Group A Streptococcus induces less p65 nuclear translocation and non-classical nuclear factor kappa B activation in macrophages, which possibly leads to a weaker inflammatory response

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**S U M M A R Y**

**Objectives:** The aim of this study was to explore the pathogenic mechanism of group A Streptococcus (GAS) and to investigate how GAS evades phagocytosis by immune cells.

**Methods:** The classical inflammatory signaling pathway of macrophages infected with GAS was investigated by protein microarray, real-time PCR, Western blot, immunoprecipitation, and flow cytometry.

**Results:** GAS induced a lower level of inflammatory mediators in macrophages than either the Gram-positive *Staphylococcus aureus* or the Gram-negative *Escherichia coli*. Therefore, the conventional inflammatory signal pathway was investigated. It was found that GAS and *S. aureus* induced both toll-like receptor (TLR)2 and TLR4 expression, while Gram-negative *E. coli* only activated TLR4 in RAW264.7 cells. Although MyD88, the main adaptor protein, was activated by the three pathogens, there was no difference in MyD88 expression in macrophages. Nuclear factor kappa B (NF-κB) is the classical transcription factor of inflammatory signals, and the results of the present study showed that GAS, similar to *E. coli*, induced a weaker p65 nuclear translocation compared to *S. aureus*. Interestingly, GAS activated NF-κB by inducing p65–p52 heterodimer, but not the classical heterodimer of NF-κB (p65–p50). While *E. coli* activated NF-κB by inducing both p65–p50 and p65–p52 heterodimers.

**Conclusions:** Compared to *S. aureus* and *E. coli* infection, GAS induced a weaker nuclear translocation and distinct combination of NF-κB subunits in macrophages, which probably leads to a weak inflammatory response.

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1. **Introduction**

Group A Streptococcus (GAS), also called *Streptococcus pyogenes*, is a prevalent and versatile human pathogen responsible for a broad spectrum of human diseases, ranging from superficial skin infections to an occasionally lethal syndrome. GAS usually persists in the host by evading immune attack, which can lead to recurrent infections under appropriate conditions. Basically, the severity and consequences of the infections caused by various pathogens depend on the ability of the host innate immune mechanisms to control bacterial growth and therefore to limit the further spread of the pathogen beyond the site of infection. However, the underlying mechanisms of persistent infections by live *S. pyogenes* are not well understood.

Macrophages are known to play an essential role in mounting an adequate defense against infections in human innate immunity. Usually, macrophages become activated by human pathogens, thus leading to an inflammatory response, which will lead to the recruitment of neutrophils and more macrophages to promote

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clearance of the invasive pathogens. However, data on macrophage activation after live GAS infection that mimics the actual clinical condition are scarce. Günther et al. compared the activation of key immune pathways in macrophages between heat-killed Staphylococcus aureus and Escherichia coli, and others have evaluated the activation of macrophages induced with bacterial components such as lipopeptides, lipopolysaccharide (LPS), and peptidoglycan.\(^4\)\(^-\)\(^7\) Studying how macrophages respond to live GAS at the molecular level will help us to understand the signaling pathways by which GAS evades immunosurveillance and may help us to develop strategies to prevent recurrent GAS infections. The transcription factor nuclear factor kappa B (NF-κB) is critical for the inducible expression of many genes involved in the immune and inflammatory response, including interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, and TNF-β.\(^8\)\(^-\)\(^10\) Moreover, NF-κB DNA binding activity typically involves two subunits, a 50-kDa protein (p50)\(^9\)\(^-\)\(^10\) and a 65-kDa protein (p65).\(^11\)\(^-\)\(^12\) In this study, the classical inflammatory signaling pathway in macrophages following GAS infection was investigated, with S. aureus (Gram-positive microbe) and E. coli (Gram-negative microbe) used as controls. It was found that when macrophages were infected by GAS, both NF-κB activity and the expressions of proinflammatory molecules were lower than those infected by either S. aureus or E. coli. This is detrimental to the production of an efficient inflammatory response and instead probably leads to persistent GAS infection.

