Serum cytokine modulation after *Staphylococcus hyicus* infection in BALB/c mice

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ABSTRACT. Staphylococcal infection is a severe bacterial infection. Finding satisfactory predictable biological markers is essential for the treatment of this condition. In this study, we applied a 32-marker sandwich ELISA-based antibody array to evaluate cytokine changes in *Staphylococcus hyicus*-inoculated BALB/c mice at 24 and 48 h post infection. Among the cytokines detected, the expression levels of granulocyte colony stimulating

factor (G-CSF), interleukin 6 (IL-6), macrophage inflammatory protein 2, and keratinocyte chemoattractant (KC) were increased to levels more than twice higher than those in the control group 24 h after infection, while the expression of interleukin 12p40p70 (IL-12p40p70) was decreased to less than half the level measured in the controls. The expression of G-CSF, IL-6, monocyte chemoattractant protei-5, chemokine ligand 11, and KC was upregulated 48 h post infection, whereas IL-12p40p70 expression was still significantly lower ($P < 0.05$). Among the detected cytokines, the expression levels of G-CSF, IL-6, and KC were constantly upregulated, while IL-12p40p70 was downregulated. This result was then validated by an ELISA assay analysis, which confirmed that G-CSF, IL-6, KC, and IL-12p40p70 expression levels were specifically modulated after an *S. hyicus* bacterial infection, while granulocyte monocyte colony stimulation factor, IL-12, and IFNγ levels were significantly increased after a viral infection. Our study indicated the potential of cytokines G-CSF, IL-6, KC, and IL-12p40p70 as markers for detecting *S. hyicus* infection. The results of this study may provide useful data for the appropriate use of medication following *S. hyicus* infection.

Key words: *Staphylococcus hyicus*; Cytokine; Antibody array; Mice; Disease marker

INTRODUCTION

Staphylococcus hyicus is a coagulase-variable species comprising both coagulase-positive and coagulase-negative isolates. *S. hyicus*, producing exfoliative toxins (ETs), is primarily known as the main causative agent of exudative epidermitis (EE) in piglets (Tanabe et al., 1996; Andresen, 2005). The disease is characterized by the separation of the cells in the upper stratum of the epidermis, exfoliation of the skin, erythema, and the formation of a thick, greasy, brown exudation (L'Ecuyer and Jericho, 1996; Ahrens and Andresen, 2004). *S. hyicus* has been isolated from pigs with septic polyarthritis and bovine mastitis and from White Leghorn laying chickens experiencing skin and feather problems (Phillips et al., 1980; Roberson et al., 1996; Chenier and Lallier, 2012). To the best of our knowledge, two cases of human infection have been reported: as early as 1997, the first case of a wound infection following a donkey bite was reported (Osterlund and Nordlund, 1997); recently, *S. hyicus* was isolated from a septic patient-a farmer who had been in close contact with piglets (Casanova et al., 2011). ETs produced by staphylococci are reported to be involved in some subcutaneous infections in mammals. In *S. hyicus*, five different ETs, including SHETB, ExhA, ExhB, ExhC, and ExhD, have been reported, with amino acid sequences homologous to ETA and ETB of *Staphylococcus aureus*, as they all belong to the serine protease-like group of ETs (Ahrens and Andresen, 2004; Futagawa-Saito et al., 2007; Nishifuji et al., 2008; Iyori et al., 2010). It was reported that the four Exh isoforms selectively digest a single peptide bond in the extracellular region of swine desmoglein 1 (Dsg1), a desmosomal cadherin-type cell–cell adhesion molecule, inducing skin exfoliation in piglets (Fudaba et al., 2005; Tosi, 2005).

Cytokines are a heterogeneous group of soluble small polypeptide or glycoprotein mediators, forming part of a complex network that helps regulate the immune and inflammatory

responses (Hill and Martins, 2006). Many biological processes such as apoptosis, inflammation, angiogenesis, immune response, and migration often accompany changes in cytokine expression levels. Immune response also varies between pathogenic insults, such as bacterial infection or viral infection. Cytokines mediate the activity of Th1 cells that induce cell immunity from viral infections or of Th2 cells that induce humoral immunity in defense against bacterial infections (Zhao et al., 2012).

