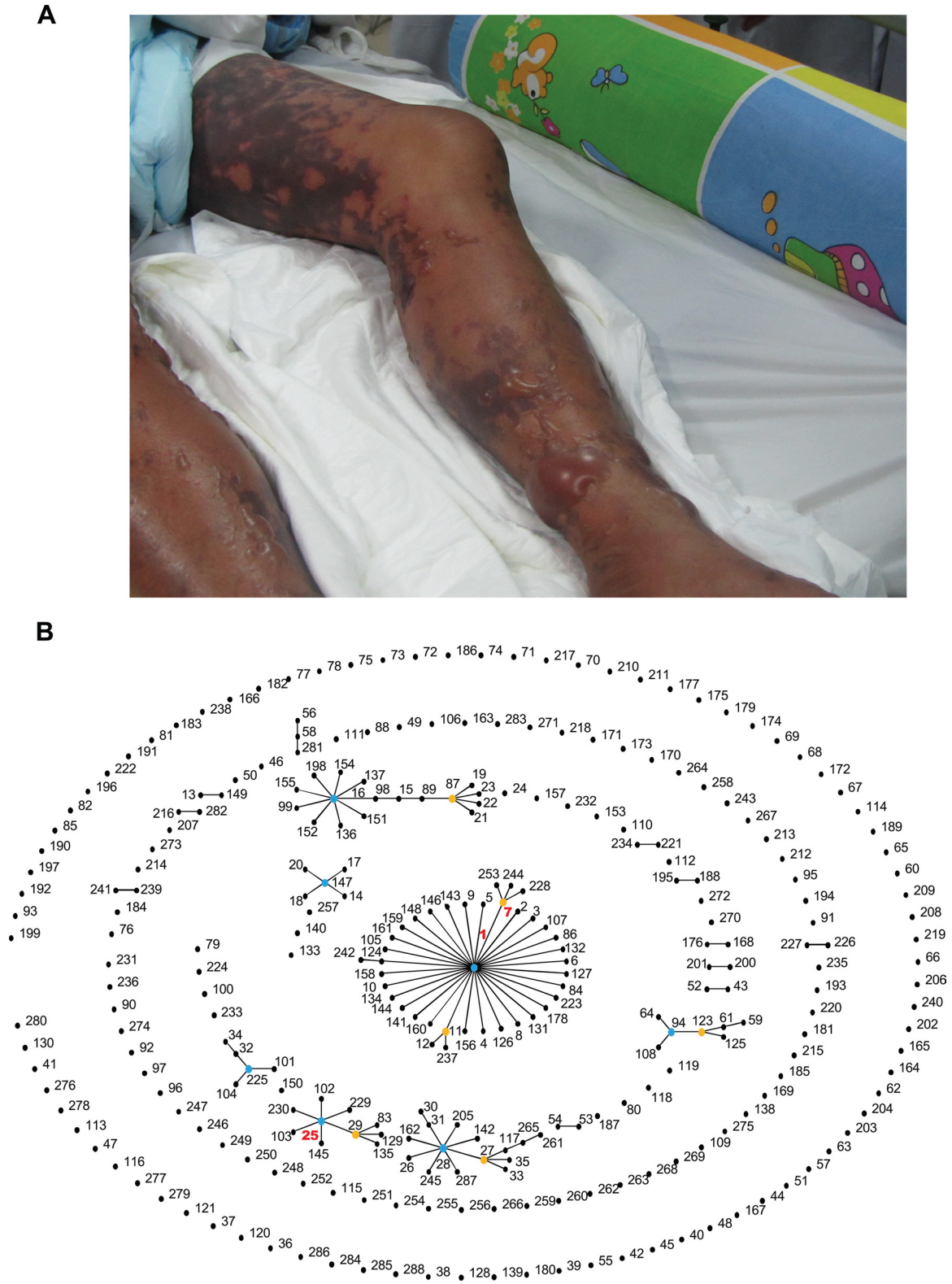

FIG 1 (A) A patient infected with the epidemic Chinese strain of *S. suis* presenting clinical signs of STSLS, featuring subcutaneous hemorrhage (purpura) in the leg. (B) MLST relationship of *S. suis* serotype 2 North American intermediately pathogenic strain 89-1591 (ST25), European highly pathogenic strain P1/7 (ST1), and Chinese epidemic strain SC84 (ST7). The whole *S. suis* MLST database (288 STs in total) is shown as a population snapshot obtained by eBURST (version 3). Each ST was represented by a single strain in the input population profile data. The linked clusters within the diagram represent clonal complexes. Primary founders and subgroup founders of these linked clusters are colored blue and yellow, respectively. Other unlinked individual STs are colored black. The three most important STs, ST7, ST1, and ST25, are labeled with red text to emphasize their significance.

discrete genomic regions and polymorphisms to the different virulence traits of the MLST types remains speculative.

The severity and outcome of infections caused by *S. suis* likely depend on the ability of host immune mechanisms to control bacterial growth and to limit the spread of the pathogen without causing excessive inflammation. Our laboratory previously developed a mouse model of *S. suis* systemic infection (20, 21). Infected animals can succumb rapidly, and this mortality can be associated with septic shock symptoms. Animals that survive the acute phase of infection might later develop clinical symptoms of meningitis. The characteristic type 1 inflammatory response that is witnessed following host infection by *S. suis* involves a complex proinflammatory cascade that includes the activation of various immune cells, as demonstrated *in vitro* and *in vivo* (1). As such, it has been suggested that increased expression of certain inflammatory mediators by the Chinese ST7 strain might be responsible for the severity of cases and STSLS (16). However, the patterns of the host response that drive the *S. suis* infection to a benign path, with eventual clearance of the pathogen, or an aggressive path, resulting in disease development, are poorly understood. Therefore, the goal of this study was to compare the innate immune response of the host following an acute infection by three different strains of *S. suis* having dissimilar virulence potentials: a representative typical intermediately pathogenic ST25 North American strain (89-1591) (22), a representative highly pathogenic ST1 European strain (P1/7) (23), and the epidemic Chinese ST7 strain responsible for the STSLS outbreaks in China (SC84) (16). A whole-genome approach was thus undertaken to evaluate and understand the modulation of host genes that might be crucial during the initial step of the host immune response to an infection by these three different *S. suis* strains, especially the highly virulent Chinese epidemic strain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. suis* serotype 2 strains 89-1591 (ST25, an intermediately pathogenic strain), P1/7 (ST1, a highly pathogenic strain), and SC84 (ST7, an epidemic strain) were used in this study. Strain 89-1591 was recovered from a case of pig septicemia in Canada (22), strain P1/7 was isolated from a case of pig meningitis in the United Kingdom (17), and strain SC84 was isolated from a patient with STSLS during the Chinese outbreak in 2005 (15). All the strains are encapsulated under the culture and assay conditions used. Bacteria were grown overnight on sheep blood agar plates (PML) at 37°C, and three colonies isolated from each strain were inoculated into 5 ml of Todd-Hewitt broth (THB; Difco Laboratories), which was incubated for 8 h at 37°C without agitation. Working cultures were prepared by transferring 10 μl of 1/1,000 dilutions of 8-h cultures into 30 ml of THB, which was incubated for 16 h at 37°C. Bacteria were washed twice in phosphate-buffered saline (pH 7.3). The bacterial pellet was then resuspended and adjusted to a concentration of 5×10^8 CFU/ml. The inoculum for experimental infection was then diluted in THB to obtain a final concentration of 1×10^7 CFU/ml. This final suspension was plated onto sheep blood agar plates to accurately determine the number of CFU/ml and to verify the purity of the inoculum.