2. Materials and methods

2.1. Mice

Female BALB/c mice, aged 7–8 weeks, were purchased from the Beijing Laboratory Animal Center and housed in groups of four. They were allowed 5 days to acclimatize to the housing facility. Environmental conditions were a temperature of 23 ± 2 °C, humidity of 55 ± 10%, lighting of 300 lux, and a 12/12 light/dark cycle. Animals were kept in 595 × 380 × 200 mm cages under specific pathogen-free conditions. They were monitored once daily with regard to their health status during housing. The treatment of the animals was carried out according to the Care and Use of Laboratory Animals (Beijing) and was approved by the Ethics Committee of Hebei Medical University (DW2014001).

2.2. Bacteria

Streptococcal strain GAS SF370 M1 and S. aureus were stored at -80 °C and grown routinely at 37 °C in Todd–Hewitt broth supplemented with 0.5% yeast extract (THY; Difco, MI, USA). E. coli was grown routinely at 37 °C in lysozyme broth culture.

2.3. Cells

The murine macrophage cell line RAW264.7 (ATCC TIB-71) and the acute monocytic leukemia cell line THP-1 (obtained from the resource center of Peking Union Medical College Hospital, China) were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 100 U/ml penicillin and streptomycin and 10% fetal bovine serum (FBS) (Gibco BRL Co. Ltd, USA) (complete medium). Before each test, THP-1 differentiated to macrophages (mTHP-1) after being induced by phorbol 12-myristate 13-acetate (PMA; 40 ng/ml; ENZO Life Sciences, Inc., USA) for 48 h. Mouse bone marrow-derived macrophages (BMDMs) were generated from bone marrow cells obtained from mouse femurs after the animals were sacrificed by anesthesia. Bone marrow cells (3 × 10⁶) were cultured with DMEM complete medium containing 10 ng/ml macrophage colony-stimulating factor (M-CSF, Thermo Fisher Scientific Inc., USA). After 7 days of culture, the fully differentiated BMDMs were used for the experiments.

2.4. Proliferation analysis

The multiplicity of infection (MOI) was optimized by measuring the growth capability of GAS-infected macrophages. For this analysis, RAW cells or BMDMs were seeded at 1 × 10⁵/well in 96-well plates containing medium without antibiotics. The next day, GAS, S. aureus, and E. coli were harvested at mid-logarithmic phase and added respectively to the cells at MOI of 2, 5, 10, 20, 30, 50, or 100 at 37 °C for 2 h. RAW264.7 cell proliferation was analyzed following the instructions of Cell Counting Kit 8 (CCK8; Dojindo). RAW264.7 cells were also co-cultured with LPS as a positive control group.

2.5. GAS infection of macrophages

RAW cells, or THP-1 or BMDMs, were seeded at 1 × 10⁶/well in 6-well plates containing medium without antibiotics, and infected with GAS, S. aureus, or E. coli at MOI of 10. After 2 h of incubation at 37 °C, non-adherent extracellular bacteria were eliminated by removing the culture medium and washing with phosphate buffered saline (PBS). Adherent extracellular bacteria were subsequently killed by incubation with fresh medium containing 100 μg/ml penicillin G/streptomycin. At specific time points after infection, supernatants were collected for ELISA, and the cells or lysates of cells were retained for quantitative real-time PCR (qRT-PCR), protein microarray technique, Western blot analysis, and co-immunoprecipitation.

2.6. Protein microarray technique

RAW264.7 cells infected with GAS, E. coli, or S. aureus at MOI of 10 were collected at 72 h and washed twice with PBS. The whole cell extracts were used to investigate inflammation-associated proteins by protein array in agreement with the RayBio mouse cytokine antibody array protocol (RayBiotech, Inc., USA). Briefly, array membranes were blocked with blocking buffer at room temperature for 30 min, following which blocking buffer was decanted from each container. The membranes were then incubated with 1 ml (500 ng) of cell lysates at room temperature for 1.5 h and washed three times with wash buffer. Subsequently, the array membranes were incubated with biotin-conjugated antibodies and horseradish peroxidase (HRP)-conjugated streptavidin in sequence at room temperature, each for 1.5 h, respectively. Finally, the detection reaction was performed by chemiluminescence imaging system. Data shown represent a single experiment or representative of three independent experiments with similar variation tendency.