Because of the extensive cross-communication between cytokines, multiplex assays must be used to obtain a complete picture of the biological responses and functions. Cytokines are known to play many critical roles in innate and adaptive immunity, apoptosis, angiogenesis, cell growth, and differentiation (Tosi, 2005; Jin et al., 2008; de la Fuente et al., 2009; McDonald et al., 2011). Detection of expression levels of multiple cytokines can provide new insights into the development of *S. hyicus* infection and help discover key factors in pathogenic progress.

Antibody array technology has drawn great attention due to its high-throughput, flexibility, and cost-effectiveness. The three platforms for antibody array approaches include sandwich-based, label-based, and competition-based antibody arrays (Borrebaeck and Wingren, 2007). Antibody array technology has emerged as a promising technology for disease biomarker discovery. Moreover, antibody array screening improves the chances of discovering key factors, disease mechanisms, and biomarkers related to cytokine signaling (Kim et al., 2009; Jiang et al., 2013). Cytokine levels have been assessed in *S. aureus*- infected mice to find biomarkers that indicate potentially fatal outcomes following infection (van den Berg et al., 2013). However, until now, little was known about cytokine expression profiles during *S. hyicus* infections.

In this study, sera from BALB/c mice infected with *S. hyicus* were screened for 32 different cytokines at prescribed time points during infection. Protein expression assays were used to determine changes in the cytokine profile after infection.

MATERIAL AND METHODS

Bacteria

The clinical strain *S. hyicus* ZC-4 (JQ728535) was used. This strain was isolated in pure culture from the pericardial fluid of a diseased piglet with serious EE from a conventional pig-rearing farm in the Guangdong province of China. Notably, *S. hyicus* ZC-4 was a virulent strain carrying the exfoliative toxin ExhA. Cultures of *S. hyicus* grown in nutrient broth were stored at -80°C.

Animals and experimental design

Specified opportunistic pathogen-free female BALB/c *S. hyicus*-free mice (four weeks old on the day of infection), purchased from Southern Medical University, were used. All procedures were in accordance with the guidelines for the care and use of laboratory animals by the Institute of Experimental Animals of Guangdong Academy of Agriculture Sciences. Experiments were performed in two groups. In the control group of 16 mice, each mouse received a 0.2 mL subcutaneous injection of phosphate buffered saline (PBS). In the *S. hyicus* treated group of 16 mice, each mouse received *S. hyicus* at a dose of 1.0×10^8 CFU via subcutaneous injection. All mice were closely monitored throughout the study for clinical signs. At both 24 and 48 h after

infection, eight mice were sacrificed via decapitation for organ extraction, bacterial culture, and serum collection. The serum samples were stored at -80°C until needed. The livers, spleens, kidneys, and lungs were removed from the infected mice at different time points after infection, and organ homogenates were examined for bacterial presence.

Identification of the clinical isolates

DNA was isolated using the Easy-DNA™ gDNA Purification Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer protocols. Specific primers (5'-ATGGTTTTGGTAGAATTGGTCGTTTA-3') and (5'-GACATTTCGTTATCATACCAAGCTG-3') were used to amplify a 931 bp gap gene. The DNA was amplified by polymerase chain reaction (PCR), with an initial denaturation at 94 °C for 5 min, followed by 30 cycles at 94°C for 30 s, 56°C for 1 min, 72°C for 30 s, and finally, extension at 72°C for 10 min.

Examination of the genes encoding staphylococcus exfoliative toxins by PCR

The PCR was performed according to the general steps using the primers 5'-ATAGAGGAGAAATCAACATG-3' (forward) and R 5'-CTATAGTTACTTGACCTCTA-3' (reverse) to amplify an 865 bp exfoliative toxin ExhA gene. The cycling conditions included an initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, and finally, extension at 72°C for 10 min.