Mice and experimental infections. Six- to 8-week-old female C57BL/6 mice (Jackson Laboratory) were acclimatized to standard laboratory conditions with a 12-h light/12-h dark cycle and unlimited access to water and rodent chow. Age-matched gamma interferon (IFN-γ)-knockout (KO) mice (B6.129S7-*Ifng*^{tm1Ts}/J) were also purchased from the Jackson Laboratory. All experiments involving mice were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care. On the day of the experiment, a 1-ml volume of either the bacterial suspension (1×10^7 CFU/ml) or the vehicle solution (sterile THB) was administered by intraperitoneal injection. Mice were euthanized at 3 and 6 h postinfection for the microarray and real-time reverse transcription-quantitative PCR (RT-qPCR) study. For cytokine and chemokine analysis, mice were euthanized at 6 h postinfection. The dose of infection and postinfection times were selected on the basis of our previous work (21).

Measurement of blood bacterial loads. At each time point (3 h and 6 h postinfection), blood was collected by cardiac puncture and mixed with 40 μl of an EDTA (0.16 M) solution to prevent clotting. Proper dilutions of 50 μl of blood samples were plated using an Autoplate 4000 automated spiral plater (Spiral Biotech Inc.). Sheep blood agar plates were incubated overnight at 37°C. Colonies were counted and expressed as the number of CFU/ml.

Spleen collection, homogenization, and extraction of total RNA. At 3 and 6 h, spleens from euthanized mice were removed, cut in small pieces, and put into 1.5 ml of RNAlater solution (Qiagen) for stabilization of total RNA. Approximately 25 mg of spleen was then disrupted and homogenized in 600 μl of lysis buffer (Qiagen) using a rotor stator homogenizer (Tissue-tearor model 398; Biospec Products). Total RNA from homogenized tissue was isolated and purified using an RNeasy minikit with on-column DNase digestion (Qiagen) and kept at −80°C until use.

Illumina microarray analysis. The microarray experiment was performed at the McGill University and Genome Quebec Innovation Centre (Montreal, Quebec, Canada), using the Illumina whole-genome expression Beadchip technology platform (Illumina). Prior to the microarray experiment, total RNA quality and quantity were assessed using an Agilent 2100 bioanalyzer (Agilent Technologies). The microarray was performed according to the manufacturer's instructions. Sample positions on the chip were randomly distributed.

Text files containing the signal and detection *P* values per probe for each sample were imported into FlexArray software v.1.6 (McGill University and Genome Quebec Innovation Centre). Data were first raw filtered in order to identify and remove probe sets that were not detected. The data with only present array features were then further preprocessed by applying a lumi filter to normalize specifically Illumina microarray data. Afterward, an analysis of variance (ANOVA) was used to search for differentially expressed genes between the infected and mock-infected groups or between infected groups in certain cases. In order to maintain manageable data sets, differentially expressed genes were defined by fold changes greater than 3-fold with an accompanying *P* value of ≤0.05.

Validation of microarray data by real-time RT-qPCR. Fourteen genes were selected for the validation of microarray results by RT-qPCR. The qPCR analysis was executed to conform to the minimum information for publication of quantitative real-time PCR experiment (MIQE) guidelines (24). The validation experiments were performed using the same RNA samples that were used for the microarray study and other supplemental samples. Extracted RNA was converted into cDNA by reverse transcription of 500 ng total RNA using an iScript cDNA synthesis kit (Bio-Rad). Then, qPCR assays were carried out using a SsoFast Evagreen supermix kit (Bio-Rad) and gene-specific primers (250 nM) on a CFX96 rapid thermal cycler system (Bio-Rad). The cycling conditions were 3 min of polymerase activation at 98°C, followed by 40 cycles at 98°C for 2 s and 57°C for 5 s. Melting curves were generated after each run to confirm the presence of a single PCR product.

All primers (Integrated DNA Technologies) that were used for detection of genes were verified to have reaction efficiencies of between 90 and 110% (see Table S1 in the supplemental material). The GeNorm applet v.3.5 (http://medgen.ugent.be/~jvdesomp/genorm/) was used to initially determine the two most stable reference genes from a set of five reference genes using random samples from the cDNA panel generated for the qPCR validation of the microarray. Therefore, normalization of the data was done using the reference genes β-actin and β2-microglobulin. The fold change of gene expression was calculated using CFX software manager v.2.1 (Bio-Rad). Samples from mock-infected mice were used as a calibrator.

Luminex quantification of plasma cytokine and chemokine levels. Blood collected from mice euthanized at 6 h and stabilized with EDTA was centrifuged at 1,300 × *g* for 10 min at 4°C. Plasma was then collected and kept at −80°C. Plasma concentrations of mouse CCL2 (monocyte chemotactic protein-1 [MCP-1]), CCL3 (macrophage inflammatory protein 1α [MIP-1α]), CCL4 (MIP-1β), CXCL1 (keratinocyte-derived chemokine [KC]), CXCL2 (MIP-2α), CXCL9 (monokine induced by IFN-γ [MIG]), CXCL10 (IFN-γ-induced protein 10 [IP-10]), IFN-γ, interleukin-1β (IL-1β), IL-6, and tumor necrosis factor (TNF) were determined using a Milliplex custom-made 11-plex cytokine panel (Millipore) according to the manufacturer's instructions. Acquisition was performed on a Luminex 100 platform (Luminex), and analysis was performed using Beadview multiplex data analysis software v.1.0 (Upstate/Millipore).

NK and DC cocultures. Untouched natural killer (NK) cells were purified from naive C57BL/6 mice using NK cell isolation kit II microbeads and magnetically activated cell sorting negative selection (Miltenyi Biotec). Purified NK cells were expanded *in vitro* for 8 days in the presence of 500 ng/ml of recombinant mouse IL-2 (Miltenyi Biotec). The resulting NK cell purity was routinely >95% $NK1.1^+$ and $CD3^-$ (as determined by fluorescence-activated cell sorting using anti-mouse NK1.1 clone PK-136 and anti-mouse CD3 clone 145-2C11; BD Pharmingen). Bone marrow-derived dendritic cells (DCs) were produced as previously described (25). DC and NK cell cocultures were established at a ratio of 1:5 in complete medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol (Invitrogen). Cocultures were infected with 2.5×10^5 CFU of different *S. suis* strains. After a bacterium-cell contact time of 6 h, gentamicin (20 μg/ml) was added to prevent cell toxicity. Supernatants were collected after a final 14 h of incubation, and IFN-γ levels were analyzed by enzyme-linked immunosorbent assay (ELISA; R&D Systems). Nonstimulated cells (medium alone) or cells stimulated with a combination of 1 μM CpG (ODN 1826; Coley Pharmaceutical Inc.) and 1 μg of lipopolysaccharide (LPS; Sigma-Aldrich) served as negative and positive controls, respectively. In addition, single DC cultures were also included as controls.

