INTRODUCTION

Streptococcus pneumoniae (S pneumoniae) is a dominant agent worldwide for pneumonia, bacteremia, and meningitis, which accounts for about one million deaths in children under 5 years¹ each year. Bacteremia and meningitis are more fatal than pneumonia, which lead to children's deaths mainly due to nervous system complications and the old people's deaths primarily due to systematic complications.
complications.2,3 S pneumoniae is frequently localized in human’s respiratory tract before its pathogenic transformation.4 Due to the poor immune response against polysaccharide of S pneumoniae, infants and the aged are more susceptible to various S pneumoniae infections.5,6 Innate immune is vital for host’s recognizing pathogenic organisms and consequent initializing protection response,7 deficit of which reduces various infections. In addition, the amount and pathogenicity of S pneumoniae constitute the other key factors. We previously verified that certain S pneumoniae virulence factors such as pneumolysin (Ply) may facilitate S pneumoniae’s invading blood system.8,9 However, other differences remain still uncertain among different S pneumoniae strains responsible for the former three types of infections. Dendritic cells, monocytes, and neutrophils are all primary guards for host’s fighting S pneumoniae, the former two of which were used for research more often.10,11 The first immune cell type to combat S pneumoniae during early infection is the alveolar macrophage,12 which derives from monocytes and is responsible for the initial detection of bacteria and subsequent modulation of the inflammatory response.13 In this study, we used THP-1 monocytes to be cultured with S pneumoniae strains from sputum, blood, and cerebrospinal fluid (CSF), and analyzed the consequent interleukin-1β (IL-1β), IL-6, IL-10, IL-8, and soluble intercellular adhesion molecule (sICAM-1) levels so as to unveil the differences among different S pneumoniae strains.

2 | MATERIALS AND METHODS

2.1 | Strains and culture

A total of 58 S pneumoniae strains were collected, of which 23 were isolated from sputum samples from patients with pneumonia at Taizhou Municipal Hospital during 2017, and 23 and 11 from blood and CSF samples, respectively, at Tangshan Maternal and Child Health Hospital in 2016 and 2017. Pneumonia was diagnosed based on the results of physical examinations, X-ray, and laboratory medicine. Another strain was ATCC49619, provided by the Chinese National Center for Medical Culture Collections. All the strains were non-repeated, that is, only one strain from each patient. They were all identified using VITEK-2 automatic microorganism analyzer (bioMérieux Co.), followed by a specific PCR method according to du Plessis.14

All the 58 strains were cultured on blood agar plates at 37°C in a 5% CO2 incubator (Thermo Electron Co.) overnight and then adjusted to 0.5 McFarland in normal saline, which were used for stimulation experiments.

2.2 | pbp2B detection

Ten to fifteen pure colonies of each S pneumoniae strain were collected from a blood agar plate and suspended in a 0.5-mL centrifuge tube containing 200 µL ddH2O. The tube was then placed in a boiling water bath for 30 minutes and transferred into a −20°C freezer for 10 minutes, followed by thawing and the centrifugation at 16 099 g for 30 seconds. The subsequent supernatant was transferred into a new 0.5-mL centrifuge tube for regular PCR of pbp2B with primers as following: forward: 5′-CTGACCATTGATTTGCTACATACTG-3′ and reverse: 5′-TTTGGCAATAGTTGCTACATCGT-3′.14 The PCR product was 682 bp. The PCR conditions were as following: 94°C denaturation for 5 minutes, 94°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute for 30 cycles, and 72°C for 10 minutes. Electrophoresis was conducted with 5 µL PCR products and 1 µL 6× loading buffer. The mixture was then loaded into 2.0% agarose gel and electrophoresed at 150 V and 75 mA for 25 minutes. The electrophoresis results were acquired by a gel camera system (Bio-Rad).

2.3 | Cell line and culture

Human THP-1 monocytes were bought from the Cell Bank of Chinese Academy of Sciences and cultured with RPMI 1640 medium (GIBCO) and 10% fetal bovine serum (Zhejiang Tianhang Biological Technology Co.) plus 1% strep-penicillin (Beijing Solarbio Science &Technology Co.) at 37°C in a humidified incubator (Thermo Electron Co.) with 95% air and 5% CO2. THP-1 monocytes under seven passages were then collected and resuspended at 5.0 × 10⁵/L using RPMI 1640 medium and 10% fetal bovine serum inactivated at 60°C for 30 minutes. Viability of THP-1 monocytes was over 95% as determined by trypan blue exclusion. This study was conducted between April and June 2018 at Zhejiang Provincial Demonstration Center of Laboratory Medicine Experimental Teaching, Wenzhou Medical University.