Cytokine antibody array analysis

The following cytokines were assessed in the sera of mice infected with *S. hyicus* at 24 and 48 h after infection: granulocyte colony stimulating factor (G-CSF), granulocyte monocyte colony stimulating factor (GM-CSF), interferon (IFN) γ, tumor necrosis factor (TNF) α, tissue inhibitor of metalloproteinase 1 (TIMP-1), Leptin (Ob), thrombopoietin (TPO), thymus activation regulation chemokines (CCL17), stem cell factor (SCF; Kit ligand), soluble TNF receptor type 1 (sTNF R1; TNFRS1A), and the interleukins (IL) -2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, and IL-17. The following chemokines were selected: keratinocyte chemoattractant (KC; CXCL1), monocyte chemoattractant protein 1 (MCP-1; CCL2), MCP-5 (CCL12), macrophage inflammatory protein 1a (MIP-1a; CCL3), MIP-2 (CXCL2), MIP-3β (CCL19), the chemokine regulated-upon-activation normal T-cell expressed and secreted (RANTES; CCL5), Eotaxin (CCL11), cutaneous T-cell-attracting chemokine (CTACK/CCL27), secondary lymphoid tissue chemokine (SLC; 6Ckine; CCL21), and vascular endothelial growth factor (VEGF). Selected cytokines and chemokines were representative of both pro- and anti-inflammatory cytokines. Cytokine levels were determined as per the protocol for using the RayBiotech mouse cytokine antibody array G2 (AAM-CYT-G2-4; RayBiotech, Norcross, GA, USA). The array consisted of glass slides that were coated with antibodies, including the positive and negative controls. The signals were scanned with a GenePix 4000B fluorescence laser scanner (Axon Instruments, Foster City, Cal.). The results were analyzed with the RayBiotech analysis tool specifically designed to analyze RayBiotech G-Series Antibody Arrays (RayBiotech Inc, Norcross, GA, USA). The signals were normalized using the positive and negative controls included on the array.

Enzyme-linked immunosorbent assays (ELISA)

ELISA was performed according to the RayBio® ELISA manual (RayBiotech, Inc., Norcross, GA, USA).

Statistical analysis

The data are expressed as the mean ± SEM. The data were analyzed using the unpaired two-tailed Student t-test for comparison between two groups. Differences with a value of $P < 0.05$ were considered statistically significant.

RESULTS

Clinical signs

All challenged mice displayed a range of clinical signs 24 h after infection, including depression, mild inflammatory exudation, rough fur coats and fur loss. Forty-eight hours after infection, all of the inoculated mice exhibited typical clinical signs, obvious inflammatory exudation, and serious hair loss. All control mice remained healthy during the study period.

Isolation and identification of *S. hyicus*

A strain of *Staphylococcus* was isolated in pure culture from the mouse organs and identified as *S. hyicus* by morphological and biochemical examination. The isolate displayed the morphology of *Staphylococcus* under the microscope, were shaped as gram-positive cocci in clumps, and showed the biochemical characteristics of *S. hyicus*, such as glucose decomposition and a positive outcome in the indole test. To confirm the results of the biochemical tests, we analyzed the *S. hyicus* ZC-4 special gene at a molecular level using PCR, examined the gap gene in the control, and the exfoliative toxins in the bacterium.

Cytokine arrays results

The serum levels of the 32 selected cytokines were assessed using antibody array technology. The reproducibility for the glass-slide-based arrays was assessed by testing replicate aliquots of the samples with two sub-arrays printed on the same slide and assayed at the same time. After subtracting background signals, the duplicate values were averaged and normalized with reference to positive controls. The comparison of signal intensities for antigen-specific antibody spots in array images of each cytokine between mice infected with *S. hyicus* and the control group was used to determine relative differences in expression levels. In this study, all cytokines with a significant difference ($P < 0.05$) in expression were selected. Then, any ≥ 2-fold increase or ≥ 2-fold decrease (≤ 0.5 of the expression level of control mice) in signal intensity for a single cytokine between mice infected with *S. hyicus* and the control group was considered a measurable and significant difference in expression. Significant differences are observed between *S. hyicus*-infected mice versus mice in the control group 24 h and 48 h after *S. hyicus* infection; the data are shown in Table 1. Data are expressed as 2-, 4-, 6-, 8-fold or greater increases, or 2-fold decreases, in mice infected with *S. hyicus* over control mice.