Statistical analysis. Data are presented as survival curves, obtained using the Kaplan-Meier method; the mean ± standard error of the mean (SEM); or the geometric mean with 95% confidence interval, where appropriate. Prism v.5 software (GraphPad) was used for data analysis. One-way ANOVA followed by Student-Newman-Keuls secondary analysis was performed to find statistical differences between groups. Log-rank (Mantel-Cox) tests were used to compare the survival curves of the different groups studied, and alpha levels were adjusted to a Bonferroni-corrected threshold for multiple comparisons. *P* values of <0.05 were considered statistically significant.

Microarray data accession numbers. The microarray raw data files were deposited and are available in the Gene Omnibus Expression database under accession numbers GSE41520, GSE41521, and GSE41522.

RESULTS

Difference in virulence among North American, European, and Chinese representative strains of *S. suis* serotype 2. It is hypothesized that the epidemic Chinese SC84 strain is more virulent than the highly pathogenic European P1/7 strain, with the intermediately pathogenic North American 89-1591 strain being the least virulent. However, the virulence of these representative strains of the ST7, ST1, and ST25 MLST types has never been evaluated in a well-standardized comparative experimental mouse model. Using our C57BL/6 mouse model of systemic infection (21), we showed that the survival of mice infected with the North American 89-1591 strain was not significantly affected and thus similar to that of mock-infected mice ($P = 0.5$). These mice showed mild or no clinical signs of disease (Fig. 2A). However, the survival of mice infected with *S. suis* European highly pathogenic strain P1/7 or

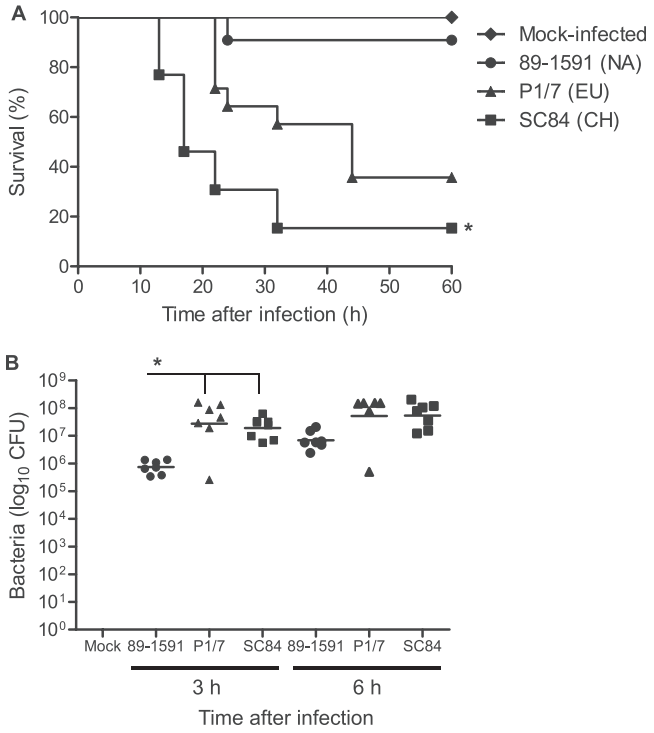

FIG 2 The *S. suis* epidemic Chinese strain is more virulent than the highly pathogenic European strain and the intermediately pathogenic North American strain. (A) Survival curves for C57BL/6 mice intraperitoneally infected with 1×10^7 CFU of *S. suis* strains originating from North America (89-1591), Europe (P1/7), or China (SC84). As controls (mock infected), animals were injected with the same broth that was used to grow the bacterial strains (Todd-Hewitt broth) ($n = 14$ for mice infected with each of *S. suis* strains; $n = 5$ for mock-infected mice). *, significantly different ($P \leq 0.05$) compared to P1/7-, 89-1591-, or mock-infected mice, as determined by the log-rank (Mantel-Cox) test. (B) Blood bacteremia of C57BL/6 mice infected with the North American (89-1591), the European (P1/7), or the Chinese (SC84) strain of *S. suis*. C57BL/6 mice ($n = 7$ per group) were intraperitoneally infected as described for panel A. At 3 h and 6 h postinfection, mice were euthanized, bacteria from blood were plated, and colonies were counted and expressed as the number of CFU/ml. *, significantly different ($P \leq 0.05$) compared to mice infected for 3 h with the European P1/7 or the Chinese SC84 strain of *S. suis*, as determined by one-way ANOVA.

Chinese epidemic strain SC84 was significantly different from that of mock-infected mice, with *P* being equal to 0.026 and 0.004, respectively. Similarly, mice infected with these strains had significantly different survival than mice infected with North American strain 89-1591 ($P = 0.008$ and $P = 0.0002$ for P1/7 and SC84, respectively). Finally, there was also a significant difference in the survival of mice infected with the European P1/7 strain and mice infected with the Chinese SC84 strain ($P = 0.024$). Chinese strain-infected mice had a 50% survival rate at 17 h, while mice infected with the European strain had a 50% survival rate at 44 h (Fig. 2A). Hence, our infection data support the hypothesis that *S. suis* strain ST7 is more virulent than an ST1 European strain, with the ST25 North American strain being the least virulent of all three strains.

ST-specific virulence degrees are not related to differences in bacteremia levels. Having shown intrinsic variations in virulence among ST types of *S. suis*, we further investigated whether these differences correlate with bacterial capacity to survive and disseminate in the bloodstream. First, we confirmed *in vitro* that growth rates in THB culture medium were similar among the 89-1591,

P1/7, and SC84 strains during either the exponential or the stationary phase of growth (see Fig. S1 in the supplemental material). Next, blood bacteremia was assessed in 89-1591-, P1/7-, or SC84-infected mice. At 3 h postinfection, all mice were bacteremic and the blood bacterial burden was similar in mice infected with either the European or the Chinese strain (Fig. 2B). Mice infected with the North American 89-1591 strain had an approximately 1-$\log_{10}$-unit lower blood bacterial burden than the other two groups of infected mice ($P < 0.001$). However, at 6 h postinfection, *S. suis* bacteremia levels were similar in mice infected with either of the evaluated strains, with no significant differences being detected between the three groups (Fig. 2B). These data suggest a delayed capacity of strain 89-1591 to establish infection, rather than a higher susceptibility of this strain to bacterial clearance by the host.

In agreement with the findings of previous studies (20, 21, 26), patterns of bacterial burdens observed in spleen and liver were similar to those observed in blood samples, with no significant differences being detected among strains (data not shown). These results suggest that the increased virulence of *S. suis* ST7 cannot be explained by an increase in blood bacterial burden and that other factors account for its pathogenic potential.

The number and level of upregulated proinflammatory genes correlate with *S. suis* virulence degree. To explore the molecular basis of the increased virulence of the *S. suis* ST7 epidemic strain, a gene expression microarray study was undertaken to provide a global view of the gene expression profile in *S. suis*-infected mice. An Illumina whole-genome microarray assay was performed on spleen samples from C57BL/6 mice infected with the 89-1591, P1/7, or SC84 *S. suis* strain. Mock-infected mice were used as controls. Mice were euthanized at 3 or 6 h postinfection, and total RNA was isolated from the spleen and used for the microarray analysis in order to explore early innate immune mechanisms that might be associated with the rapid onset of death observed after infection with the Chinese strain (Fig. 2A).