2.4 | Infection of THP-1 cell line with S pneumoniae cultures

One hundred and seventy-seven milliliters of THP-1 cells were inoculated in 177 wells of 12-well culture plates, which were 5.0 × 10⁹/L, one milliliter per well. One hundred microliters of the aforementioned S pneumoniae suspensions at 0.5 McFarland was then seeded into each one of 174 wells. Normal saline was used as control, 100 µL per well for the other three wells. All the wells were cultured at 37°C in 5% CO2 for 4 hours (59 wells), 8 hours (another 59 wells), and 12 hours (another 59 wells). At the end of the experiments, all of the cell cultures plus controls were collected in 1.5-mL centrifuge tubes and centrifuged for 10 minutes at 4500 g. The supernatants were for the analysis of interleukins and adhesin. The experiments were repeated thrice.

2.5 | Analysis of interleukins and adhesin

IL-1β, IL-6, IL-10, IL-8, and sICAM-1 concentrations were analyzed using an ELISA method, according to the manufacturer’s protocols (Shanghai Xitang Biotechnology Co.).

2.6 | Statistical analysis

All the data analyses were made using SPSS 19.0 statistical software (SPSS). The normality analysis was performed by Kolmogorov-Smirnov test and homogeneity of variance analysis between two groups was by Levene’s test. The mean difference analysis was
FIGURE 1  Concentrations of IL-1β secreted by THP-1 cells stimulated by *S pneumoniae* groups. (ATCC, sd-SP, bd-SP, and cd-SP). ATCC, ATCC49619; bd-SP, blood-derived SP; cd-SP, cerebrospinal fluid-derived SP; IL-1β, interleukin-1β; sd-SP, sputum-derived SP

FIGURE 2  Concentrations of IL-6 secreted by THP-1 cells stimulated by *S pneumoniae* groups

conducted by the single-sample t test or analysis of variance for factorial designs. *P* > 0.10 in the normality test and *P* < 0.05 in mean comparison were considered statistically significant.

3 | RESULTS

As shown in Figure 1, the concentrations of IL-1β in clinical *S pneumoniae* groups (sd-SP, bd-SP, and cd-SP) were all higher than ATCC49619 by the single-sample t test. Variance for factorial designs was used to determine the differences among the three clinical *S pneumoniae* groups. No significant difference was found among three clinical groups (F = 2.791, *P* = 0.064), which was found among three stimulation time points (F = 70.559, *P* = 0.000). Interaction effect was not found in three clinical groups and tree stimulation time points (F = 0.457, *P* = 0.767), suggesting no interaction effect between *S pneumoniae* groups and stimulation time points.

As shown in Figure 2, the concentrations of IL-6 in clinical *S pneumoniae* groups were lower than ATCC49619 except at 8 hours for sd-SP group by the single-sample t test. Variance for factorial designs was used to determine the differences among the three clinical *S pneumoniae* groups. No significant difference was found among three clinical groups (F = 2.841, *P* = 0.061), which was found among three stimulation time points (F = 67.312, *P* = 0.000). Interaction effect was not found in three clinical groups and tree stimulation time points (F = 0.334, *P* = 0.855), showing no interaction effect between *S pneumoniae* groups and stimulation time points.

As shown in Figure 3, no significant difference was found between the concentrations of IL-10 in clinical *S pneumoniae* groups and ATCC49619 group by the single-sample t test. Variance for factorial designs was used to determine the differences among the three clinical *S pneumoniae* groups. No significant difference was found among three clinical groups (F = 0.968, *P* = 0.382), which was found among three stimulation time points (F = 170.524, *P* = 0.000). Interaction effect was not found in three clinical groups and tree stimulation time points (F = 0.019, *P* = 0.999), suggesting no interaction effect between *S pneumoniae* groups and stimulation time points.

As shown in Figure 4, the concentrations of IL-8 in clinical *S pneumoniae* groups were lower than ATCC49619 except at 8 hours point for bd-SP group by the single-sample t test. Variance for factorial designs was used to determine the differences among the three clinical *S pneumoniae* groups. No significant difference was found among...
three clinical groups ($F = 1.485, P = 0.230$), which was found among three stimulation time points ($F = 64.852, P = 0.000$). Interaction effect was not found in three clinical groups and tree time points ($F = 0.040, P = 0.997$), showing no interaction effect between \emph{S pneumoniae} groups and stimulation time points.

As shown in Figure 5, the concentrations of sICAM-1 in clinical \emph{S pneumoniae} groups were all higher than ATCC49619 by the single-sample t test. Variance for factorial designs was used to determine the differences among the three clinical \emph{S pneumoniae} groups. Significant difference was found among three clinical groups...
(F = 7.669, P = 0.001), which was also found among three stimulation time points (F = 60.898, P = 0.000). Interaction effect was not found in three clinical groups and tree time points (F = 0.751, P = 0.559), suggesting no interaction effect between S pneumoniae groups and stimulation time points. However, Figure 5 shows lower sICAM-1 concentrations in cd-SP group than sd-SP and bd-SP groups at 4 and 8 hours points, equal at 12 hours.

As shown in Figure 6, rapid accumulation of THP-1 cells was captured after S pneumoniae was added. With the stimulation time progressing, THP-1 cells tended to show similar morphisms while S pneumoniae kept decreasing. No significant difference was found among the three clinical S pneumoniae groups for morphological changes.