2.7. qRT-PCR

Cells collected at various time points were used to isolate total RNA using a RNeasy kit (Takara). The qRT-PCR analysis was performed following a modified protocol. Briefly, cDNA samples converted from 1 μg total RNA were diluted at several concentrations. Diluted cDNA was mixed with a pair of primers (10 μM) targeting mouse monocyte chemotactic protein (MCP)-1, RANTES, IL-6, IL-1β, TNF-α, IL-12, toll-like receptors (TLRs; TLR2 and TLR4), and β-actin cDNA sequences at an annealing temperature of 60 °C and 35 amplification cycles, following the manufacturer’s instructions. The following primers were used for PCR amplification: MCP-1-F: GCTGACCCCAAGAAGGAATG; MCP-1-R: GAAGACCCTAGGGCATGCTA; RANTES-F: GCACAGCACAACCCAGAAAAT; RANTES-R: ACTTTGTGCCCAAGAGGT; IL-6-F: AAGGACCTTAGGGCATGCTA; IL-6-R: AGAGAATGGAGCTGCTGCTG; IL-1β-F: GGTGGTCCCAAGTCACTG; IL-1β-R: TCACCACACTGAGTATGTTG; TLR2-F: TGGGCTGAGAAGAGAGGG; TLR2-R: CAGCTTCAGAAGTGGAGAG; TLR4-F: CAGAAGGAACACAGGGTG; TLR4-R: GGCAGGTTGGGAGAGAAG; β-actin-F: AGAGAATGGAGCTGCTGCTG; β-actin-R: TCACCACACTGAGTATGTTG; IL-12-F: GGTGGTCCCAAGTCACTG; IL-12-R: TCACCACACTGAGTATGTTG.
CTA AGG ACC AA; IL-6-R: GTT TGC GTA GAT CTC AAA; IL-1β-F: TTC CTT GTG CAA GTG TCT GAA G; IL-1β-R: CAC TGT CAA AAG GTG GCA TTT; TNF-α-F: TGCAGTGGAGTTGAGAAGTTAG; TNF-α-R: TGGGCCCTAGAATGTGAGA; IL-12-F: GTAACCAAGAAGTG CGTTCC; IL-12-R: CTGAC GC 3GACGACAATC; β-actin-F: TACCG CAGCT ATTTGCTGACAG; β-actin-R: ACTTTGCGGTGCAAGATGGA; TLR2-F: TTCAACAAAGATCTACATT G GC; TLR2-R: CAAGACTGCTCAGAGATA AAG G; TLR4-F: CTGATGACTCTT CTTCACC; TLR4-R: TTTCCTGCATTACATGATTTGAG.

2.10. Flow cytometry analysis

The RAW264.7 cells were stimulated by GAS, S. aureus, E. coli, or PBS at 37 °C for 2 h, and then collected and adjusted to 10^6 cells/ml with DMEM culture medium. Next, 100 μl of the suspended cells was incubated for 30 min at room temperature in a dark room with fluorescein isothiocyanate (FITC)-labeled anti-mouse CD282 (TLR2) and phycoerythrin (PE)-labeled anti-mouse CD80/CD86 (MD-2) complex antibodies (eBioscience Inc., USA). The mouse IgG isotypes were used as controls. Suspensions were washed twice with PBS and analyzed by flow cytometry (Becton, Dickinson and Company, USA). Data were analyzed using CellQuest software.

2.9. Blockade of TLR2 and TLR4

To verify the roles of TLR2 and TLR4 in GAS infection, RAW264.7 cells were blocked with anti-mouse neutralizing antibodies according to a method described elsewhere. Briefly, 1 × 10^6 RAW264.7 cells were dispensed into the wells of 6-well plates (Costar Electronics Inc., Taipei, Taiwan) and incubated at 37 °C, 5% CO₂ for 24 h. Following this, they were washed twice with PBS and the cells were then pre-incubated for 1 h at 37 °C with 1 ml of anti-TLR2 or anti-TLR4 neutralizing antibodies, or IgG isotypes as control (10 μg/ml; eBioscience Inc., California, USA) before stimulation by GAS, S. aureus, or E. coli for 2 h. The cells and the supernatant were then collected and frozen in aliquots at −80 °C until assay.