Twenty-four hours after inoculation, the levels of 4 out of 32 cytokines (G-CSF, IL-6, KC [CXCL1], MIP-2 [CXCL1]) were at least twice higher compared to those of the controls. The G-CSF level in serum was 193.81 times higher than that of the controls. The level of IL-6 increased 6.2-fold, KC 24.74-fold, and MIP-2 (CXCL1) 3.8-fold compared to those of the controls. Among the cytokines detected, only the expression of IL-12p40p70 was significantly decreased. Expression of IL-12p40p70 in the infected mice was only 0.41 times the level of the control 24 h after infection (Table 1 and Figure 1).

Table 1. Changes in the level of serum cytokines in BALB/c mice in response to *S. hyicus* infection ($P < 0.05$).

Cytokine	Fold change (ZC-4/PBS)	
	24 hours post infection	24 hours post infection
6Ckine	0.88	1.08
CTACK	0.85	1.16
CCL11	1.25	2.69↑
G-CSF	193.81↑↑↑↑	343.41↑↑↑↑
GM-CSF	0.85	0.94
IL-2	0.94	1.14
IL-3	0.81	0.99
IL-4	0.97	0.91
IL-5	0.86	0.96
IL-6	6.20↑↑↑	2.44↑
IL-9	0.69	1.07
IL-10	0.88	0.97
IL-12p40 p70	0.41↓	0.47↓
IL-12p70	0.77	1.03
IL-13	0.81	1.09
IL-17	0.96	1.01
IFN-γ	0.87	0.87
KC	24.74↑↑↑↑	6.30↑↑↑
Leptin (Ob)	0.69	1.08
MCP-1/CCL2	0.66	1.27
MCP-5/CCL12	1.45	3.43↑
MIP-1 alpha/CCL3	1.12	1.21
MIP-2/CXCL1	3.80↑	1.68
MIP-3 beta/CCL19	0.93	0.98
RANTES/CCL5	0.84	0.98
SCF/Kit Ligand (KITLG)	1.34	0.98
sTNFR1	1.05	1.19
sTNFR2	1.15	0.94
TIMP-1	1.52	1.90
TNF-α	0.99	0.96
Thrombopoietin	1.01	1.07
VEGF	0.50	1.22

Expression of cytokines in the serum at two time points after infection. The controls are BALB/c mice injected with PBS. Values are expressed as 2, 4, 6, or ≥8 times greater or lower than controls: ↑ 2 times greater than control; ↑↑ 4 times greater than control; ↑↑↑ 6 times greater than control; ↑↑↑↑ 8 times greater than control; ↓ 0.5 times lower than control.

Forty-eight hours after infection, the expression levels of 5 of 32 cytokines (G-CSF, IL-6, KC [CXCL1], CCL11, and MCP-5 [CCL12]) were significantly higher than the control levels. The amount of G-CSF was 343.41-fold higher than in the control. The level of IL-6 was 2.44-fold that of the control. KC expression was 6.30-fold higher than that of the control. CCL11 levels were elevated 2.69-fold compared to those in the controls. MCP-5 (CCL12) levels were increased 3.43-fold over those in the control. Expression of IL-12p40p70 was only 0.47 times the level of the control at 48 h after infection (Figure 2 and Table 1).