Gene expression was considered significantly modified when their corrected *P* values were <0.05 and the change of expression was more than 3-fold compared to that in mock-infected mice. At 3 h postinfection, the majority of changes in gene expression were due to upregulation, while very few genes were downregulated (Fig. 3A and B). Interestingly, the number of genes specifically upregulated at 3 h postinfection with the Chinese SC84 strain was higher than that observed in the other two groups of infected mice (Fig. 3B). At 6 h postinfection, the majority of changes in gene expression were also due to upregulation, with the total number of modified genes being much higher than that observed at 3 h for mice infected with either of the *S. suis* strains (Fig. 3C and D). Furthermore, there was an even more pronounced difference in the total number of genes exclusively modified at 6 h in mice infected with the Chinese SC84 strain than mice infected with either the North American 89-1591 strain or the European P1/7 strain. These observations might correlate with the ability of the Chinese epidemic strain to induce a rapid onset of death after infection, as shown in Fig. 2A.

Gene expression modifications in infected mice were then clustered by functions. Table 1 provides important genes which were upregulated at 6 h postinfection. The entire list of genes modified at 3 h postinfection can be found in Tables S2 and S3 in the supplemental material. Genes upregulated or downmodulated at 6 h postinfection are displayed in Tables S4 and S5 in the supplemental material. Downregulated genes did not provide useful insights into the immunopathogenesis of *S. suis* infection. On the other hand, upregulated gene functions predominantly related to immune response, such as cell adhesion and migration, transcriptional and translational regulation, cytokine signaling, and expression of cytokines, chemokines, and related receptors, were found. In particular, we observed that expression of the proinflammatory mediators CCL2, CCL3, CCL4, CCL7, CXCL1, CXCL2, IL-1α, IL-1β, and IL-6 was overall higher in mice infected with the Chinese SC84 strain than mice infected with either of the other strains (Table 1; see Tables S2 and S4 in the supplemental material). Moreover, mice infected with the North American strain presented in general the lowest levels of expression of proinflammatory cytokine, chemokine, and related genes. From these data, we might assume that mice infected with the Chinese ST7 strain develop a stronger and earlier proinflammatory response than mice infected with ST1 or ST25 *S. suis* strains.

Gene expression was next analyzed by real-time RT-qPCR to confirm and validate the results obtained from the Illumina microarray assay. As shown in Table 1, our RT-qPCR results for selected genes followed the same trends obtained by microarray analysis. Differences in gene expression among groups of infected mice were confirmed or even amplified using this more powerful and quantitative technique, as reported previously (27). In accordance with our microarray data, mice infected with the Chinese

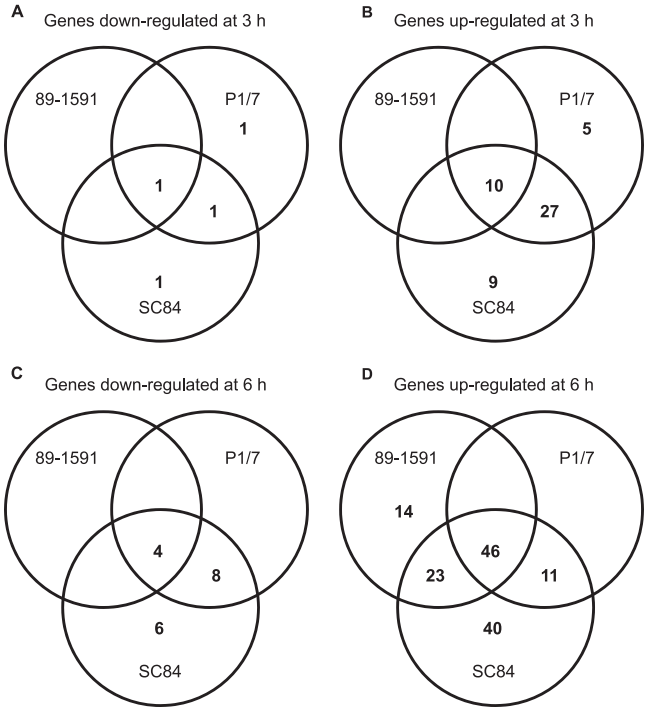

FIG 3 Host genes are modified in greater numbers in C57BL/6 mice infected with the epidemic Chinese strain of *S. suis* than mice infected with either the highly pathogenic European or intermediately pathogenic North American strains. (A to D) Venn diagrams showing the total number of genes modified in C57BL/6 mice ($n = 4$ per group) infected for 3 and 6 h with different strains of *S. suis* (North American [89-1591], European [P1/7], or Chinese [SC84]) compared to their expression in mock-infected mice, as determined by the Illumina microarray study. Differentially expressed genes were defined by a fold change greater than 3-fold (upregulation or downregulation) with an accompanying *P* value of ≤0.05.

TABLE 1 Validation of microarray gene expression using real-time RT-qPCR in C57BL/6 mice infected with different *S. suis* strains for 6 h

Gene	RNA quantification method	Fold increase in expression[a]		
		89-1591	P1/7	SC84
Cxcl1 (*Kc*)	Microarray	12	20	35
	qRT-PCR	44 ± 5	98 ± 11	142 ± 16
Cxcl2 (*Mip-2*)	Microarray	5.2	6.6	16
	qRT-PCR	74 ± 14	230 ± 23	326 ± 43
Ccl3 (*Mip-1*α)	Microarray	24	28	46
	qRT-PCR	62 ± 9	108 ± 5	143 ± 11
Ccl4 (*Mip-1*β)	Microarray	33	38	53
	qRT-PCR	55 ± 8	97 ± 5	115 ± 8
Ccl7 (*Mcp-3*)	Microarray	4.6	3.8	11
	qRT-PCR	30 ± 5	34 ± 3	79 ± 6
*Il1*β	Microarray	2.9	2.5	4.3
	qRT-PCR	2.5 ± 0.3	1.8 ± 0.3	3.7 ± 0.3
Il6	Microarray	2.7	2.2	6.0
	qRT-PCR	284 ± 40	680 ± 76	1,003 ± 108
Tnf	Microarray[b]	3.9	9.3	10
	qRT-PCR[b]	3.1 ± 1.1	9.0 ± 1.4	13 ± 3.1

[a] Values represent the mean fold increase in expression in spleens of infected mice compared to that in spleens of mock-infected C57BL/6 mice for the microarray analysis and the mean fold increase ± SEM for RT-qPCR. Data are for 4 mice per group for the microarray analysis and 7 mice per group for the RT-qPCR assay. The strains were from North America (89-1591), Europe (strain P1/7), and China (strain SC84).
[b] For *Tnf* gene expression, data presented are from mice infected for 3 h.

strain had the highest expression of genes for proinflammatory mediators. European strain-infected mice for the most part had an intermediate level of expression of these genes, and North American strain-infected mice presented the lowest level of expression of these genes (Table 1). These differences in gene expression were confirmed by Luminex analysis of secreted protein levels in plasma at 6 h postinfection (Fig. 4).

TNF is a powerful proinflammatory cytokine usually associated with septic shock, and it is produced very fast upon infection (28). Maximal mRNA expression levels were observed at 3 h postinfection in mice infected with the Chinese or the European strain, reaching levels higher than those observed in the North American strain-infected mice (Table 1). In fact, induction of TNF gene expression was delayed in the latter group of mice (see Tables S2 and S4 in the supplemental material). As for most other cytokines, TNF protein levels in plasma were higher in mice infected with the Chinese SC84 strain than the two other groups of mice (Fig. 4).