2.10. Western blot analysis

The whole cell protein was extracted from the cells infected by GAS or E. coli by radioimmunoprecipitation assay (RIPA) protein extraction kit (Beyotime, China). Briefly, cells infected by GAS or E. coli were washed twice and mixed well with appropriate RIPA lysis buffer; they were then centrifuged at 12 000 rpm for 5 min at 4 °C. Next, the lysate was collected and the protein concentration was determined using the Bradford assay. The whole cell lysates were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking, blots were developed with rabbit monoclonal antibodies against MyD88 (Abcam Inc., UK), p65, p-p65 (Cell Signaling Inc., USA), p-p105, or p-p100 (Cell Signaling Inc.). Histone H or β-actin (Cell Signaling Inc.) was used as the internal control. Blots were then hybridized with HRP-conjugated goat anti-rabbit IgG, incubated with enhanced chemiluminescence (ECL) solution (Perkin Elmer Life Sciences, USA), and exposed to X-ray film.

2.11. Co-immunoprecipitation

Immunoprecipitation was carried out using Protein G PLUS-Agarose (GE Healthcare, USA) following the manufacturer's instructions. Briefly, 500 μg of protein extract was incubated with 2 μg of special antibody overnight at 4 °C under gentle agitation, and then placed into a tube containing pre-cleared 50 μl of Protein G PLUS-Agarose. This mixture was incubated for 4.5 h at 4 °C with stirring. After washing three times with PBS, precipitated complexes were solubilized by boiling in SDS buffer, fractionated by 12% SDS-PAGE, and transferred to a PVDF membrane for Western blotting.

2.12. Statistical analysis

The differences between groups were analyzed by Mann–Whitney U-test and by Kruskal–Wallis analysis of variance test, as appropriate. The SD of the mean is shown unless indicated otherwise. Alpha values of p < 0.05 were considered to indicate a statistically significant difference between the data compared.

3. Results

3.1. Streptococcus pyogenes induced less expression of inflammation-associated proteins in both murine peritoneal macrophages and RAW cells

In order to determine the optimal MOI of GAS for infection, macrophages were infected with different MOI ranging from 2 to 100. When the MOI was <30, it had similar effects on macrophage proliferation (Figure 1A). Thus, an MOI of 10 was used to measure the inflammation-associated molecules in murine peritoneal macrophages or RAW264.7 cells. In line with previous reports, several inflammatory cytokines and chemokines were induced by these three bacteria. However, compared to S. aureus and E. coli infections, macrophages infected with GAS generally produced much lower levels of cytokines, including soluble TNF receptor 1 (sTNFR1), TNF-α, RANTES, MCP-1, and IL-1α (Figure 1B). A low mRNA expression of these cytokines and chemokines was also displayed in cells infected by GAS compared to S. aureus and E. coli infections (Figure 2). These data indicate that GAS elicits a lesser cytokine response in macrophages compared to S. aureus and E. coli.

3.2. TLR2, but not TLR4 or MyD88, mediated the different expressions of inflammatory cytokines induced by GAS, Staphylococcus aureus, and Escherichia coli

In order to explore the mechanisms of the differential expression of inflammatory cytokines, the expression of TLR2 and TLR4 were analyzed in GAS-, S. aureus-, and E. coli-infected macrophages by flow cytometry and real-time PCR. As shown in Figure 3, TLR2 expression levels were much higher in GAS- and S. aureus-infected macrophages than in E. coli-infected macrophages. There were no significant differences in TLR4 expression among the three groups. There was a significant difference between the infected groups and the PBS negative control. In agreement with the fluorescence-activated cell sorting results, a similar pattern of TLR2 and TLR4 expression was also observed by real-time PCR, except for S. aureus, which induced the highest TLR4 mRNA level compared to the others. These results suggest that both GAS and S. aureus can induce high expression of TLR2 and TLR4, while E. coli solely induces high expression of TLR4 in macrophages.

To further investigate the role of TLR2 and TLR4 in the production of proinflammatory cytokines by GAS, S. aureus, and E. coli, RAW cells were pre-incubated with anti-TLR2 neutralizing antibody for 1 h, followed by infection with GAS, S. aureus, or E. coli. Blocking of TLR2 significantly down-regulated the expression of IL-1β (Figure 4A) and IL-6 (Figure 4B), but not TNF-α (Figure 4C) in GAS- and S. aureus-induced RAW cells. Interestingly, anti-TLR2 neutralizing antibody had almost no effect on the expression of IL-1β and IL-6, while TNF-α expression was
increased in E. coli-induced RAW cells (Figure 4A–C). In contrast to the effects of blocking TLR2, blockage of TLR4 showed a limited influence on the expression of IL-1β, IL-6, and TNF-α in GAS- and S. aureus-induced RAW cells (Figure 4D–F), while these three cytokines were decreased significantly compared with unblocking TLR4 in E. coli-infected RAW cells. These results indicate that the production of IL-1β and IL-6 stimulated by GAS or S. aureus is mediated mainly by TLR2 and not TLR4. In contrast, TNF-α production is mediated mainly by TLR4 following E. coli infection.