From the data above, G-CSF, IL-6, KC (CXCL1), and IL-12p40p70 were consistently at significantly different levels in comparison to the controls at both 24 and 48 h after infection.
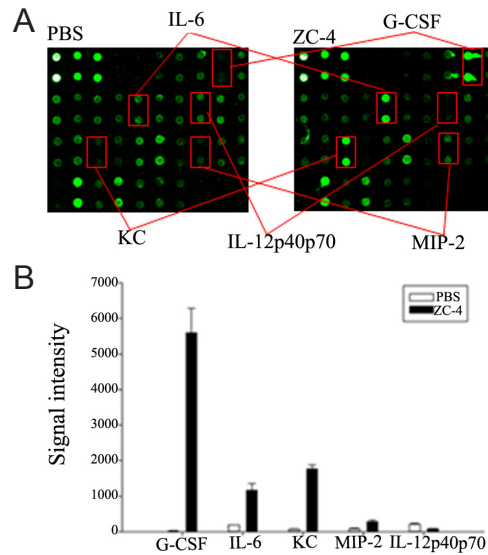
Figure 1. Cytokine profiles in sera from BALB/c mice with *S. hyicus* and control mice at 24 hours after infection. **A.** Representative cytokine antibody arrays. The spots for G-CSF, IL-6, KC, MIP-2 and IL-12p40p70 showed a marked density increase (or reduction) compared to the control. **B.** Expression of cytokines was found to be 2-fold higher or lower than that of the controls. All P < 0.05.
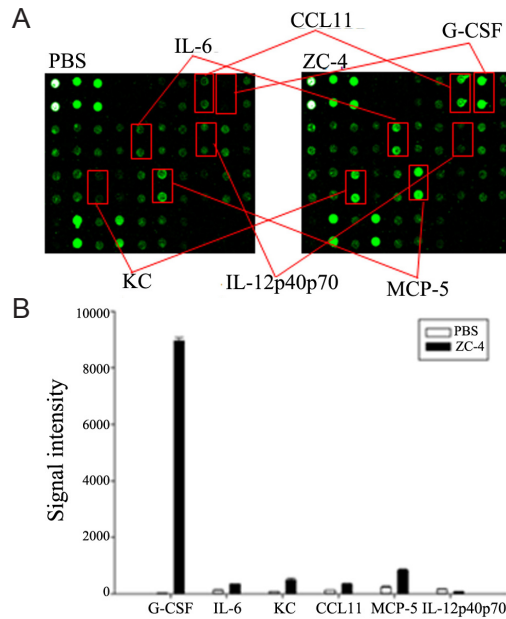

Figure 2. Cytokine profiles in sera from BALB/c mice with *S. hyicus* and control mice at 48 h after infection. **A.** Representative cytokine antibody arrays. The spots for G-CSF, IL-6, KC, CCL-11, MCP-5, and IL-12p40p70 showed a marked density increase (or reduction) compared to the control. **B.** Expression of cytokines was found to be at least twice higher or less than that of the controls. All P < 0.05.

Validation of cytokines with the ELISA assay

To confirm our observations from the cytokine antibody array studies, an ELISA assay was used to quantitatively measure the expression levels of G-CSF, IL-6, KC, and IL-12p40p70. At both 24 and 48 h after infection, the IL-6, KC, and G-CSF expression levels were all significantly upregulated in the sera of mice inoculated with *S. hyicus* compared to the controls, as indicated in Figure 3. For IL-12p40p70 detection, the expression level was also significantly downregulated. Figure 3 shows representative ELISA data for cytokines IL-6, KC, G-CSF, and IL-12p40p70 24 h after infection.
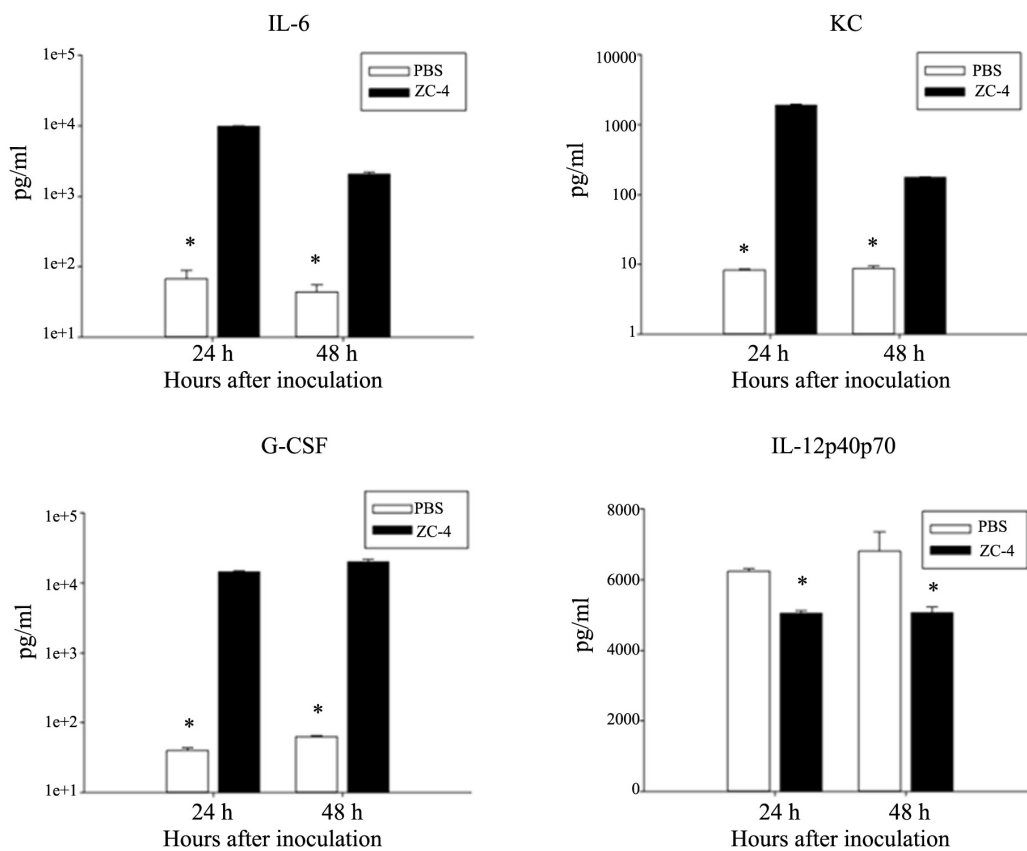