A balance between type I and type II interferon responses determines the capacity to control infection or the development of fatal STSLS. Aforementioned data highlighted the capacity of an epidemic strain to induce an exacerbated proinflammatory response that might be associated with STSLS. Since to date none of the usual superantigens that are typically responsible for this clinical manifestation have been shown to be produced by the ST7 strain responsible for the outbreak (9, 17), other mechanisms might be involved. Microarray data indicated a stronger induction of type II interferon (IFN-γ) gene expression in Chinese strain-infected mice than the other infected mouse groups (see Table S4 in the supplemental material). Thus, we hypothesized that a cascade of IFN-γ-related inflammatory events might be responsible for STSLS. Using both RT-qPCR and the Luminex assay, we demonstrated that significantly higher levels of IFN-γ are produced in mice infected with the epidemic SC84 strain than mice infected with either the North American 89-1591 strain or the European P1/7 strain (Fig. 5A and C). Since IFN-γ is known to be a master regulator of inflammation (29), we decided to further explore other genes that might be related to this increased IFN-γ expression.

Interferon-regulatory factor 1 (IRF1), a transcription factor that is mainly induced by the IFN-γ signaling pathway, is involved in many aspects of innate and adaptive immune responses. IRF1 drives inducible expression of many target genes through interaction with the IRF-binding sequence elements (IRF-Es). This specificity overlaps that of IFN-stimulated response elements (ISREs). In this way, IRF1 is able to induce a subset of the full spectrum of IFN-inducible genes (29, 30). Compared to mock-infected mice, IRF1 mRNA expression was upregulated only in the Chinese strain-infected mice in the microarray assay (see Table S4 in the supplemental material). A significantly higher IRF1 expression in mice infected with this epidemic SC84 strain than in the other two groups of mice was confirmed by RT-qPCR (Fig. 5E), a condition that is analogous to the IFN-γ expression data. Furthermore, the expression of CXCL9, a chemokine induced by the IFN-γ cascade (31), was also significantly higher in strain SC84-infected mice than European strain-infected mice, as determined by RT-qPCR and Luminex assay (Fig. 5G and I). CXCL10, a chemokine gene that can be upregulated in response to the IFN-γ/IRF1 signaling pathway (32), also saw its expression significantly upregulated in the epidemic SC84 strain-infected mice compared to the level of expression in the European strain-infected mice (Fig. 5F and H). Altogether, these findings suggest a role of the IFN-γ loop in the exacerbated inflammatory response induced by the Chinese strain.

Unexpectedly, CXCL9 and CXCL10 were also expressed at a significantly higher level in North American 89-1591 strain-infected mice than in European P1/7 strain-infected mice (Fig. 5F to I). The higher gene and protein expression levels of these two chemokines in the group of mice infected with the North American 89-1591 strain could not be explained by an increase in IFN-γ levels, because IFN-γ expression was similar to that in mice infected with the European P1/7 strain (Fig. 5A and C).

To better understand the immunopathogenesis of an ST25 *S. suis* strain, we searched for the expression of genes for type I interferons since it has been demonstrated that they can also contribute to CXCL9 and CXCL10 induction (33, 34). RT-qPCR analysis confirmed our hypothesis, as extremely high levels of IFN-β mRNA expression were observed in mice infected with the North American 89-1591 strain compared to those observed in the other infected mouse groups (Fig. 5B). In agreement with these results, expression of IRF7, the master regulator of type I interferon-dependent immune responses (35), was also markedly increased in North American 89-1591 strain-infected mice compared to animals infected with either the ST1 or the ST7 strain (Fig. 5D; see Table S4 in the supplemental material). These data suggest preferential induction of the type I interferon loop during ST25 *S. suis* infection.

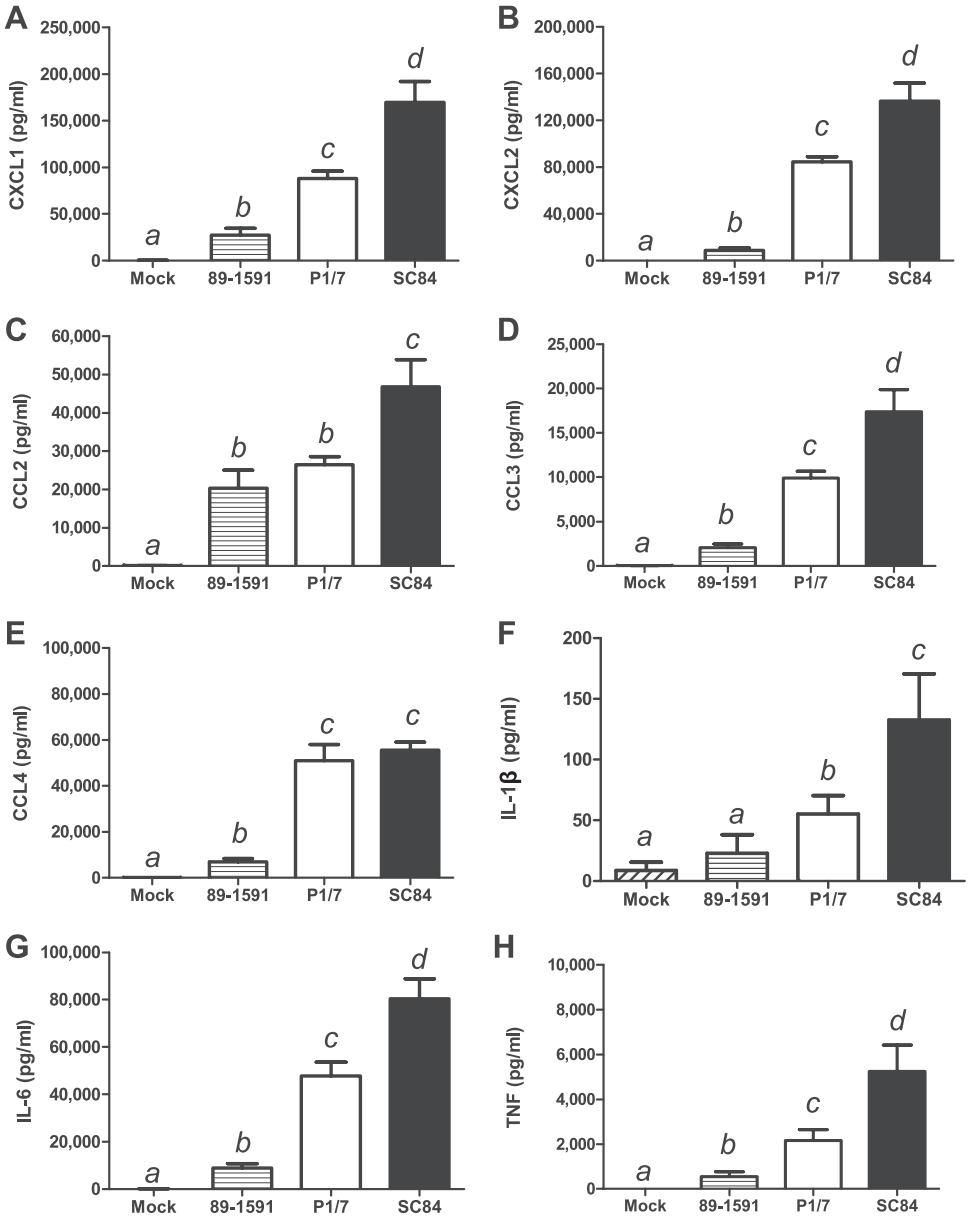

FIG 4 Proinflammatory cytokines and chemokines are highly expressed in mice infected with the epidemic Chinese strain of *S. suis*. Plasma levels of CXCL1 (A), CXCL2 (B), CCL2 (C), CCL3 (D), CCL4 (E), IL-1β (F), IL-6 (G), and TNF (H) proteins in C57BL/6 mice ($n = 7$ per group) infected with 1×10^7 CFU of the intermediately pathogenic North American (89-1591), highly pathogenic European (P1/7), or epidemic Chinese (SC84) *S. suis* strain for 6 h, as quantified by Luminex assay, are shown. Data represent mean values (in pg/ml) ± SEMs. Groups that are significantly different ($P \leq 0.05$), as determined by one-way ANOVA, are indicated by letters (*a*, *b*, *c*, and *d*).