4 | DISCUSSION

Our data showed the different impact on THP-1 cells by different S pneumoniae strains. ATCC49619 was remarkably different from clinical S pneumoniae strains, while the latter themselves showed similar traits except the reduction of sICAM-1.

The forebody of IL-1β is 34 KD, which is spliced as 17KD. IL-1β plays an important role in the process of various infectious and chronic inflammatory diseases. IL-1β is primarily secreted by neutrophils, monocytes/macrophages, and dendritic cells, which is also induced by Ply. IL-6 is mainly secreted by neutrophils and monocytes/macrophages, the lack of which could result in the deficit of innate CNS immune against S pneumoniae and consequent rising mortality. IL-10 is a kind of anti-inflammation cytokine that is predominantly secreted by neutrophils, monocytes/macrophages, dendritic cells, and lymphocytes and is vital in acute and persistent bacterial infections. IL-10 could prevent pulmonary inflammation from aggravation induced by S pneumoniae, the lack of which may cause the rising mortality. IL-8 is also called chemokine 8, which is secreted by monocytes/macrophages and dendritic cells. IL-8 could promote inflammatory cells' accumulation and activation, the release of inflammatory mediators, and chemotaxis of neutrophils to inflammatory sites so as to regulate host's inflammatory response. Cytokines are classified as proinflammatory cytokines (IL-1β, IL-6, and IL-8, etc) and anti-inflammatory ones (IL-4 and IL-10, etc). ICAM-1 could bind the lymphocyte function-associated antigen 1 (LFA-1) widely distributed in lymph node and tonsil vascular endothelial cells, thymic dendritic cells, tonsil and glomerular epithelial cells, leukocytes, macrophages, and fibroblasts. ICAM-1 could also promote leukocytes' accumulation toward S pneumoniae-infected sites, especially extravascular sites.

As opposed to IL-10, as shown in Figures 1-5, IL-1β, IL-6, IL-8, and sICAM-1 levels all increased first and then decreased, which reflects the expanding to diminishing of inflammatory response. The concentrations of IL-1β and sICAM-1 in the group ATCC49619 were both higher than all the clinical S pneumoniae groups, IL-10 equal, IL-6 and IL-8 versa. ATCC49619 is a standard S pneumoniae strain, which is less virulent than clinical ones as shown in our previous study. The intriguing difference among clinical S pneumoniae groups lies in sICAM-1 except IL-1β, IL-6, IL-10, and IL-8.
cd-SP group showed lower sICAM-1 concentrations than sd-SP and bd-SP groups at both 4 and 8 hours, similar at 12 hours. For early infection, lower sICAM-1 concentration is infaus for the accumulation of leukocytes in infectious sites so as to aid in S pneumoniae’s surviving and progressing in CSF. The concentrations of such interleukins and adhesin were equal between sd-SP and bd-SP groups, while our previous study proved sd-SP is less virulent than bd-SP.\textsuperscript{8,9} The data in this study were some different from those in our previous study,\textsuperscript{23} which was due to the different concentrations of S pneumoniae and THP-1 cells. In this study, the ratio of S pneumoniae and THP-1 cells was much lower, which resulted in the faster clearance of S pneumoniae. The 16S rRNA cycle threshold \textgreater 32.0 in qRT-PCR also suggested much poorer survival of S pneumoniae (data not shown). And Figure 6 also confirms the scarcity of S pneumoniae and more healthy conditions of THP-1 cells. Rapid accumulation of THP-1 cells was captured after S pneumoniae was added. With the stimulation time progressing, THP-1 cells tended to show similar morphisms while S pneumoniae kept decreasing. In this study, THP-1 cells showed rapid clearance ability of S pneumoniae, in agreement with another report.\textsuperscript{24} Our study had some limitations. Many types of cell lines are involved in S pneumoniae-host interactions, the amount and concentrations of which are hard to determine. However, only one fixed concentration of THP-1 cells was used to simulate actual interactions. Furthermore, THP-1 cell line derived from leukemia cells, which is capable of self-proliferation and owns different ability of phagocytosing and killing S pneumoniae from normal monocytes.

In summary, our data demonstrated lower secretions of sICAM-1 from monocytes may facilitate S pneumoniae’s invading CSF. And further investigations need to be conducted.

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AUTHORS’ CONTRIBUTIONS

Ping Ren and Dakang Hu conceived of the study and were involved in the stimulation test. Lianhua Yu and Chunyan Gao were dedicated to strains’ collection and identification. Jin Zhang carried out PCR and ELISA tests. Ying Qu and Xinyu Jiang analyzed the data. Jin Zhang and Chunyan Gao wrote the manuscript, which was revised by Dakang Hu and Yixia Zhou. All authors read and approved the final manuscript.

ETHICAL APPROVAL

This work was approved by the Ethics Committee of Taizhou Municipal Hospital.

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