Figure 3. IL-6, KC, G-CSF, and IL-12p40p70 expression in sera of *S. hyicus*-infected mice compared to controls. For IL-6, KC, G-CSF, and IL-12p40p70, animals at all times post-infection had significantly different (*) cytokine levels compared to controls. ELISA data are shown as the mean protein concentration (pg/mL). *P < 0.05.

DISCUSSION

Staphylococcus hyicus is the main causative agent of EE in piglets (Tanabe et al., 1996; Andresen, 2005). In this study, an *S. hyicus*-infected model was established in BALB/c mice. The

cytokine expression profile in sera from *S. hyicus* infected in BALB/c mice was then analyzed by the semi-quantitative, sandwich-based antibody array technology.

Our study is the first report of the application of sandwich-based antibody array technology for cytokine detection in the sera of BALB/c mice inoculated with *S. hyicus*. Among the 32 cytokines detected, levels of CCL11, G-CSF, IL-6, KC, MIP-2/CXCL1, and MCP-5/CCL12 increased to at least twice those of the controls and levels of IL-12p40p70 decreased to less than half that of the controls at either 24 hours or 48 hours post infection. Among the above cytokines, G-CSF, IL-6, and KC were consistently upregulated at the two time points studied, and IL-12p40p70 was consistently downregulated. These results were validated with an ELISA assay.

Interleukin 6 (IL-6) is a pleiotropic cytokine involved in the regulation of acute-phase reaction, inflammation, hematopoiesis, and the transition from innate to acquired immunity (Fielding et al., 2008; Abajo et al., 2012). Endogenous IL-6 plays a crucial anti-inflammatory role in both local and systemic acute inflammatory responses in mouse models. The viable bacterial count in the livers of IL-6-deficient mice was higher than those of control mice (Onogawa et al., 2013). Furthermore, elevated IL-6 levels in the serum and increased expression of IL-6 mRNA in the skin were detected in response to *S. aureus* ETA exposure (Dalrymple et al., 1996). G-CSF is a pleiotropic cytokine best known for its specific effects on the proliferation, differentiation, and activation of hematopoietic cells of the neutrophilic granulocyte lineage (Cheers et al., 1988; Rolle et al., 2013). *In vitro*, G-CSF stimulates growth, differentiation, and other functions in cells of the neutrophil lineage (Kawakami et al., 1990; Demetri and Griffin, 1991). KC, a member of the alpha (CXC) chemokine sub-family, is the murine functional homologue of human IL-8 (Kawakami et al., 2004), and appears to have an important role in inflammation, based on the pattern of KC expression in a number of inflammatory disease models. Similar to our findings, KC was assessed in a study of *S. aureus* infection in mice and reported as one of the biomarkers predicting fatal outcome of infection (van den Berg et al., 2013).