The STSLS phenotype of the epidemic ST7 *S. suis* strain is reversed in IFN-γ-deficient mice. In order to confirm our hypothesis that the IFN-γ pathway plays a major role in the pathology of the disease caused by an infection with the *S. suis* epidemic Chinese SC84 strain, IFN-γ-KO mice were infected with either the SC84 strain or the European P1/7 strain and survival was monitored. As expected, C57BL/6 mice infected with the Chinese strain quite rapidly succumbed to the infection (Fig. 6A). Remarkably, IFN-γ-KO mice showed increased resistance to an infection with the epidemic strain. Indeed, at 48 h postinfection, 50% of the IFN-γ-KO mice were still alive ($P < 0.05$ compared to Chinese strain-infected C57BL/6 mice). In contrast, IFN-γ-KO mice infected with the European ST1 strain of *S. suis* had a survival curve similar to that of their infected C57BL/6 mouse counterparts ($P = 0.89$; Fig. 6A). This finding is in agreement with the low levels of IFN-γ production induced by the European P1/7 strain, as shown in Fig. 5.

It should be noted that no significant difference in survival levels was observed between C57BL/6 mice infected with the European strain and IFN-γ-KO mice infected with the Chinese strain ($P = 0.54$). These data indicate that the absence of IFN-γ led to a reversal of pathological symptoms in mice infected with the ST7 epidemic strain, as they reacted phenotypically in a way similar to that for wild-type mice infected with an ST1 European strain

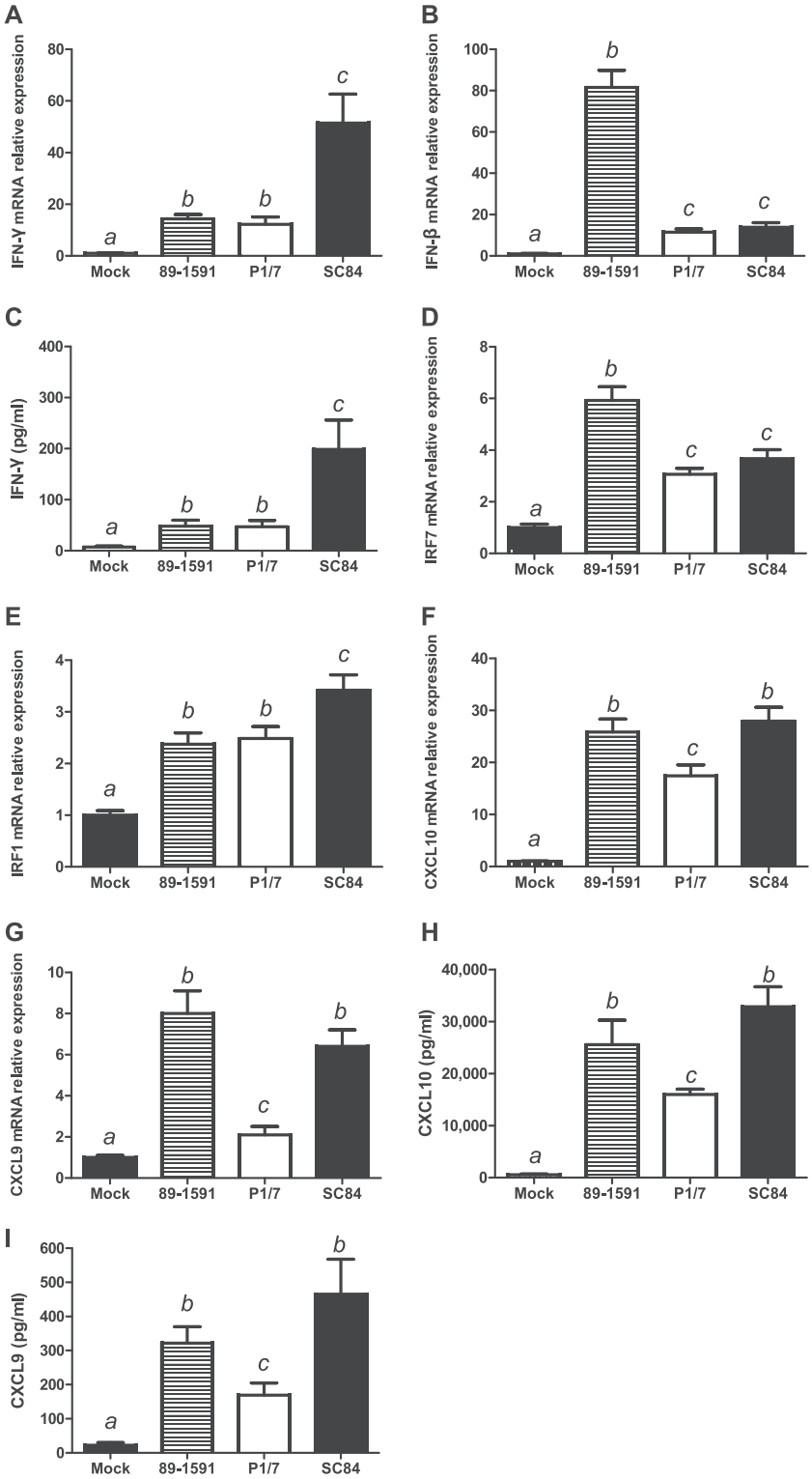

FIG 5 Type I and II IFN pathways are differently expressed in C57BL/6 mice infected with the intermediately pathogenic North American, the highly pathogenic European, or the Chinese epidemic strain of *S. suis*. (A, B, and D to G) Quantitative PCR analysis of expression of *Ifn*γ, *Ifn*β, and related genes in C57BL/6 mice ($n = 7$ per group) infected with 1×10^7 CFU of the North American (89-1591), the European (P1/7), or the Chinese (SC84) strain of *S. suis*. Total RNA was isolated from spleen samples at 6 h postinfection. Data represent mean values ± SEMs of the relative fold expression in infected groups compared to that in the reference mock-infected group. (C, H, and I) Plasma levels of IFN-γ (C), CXCL10 (H), and CXCL9 (I) proteins in C57BL/6 mice infected for 6 h with 1×10^7 CFU of the North American, the European, or the Chinese strain of *S. suis* ($n = 8$ per group). Data represent mean values (in pg/ml) ± SEMs. Groups that are significantly different ($P \leq 0.05$), as determined by one-way ANOVA, are indicated by letters (*a*, *b*, *c*, and *d*).