MyD88, the common adaptor protein of the TLR signaling pathway, was detected by Western blot and real-time PCR in macrophages after infection with GAS, S. aureus, and E. coli. Although MyD88 protein was highly expressed in macrophages infected by GAS, S. aureus, and E. coli, there was no significant difference among the three groups (data not shown), suggesting that MyD88 is not the main reason for the differential expression of the inflammatory cytokines in GAS-, S. aureus-, and E. coli-infected macrophages, even though it is the major adaptor protein in the cell signaling pathway of infection. Perhaps some other pathway is also involved, which should be confirmed in future research.

3.3. GAS induced less p65 nuclear translocation than Staphylococcus aureus, but similar to Escherichia coli in macrophages

To evaluate the transcription activity of NF-κB in macrophages after infection, RAW cells were infected with GAS, S. aureus, or E. coli at MOI of 10 for 30 min or for 24 h, following which the cells were collected to extract the nucleoprotein and cytoplasmic proteins for the measurement of NF-κB by Western Blot. As shown in Figure 5A, S. aureus induced more translocation of NF-κB p65 subunit in the nucleus compared to GAS after infection of macrophages for 30 min and for 24 h, while there was no significant difference in nuclear p65 accumulation between GAS- and E. coli-infected macrophages. The level of p65 phosphorylation in GAS-, S. aureus-, and E. coli-induced THP-1 was also detected under the above conditions. As expected, similar results were observed, as shown in Figure 5B, suggesting that GAS elicits less p65 nuclear translocation than S. aureus, although both are Gram-positive microbes. However, the level of p65 nuclear translocation elicited by GAS was similar to that of E. coli in macrophage cells.

3.4. Different subunits of NF-κB in murine macrophages were activated by GAS and E. coli

As mentioned above, p65 nuclear translocation elicited by GAS was similar to that of E. coli; however, E. coli induced macrophages to express higher levels of inflammatory cytokines than GAS, especially within 24 h after infection. Consequently, the p-p65, p-p100 (the former of p-p52), and p-p105 (the former of p-p50) were detected in GAS- and E. coli-infected RAW264.7 cells within 30 min after infection. The results showed that GAS and E. coli induced similar levels of p-p65 and p-p100 expression (Figure 5B). However, the expression of p-p105 induced by E. coli was higher than that induced by GAS (Figure 5B). Thus the stimulation was extended to 90 min and it was found that E. coli induced significantly higher levels of p-p65 and p-p105 than GAS at 60 min post-stimulation. Accordingly, similar results were observed in BMDMs after the infection was repeated (Figure 6A), suggesting that activation of the NF-κB subunits was different in macrophages infected by GAS than in those infected by E. coli.

Immunoprecipitation was further performed using anti-p-p65 monoclonal antibody to bind the other subunit of NF-κB in RAW cells induced by GAS or by E. coli. As shown in Figure 6B, p-p65 mainly bound to p-p100 following GAS infection, while p-p65 bound to both p-p100 and p-p105 after RAW cells were infected by E. coli, suggesting that activated NF-κB mainly consists of p65 and p52 subunits in murine macrophages following GAS stimulation, while E. coli-induced NF-κB activation consists of p65–p52 and p65–p50 heterodimers.

4. Discussion

GAS is the most common bacterial cause of diseases with a broad range of symptoms, including rheumatic fever, erysipelas, necrotizing fasciitis, toxic shock syndrome, and acute pharyngitis; it is also known to be the cause of allergic diseases such as rheumatic heart disease and post-streptococcal glomerulonephritis.1,15 Furthermore, GAS has the capability of utilizing host complement regulatory proteins to enter epithelial cells for immune evasion and host colonization.16,17
Macrophages are the key sensory and regulatory cells of the host innate immune system and their functional status directly affects the prognosis of infection. Macrophages have the ability to secrete chemokines such as MCP-1 and RANTES, and cytokines including the major proinflammatory cytokines IL-1, TNF-α, and IL-6, which can signal and attract neutrophils, macrophages, and other immune cells to the local site of infection, leading to an inflammatory reaction that affects the prognosis of the disease. However, another crucial point is how the bacteria interact with host macrophages, leading to a persistent bacterial infection.