IL-12 was the only cytokine to decrease to less than half the level of the control at each time point. IL-12 is produced by macrophages and B-lymphocytes and has been shown to have multiple effects on T cells and natural killer (NK) cells. These effects include inducing production of IFN-γ and TNF by resting and activated T and NK cells, synergizing with other IFN-γ inducers at both the transcriptional and post-transcriptional levels (Bozic et al., 1995; Une et al., 2003). It is likely that the amplification of antigen-specific responses by IL-12 may be at least partially impaired by decreased IL-12, thus contributing to decreased IFN-γ production, although the difference was not significant.

Recently, the up-regulation of several factors including TNF-α, IL-1α, G-CSF, IL-6, MIP-1a, and KC after *S. aureus* infection was reported (van den Berg et al., 2013). Our study identifies four cytokines (G-CSF, IL-6, KC, and IL-12p40p70) that may represent useful markers for detecting *S. hyicus* infection. Cytokine modulation is crucial in activating immune responses to various pathogens. Further studies are required to arrive at a conclusion and to detect biomarkers that predict the severity of infection and its progression.

Cytokines play a critical role in the host's immune defense against infection. It has been established that intracellular pathogens, such as those involved in a viral infection, initiate a cellular immune response characterized by the activation of type 1 T lymphocytes (T1) and the production of IFN-γ, TNF-α, and IL-2. Extracellular pathogens, such as those involved in a bacterial infection, initiate a humoral immune response characterized by the activation of type 2 T lymphocytes (T2), which produce IL-4, IL-5, IL-6, and IL-10. T1 and T2 cell responses are mutually inhibitory

(Zhao et al., 2012). The differences in cytokine modulation between our results and the cytokine expression profile in a report of reovirus-infected mice (Hazlett et al., 2003) are shown in Table 2. G-CSF is known for its specific effects on the proliferation, differentiation, and activation of hematopoietic cells. The expression of G-CSF was strikingly increased in conditions of *S. hyicus* infection (193.81-fold) compared to that in reovirus infection (2.1-fold). IL-6, which promotes T2 reaction, also showed increased expression in *S. hyicus* infection. IL-12 expression was decreased in *S. hyicus* infection and increased in reovirus infection. The differences between the two cytokine expression profiles also shows that IL-10, the factor from Treg cells, and IL-17, from Th17 cells, were down-regulated in *S. hyicus* infections and up-regulated in reovirus infections. This suggests a potential role for Treg and Th17 cells in suppressing Th1 activation and shifting the Th1/Th2 balance to Th2 activation.

Table 2. Changes in cytokine levels after infection with *S. hyicus* or reovirus (for all changes, $P < 0.05$).

Cytokines	Fold Change	
	S. hyicus	Reovirus
G-CSF	193.81	2.1
GM-CSF	0.85	3.5
IL-2	0.94	1.3
IL-3	0.81	1.2
IL-4	0.97	1.1
IL-5	0.86	3
IL-6	6.2	2.2
IL-9	0.69	2.6
IL-10	0.88	7.3
IL-12p70	0.77	3.3
IL-13	0.81	1.7
IL-17	0.96	4
IFN-γ	0.87	5.9
MCP-1/CCL2	0.66	3
MCP-5/CCL12	1.45	3.1
RANTES/CCL5	0.84	1.1
SCF	1.34	2.6
sTNFR1	1.05	1.8
TNF-α	0.99	1.7
VEGF	0.5	1

In terms of limitations, this study did not explore the underlying reason for the aforementioned differences any further. After a bacterial infection, defense mechanisms give rise to immune changes to protect the body by increasing IL-6, mediated by the cellular immune response, and G-CSF, mediated by the humoral immune response. However, the pathways that are activated that result in a sophisticated pattern of cytokine expression, and their genetic regulation remain unclear.

In conclusion, by applying cytokine antibody array analysis to *S. hyicus*-infected mice, the cytokines IL-6, KC, G-CSF, and IL-12p40p70 have been detected in the expression profile as potential biomarkers for *S. hyicus* infection. In the future, further analysis and tracking of these cytokines may aid in the prognosis of *S. hyicus* infection and the determination of an appropriate treatment for this condition.

Conflicts of interest

The authors declare no conflict of interest.

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