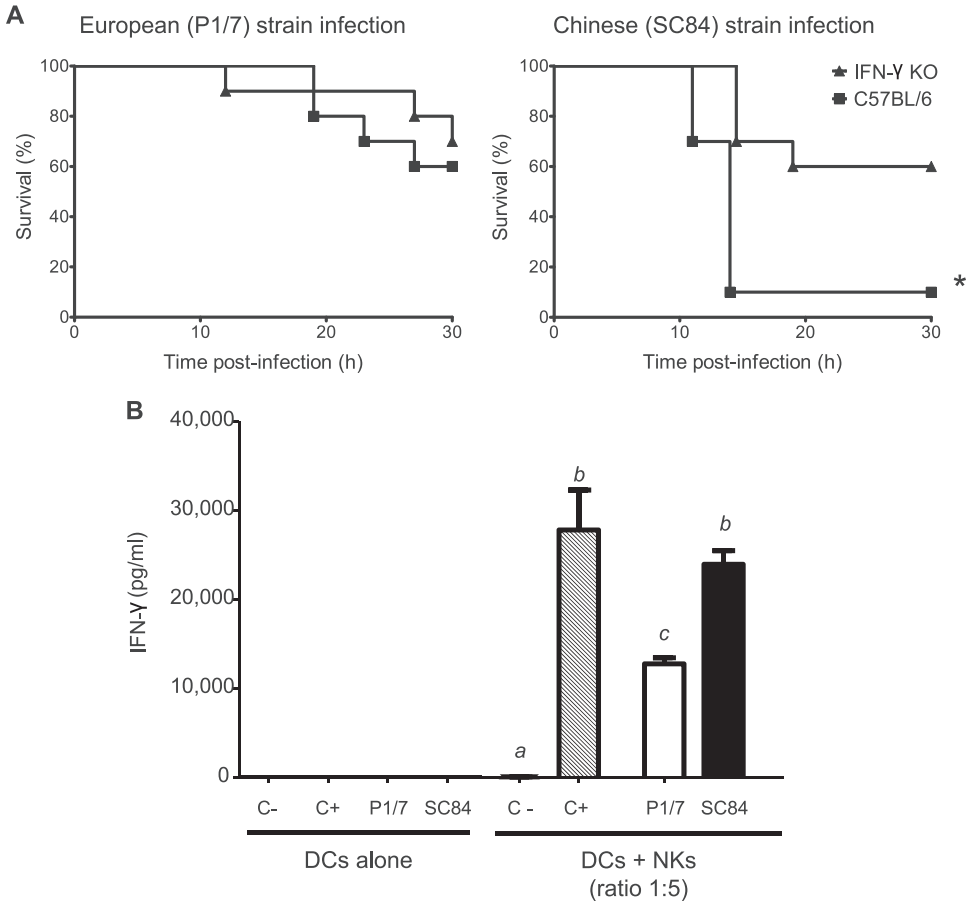

FIG 6 IFN-γ exacerbates the host response following an infection by the epidemic Chinese strain of *S. suis*: role of NK cells. (A) Survival curves for C57BL/6 or B6.129S7-*Ifng*^{tm1Ts}/J (IFN-γ-KO) mice ($n = 10$ per group) intraperitoneally infected with 1×10^7 CFU of the highly pathogenic European or the epidemic Chinese *S. suis* strain. *, significantly different ($P \leq 0.05$) compared to IFN-γ-KO mice infected with the Chinese strain of *S. suis*, as determined by the log-rank (Mantel-Cox) test. (B) IFN-γ levels were measured in DC and NK cell cocultures (ratio, 1:5) infected *in vitro* with either the epidemic Chinese *S. suis* strain (SC84) or the highly pathogenic European *S. suis* strain (P1/7) (2.5×10^5 CFU). After a bacterium-cell contact incubation time of 6 h, gentamicin was added to kill the bacteria and prevent cell toxicity. Supernatants were collected at 14 h of incubation, and IFN-γ levels were measured by ELISA. Nonstimulated cells (medium alone) or cells stimulated with a combination of CpG and LPS served as negative (C−) and positive (C+) controls, respectively. In addition, single-DC cultures were also included as controls. Data represent mean values (in pg/ml) ± SEMs of eight distinct experiments. Groups that are significantly different ($P \leq 0.05$), as determined by one-way ANOVA, are indicated by letters (*a*, *b*, and *c*).

(Fig. 6A). Taken together, these results support an important role for IFN-γ in the increased inflammatory state of the host during the acute phase of severe *S. suis* infection and suggest that its excessive expression is a major factor leading to lethal STSLS.

The ST7 epidemic strain induces a strong IFN-γ response by NK cells. IFN-γ-dependent pathology is known to contribute to lethality in bacterial superantigen-induced toxic shock syndrome (TSS), which is characterized by a robust T cell activation by the superantigens (36). Since the presence of genes homologous to typical superantigens has not been detected so far in the epidemic clone, we hypothesized that *S. suis* ST7 has developed alternative mechanisms to overactivate NK cells to induce exacerbated levels of IFN-γ during the acute phase of the infection. We thus analyzed IFN-γ levels in NK cell-DC cocultures infected *in vitro* with either the epidemic ST7 strain or the European ST1 *S. suis* strain. The Chinese SC84 strain induced a strong IFN-γ response by NK cell-DC cocultures which was as high as that induced by a combination of CpG and LPS (used as a positive control). Compared to the epidemic strain, the ST1 strain P1/7 presented a reduced capacity to activate NK cells *in vitro* (Fig. 6B). It should be noted that DCs alone (Fig. 6B) or NK cells alone (not shown) failed to secrete IFN-γ upon bacterial infection. This is in agreement with several reports indicating that activation of NK cells by extracellular pathogens (in contrast to virus-infected or transformed cells) seems to be indirect and results from signals provided by accessory cells, such as DCs (37).

DISCUSSION

TSS caused by the superantigen exotoxins of *Staphylococcus aureus* and *S. pyogenes* is characterized by robust T cell activation and a profound elevation in systemic levels of multiple cytokines, including IFN-γ, TNF, IL-1, and IL-6, followed by the dysfunction of multiple organs and often death (36, 38). Gene gain via the horizontal acquisition of mobile genetic elements is a key factor in the emergence of new pathogenic strains of streptococci. For example, acquisition of superantigen-encoding genes by *S. pyogenes* has been associated with increased morbidity and mortality in humans (39, 40). The emergence of a new, epidemic ST of *S. suis*

during the Chinese outbreak leading to unusual clinical manifestations related to STSLS allowed the hypothesis of the acquisition of superantigens by this important zoonotic pathogen to arise as well. However, and in spite of extensive whole-genome analysis (9, 16–18), no homology with known superantigens has so far been identified in the *S. suis* epidemic ST7 strain. Thus, in order to dissect the mechanisms underlying the emergence of a new pathogenic strain of *S. suis*, we performed a comparative analysis of the whole mouse genome. We demonstrated here for the first time that unbalanced host type II and type I interferon responses dictate the disease outcome and clearly discriminate between a newly emerged hypervirulent clone from the less virulent *S. suis* STs. Indeed, our study further contributed to an understanding of the mechanism(s) used by this new epidemic strain to induce STSLS and death.