In this study, it was found that the levels of MCP-1, RANTES, IL-1, TNF-α, and IL-6 expressed by GAS-stimulated macrophages were lower than those of the S. aureus- and E. coli-stimulated macrophages. These results suggest that GAS may decrease the recruitment of inflammatory cells compared with S. aureus and E. coli through the down-regulation of chemokine and cytokine expression, which is conducive to GAS escaping immune attack and promotes GAS survival in the host.

The mechanisms underlying the lower expression of cytokines in macrophages induced by GAS are not completely understood. It is not known whether the conventional inflammatory signaling pathway in macrophages elicited by GAS is different from that of other bacteria. Thus, in this study, the classical receptors TLR2 and TLR4 were first detected, since TLRs are the most important members of the pattern-recognition receptors in mammals, in particular TLR2 for peptidoglycans, lipooligosaccharides, and bacterial lipoprotein and TLR4 for LPS and lipoteichoic acid. The results showed that a significant high level of TLR2 at 30 h was induced by GAS and S. aureus and that TLR4 could be activated by all three of the pathogens (Figure 3), although TLR4 was found to have different activation levels in the three groups based on flow cytometry and real-time PCR. This implies that both TLR2 and TLR4 are involved in the recognition of Gram-positive microorganism such as GAS and S. aureus, while E. coli selectively induces TLR4 to activate the host defense mechanisms. Subsequently, TLR2 and TLR4 were blocked separately by their neutralizing antibodies and the expression of proinflammatory cytokines IL-1β, IL-6, and TNF-α was measured. As expected, after TLR2 was blocked, the expression levels of IL-1β and IL-6 induced by GAS and by S. aureus were significantly reduced. Conversely, the blockage of TLR2 did...
not affect the expression of IL-1β and IL-6 in E. coli-induced RAW cells. However, after blocking TLR4, the expression of inflammatory cytokines IL-1 and IL-6 was significantly decreased in E. coli-induced RAW cells, while the expression of these two cytokines was almost unaffected in GAS-stimulated cells. These results indicate that although both TLR2 and TLR4 are involved in the recognition of the microorganism, the production of IL-1β and IL-6 appears to be mostly TLR2-dependent in GAS- and in S. aureus-induced RAW cells and to be TLR4-dependent in E. coli-induced RAW cells. Interestingly, TNF-α generation was significantly increased by TLR2 blockage, but was inhibited by TLR4 blockage in E. coli-induced RAW cells. It is tempting to speculate that there may be crosstalk between TLR2 and TLR4 in the regulation of intracellular signals of TNF-α production and that TLR4 has primary responsibility for the generation of TNF-α.

The results of this study showed that the levels of proinflammatory cytokines induced by GAS were lower than those induced by S. aureus, although these two organisms had similar

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**Figure 3.** Analysis of TLR2 and TLR4 expression in GAS-, S. aureus-, and E. coli-stimulated macrophages. RAW264.7 cells were infected by GAS, S. aureus, or E. coli at 37 °C for 2 h, and TLR2 and TLR4 expression levels were analyzed by flow cytometry (A, B, C), and by real-time PCR (D). Each experiment was performed at least three times. Results are presented as the mean ± SD; *p < 0.05 vs. PBS control; **p < 0.05 vs. E. coli group.
capability with regard to the induction of TLR2 and TLR4 expression. It has been reported that diverse molecules are involved in intracellular signaling pathways activated by GAS and S. aureus in macrophages.\textsuperscript{26,27} Furthermore, some researchers have reported that distinct TLRs can discriminate between different pathogen-associated molecular patterns (PAMPs), and that different TLR-induced signals may also be different, which will lead to different gene activation.\textsuperscript{13} In addition, there are data indicating that the different intracellular protein complexes containing adaptor proteins are recruited by binding to TLR4/TLR4, TLR2/TLR6, and TLR2/TLR1 dimers.\textsuperscript{28} However, MyD88 is the major adaptor protein of TLRs, thus the level of MyD88 in
Figure 5. Western blot analysis of unclear translocation of p65 and p-65, p100, and p105 phosphorylation in GAS-, SA-, and E. coli-induced macrophages. Macrophages were infected with GAS, or SA, or E. coli at 10 of MOI. At different post-infection times, the level of p65 unclear translocation in RAW264.7 cells (A), and the level of p65 phosphorylation in THP1 cells (B), and the levels of p65, p100, and p105 phosphorylation in RAW264.7 cells (C) were detected by Western blot respectively. ★ p < 0.05; ★★ p < 0.01. Each experiment was repeated three times and similar results were obtained.
macrophages stimulated by GAS, S. aureus, and E. coli was further investigated.

High level expression of MyD88 was observed by Western blot, and unexpectedly there was no significant difference in MyD88 levels among the three pathogen groups. However, as mentioned above, the distinct bacteria induced different levels of cytokines and chemokines, so a possible explanation for this contradiction is that MyD88 is involved in the transduction of the signal but is not the main reason for the difference in expression of the inflammatory factors induced respectively by the three pathogens. This difference may be related to the involvement of other adaptor proteins. In fact, these signals emanating from pattern-recognition receptors and receptors of members of the TNF and IL-1 cytokine families terminally activate transcription factor NF-κB. Therefore, it was important to test the level of activated NF-κB and its subunit composition in GAS-, S. aureus-, and E. coli-stimulated macrophages.

B. In fact, these signals emanating from pattern-recognition receptors and receptors of members of the TNF and IL-1 cytokine families terminally activate transcription factor NF-κB. Therefore, it was important to test the level of activated NF-κB and its subunit composition in GAS-, S. aureus-, and E. coli-stimulated macrophages.

Figure 6. Western blot and immunoprecipitation analysis of p-p100 and p-p105 interaction with p-p65 in RAW264.7 macrophages following GAS, S. aureus, or E. coli infection. The expressions of p-p65 and p-p105 in RAW264.7 or BMDMs stimulated with GAS, S. aureus, or E. coli were detected by Western blot (A), and the interaction of p-p100 and p-p105 with p-p65 was detected by immunoprecipitation (B); *p < 0.05; **p < 0.01. Each experiment was repeated three times and similar results were obtained.
heterodimerization. p65 predominantly heterodimerizes with p50,33 which constitutes the canonical NF-κB signaling pathway; this is activated and accumulates in the nucleus after induction by most stimuli.34 Generally, different NF-κB dimers target different genes, which will be transcribed into proteins to play different roles during the course of an innate immune response. The major function of p105 appears to be as a reservoir for the production and regulation of the p50–p50 homodimer.35 Unlike p105, p100 plays an important role in regulating p65 homodimer and inhibiting the classical NF-κB dimer as well.36,37 In this study, the activation of p65, which is mainly associated with proinflammatory factor production, was investigated; the results showed that in the three groups, S. aureus induced the highest level of p65 nuclear accumulation, suggesting that S. aureus probably induces more canonical NF-κB activation, which leads to higher expression levels of proinflammatory cytokines. The level of proinflammatory cytokines induced by GAS was much lower than that induced by E. coli. It is puzzling that the p65 nuclear accumulation induced by GAS was similar to that induced by E. coli. One possible explanation for this contradiction is that GAS and E. coli activate different types of NF-κB in macrophages. Therefore, the subunits and the combinations of NF-κB were further detected. As expected, GAS induced p65–p52 heterodimer, while E. coli induced two heterodimers of NF-κB, p65–p50 and p65–p52 (Figure 7).

In summary, although GAS is a Gram-positive pathogen, GAS was found to induce a lower level of p65 activation in murine macrophages than S. aureus, which resulted in a lower level of inflammatory cytokine expression than in the S. aureus group. Compared to E. coli, GAS induced a similar level of p65 activation, which was p65–p50 heterodimer, whereas the NF-κB activated by E. coli was in the form of two heterodimers, p65–p50 and p65–p52. These results may account for the lower expression of proinflammatory cytokines induced by GAS than by E. coli.

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Conflict of interest: All authors declare that they have no conflicts of interest.

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