Innate immunity is especially important in order to mount an immediate defense in response to pathogens. This inflammatory process plays a key role in eliminating bacteria and regulates many cells through the release of cytokines and chemokines. On the other hand, excessive inflammation has damaging effects, which can lead to severe symptoms, such as septic shock. In this regard, the increased severity of *S. suis* infections in humans observed in China, including a shorter incubation time (as short as 9 h after the onset of illness), rapid disease progression (the median time from disease onset to death was 23 h), sudden death, and higher mortality (64% in patients with STSLS) (7, 9, 16), correlates with an overzealous and inappropriate inflammatory response. Clinical and epidemiologic data for patients infected during the Chinese outbreak showed that during the early phase of the disease, serum levels of IFN-γ, IL-1β, IL-6, IL-8, and TNF were extremely high (16). These observations are in agreement with our present results obtained using a mouse model of infection. Cytokine levels, but not bacterial burden, correlate with the degrees of virulence of ST7, ST1, and ST25 strains. Among several proinflammatory mediators, this study identified the IFN-γ/IRF1 pathway to be a major pathway that could be related to the STSLS induced by the epidemic strain. Indeed, in the absence of IFN-γ (KO mice), a reversion of the ST7 phenotype was observed, as KO mice presented a survival rate similar to that for mice infected with an ST1 strain. By enhancing chemotaxis and phagocytic killing, IFN-γ is expected to contribute to bacterial clearance in the infected host (29). On the other hand and as mentioned above, IFN-γ may directly mediate pathological changes and lethality, probably in conjunction with other proinflammatory mediators such as TNF (41). It is known that IFN-γ induces activation of the transcription factor STAT1, which can then trigger activation of IRF1 (29). These two proteins can subsequently activate transcription of certain IFN-γ-induced genes, such as CXCL10 (32) and CXCL9 (31), resulting in enhanced expression of these two chemokines, as shown in this study. Synergistic promoter activation can also take place following cooperation between STAT1/IRF1 and NF-κB, with the latter being activated after TNF stimulation (42–44). Furthermore, the combination of IFN-γ and TNF can induce even higher levels of IRF-1 mRNA (30). Thus, our findings of higher IRF1 gene activation and TNF production in mice infected with the epidemic strain suggest that synergistic interactions between IFN-γ and TNF might contribute to the increased mortality observed in animals infected with the epidemic *S. suis* strain. As IL-1β is also highly induced by an infection with the epidemic strain, similar synergies between the IFN-γ and IL-1β pathways may also contribute to amplification of a fatal inflammatory loop (32).

NK cells have sophisticated biological functions and are key actors in innate immunity. NK cell-derived cytokines, such as IFN-γ, enhance the innate immune response and shape the subsequent adaptive immune response (45). We showed here that these cells respond to the epidemic strain by releasing large amounts of IFN-γ, the central player in the inflammatory cascade exacerbated during infection by this strain. Moreover, the IFN-γ-induced CXCL9 and CXCL10 chemokines share the ability to signal through CXC chemokine receptor 3 (CXCR3), which is present on NK cells. Ligand binding to the receptor results in further activation and recruitment of these cells to sites of inflammation. In addition, IRF1 is also known to influence the function of NK cells (30). A critical role of NK cells in LPS-induced lethal shock in mice has been reported, with NK cells being the major IFN-γ producers (46, 47). Similarly, a clinical study reported that high NK cell counts in blood at early time points predict mortality in severe sepsis (48). This immunopathological process differs from the sepsis disease induced by a typical pathogenic European ST1 strain of *S. suis*. The fact that IFN-γ does not seem to play a fatal role during infection with an ST1 strain of *S. suis* is in agreement with the finding observed for *Streptococcus pneumoniae*-infected IFN-γ receptor-deficient (IFN-γ $R^{-/-}$) mice. In these studies, the mortality rate for IFN-γ $R^{-/-}$ mice resembled that for infected wild-type mice (49).

While investigating the participation and the contribution of IFN-γ to the aberrant host inflammatory response during infection by the Chinese *S. suis* strain, we observed similarly elevated levels of CXCL9 and CXCL10 in mice infected with a North American *S. suis* strain. In the case of the latter strain, the elevated expression of these chemokines could not be explained by elevated IFN-γ expression. Instead, these mice showed elevated expression of the type I interferon IFN-β. Historically, type I IFN responses were primarily considered to be unique to viral infections; however, this view has recently changed, as these IFNs are also induced after exposure to bacteria and pathogen-associated molecules (50, 51). There is a substantial overlap between IFN-β-inducible and IFN-γ-inducible genes; however, IFN-β can activate transcription of another set of IFN-stimulated genes via ISRE (51). In this regard, our data indicated a preferential upregulation of IRF7 over IRF1 in mice infected with the North American strain. As production of IFN-β has been shown to be regulated by a positive-feedback loop where IRF7 is implicated (35, 52), the upregulation of this IRF pathway by the North American strain may explain the enhanced type I IFN response in mice infected by this strain. Albeit IRF1 is also known to bind the IFN-β promoter and IFN-β itself can contribute to IRF1 induction (30), this pathway is not activated by an infection with the Chinese ST7 strain (which induces a very low IFN-β response), which seems to divert the system toward a mostly exclusive type II interferon response (53). Conversely, the IRF-1/IFN-β amplification loop might also contribute to the immune response against an ST25 strain in parallel with the major IRF7/IFN-β pathway. This is suggested by moderate levels of IRF1 activation in the absence of significant IFN-γ production by an ST25 infection. Similarly, *Mycobacterium tuberculosis*-infected human DCs have been shown to induce IFN-β, followed by upregulation of IRF1 and IRF7 (54). The observed upregulation of IRF7 by an ST25 *S. suis* infection differs from the gene expression observed in other pathogenic *Streptococcus* spe-

cies. For instance, *S. pneumoniae* and *S. pyogenes* induce IFN-β expression through a signaling cascade involving IRF3 (55–57).

As mentioned above, CXCL9 and CXCL10 recruit NK cells. However, these cells do not seem to play an important role during infection with an ST25 *S. suis* strain, as they are poorly activated by this strain *in vitro* (unpublished observations). As such, these two chemokines might play a different role in the context of an ST25 infection. CXCL9 and CXCL10 are also known to recruit and activate T cells, which might lead to the development of a protective response (33). In fact, the role of type I IFNs in bacterial diseases is controversial, and most studies have focused on intracellular bacteria. Signaling through the type I IFN pathway can have harmful effects in some bacterial infections and beneficial consequences in others (55, 58–62).

In conclusion, this *in vivo* whole-genome comparative study provided for the first time a deeper understanding of the host response to *S. suis* infections by showing that a newly emerged ST7 epidemic clone of *S. suis* responsible for the Sichuan outbreak in 2005 has evolved from an ST1 clone to overexpress IFN-γ. The use of a mouse model known to be poorly sensitive to superantigens and the current failure in revealing the presence of superantigens in the ST7 clone suggest that alternative mechanisms leading to increased virulence and IFN-γ production, probably by NK cells, may be used by this strain to provoke STSLS. Conversely, the less virulent ST25 *S. suis* strain (present mainly in North America) results in an IFN-β-subjugated, low inflammatory response that might be beneficial for the host in order to clear the infection.

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