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Complement activation contributes to the anti-methicillin-resistant *Staphylococcus aureus* effect of natural anti-keratin antibody

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains a major public health problem worldwide because of its strong resistance to a variety of antibiotics. Natural immunoglobulin (Ig) M antibodies have been reported to protect against microbial infections. In the present study, the function of a monoclonal natural anti-keratin antibody IgM (named 3B4) in MRSA infection was evaluated. The binding of 3B4 to MRSA was studied using immunofluorescence assay and flow cytometry (FCM). The binding of 3B4 to mannose-binding lectin (MBL) and complement activation were detected by ELISA. For the *in vivo* study, transgenic mice for the V_H gene from 3B4 (TgV_H 3B4) were used. After infection, the bacterial burden was examined in the kidney, spleen and enterocelia. Inflammatory cytokine levels and the neutrophil ratio in peritoneal lavage fluid (PLF) were assessed by ELISA and FCM, respectively. Additionally, the total serum hemolytic activity (CH50) in the early stage of infection was detected by ELISA. The results showed that 3B4 bound directly to MRSA and MBL, and the interaction between 3B4 and MRSA/MBL led to the activation of the classic and the MBL pathway *in vitro*. After 48 h of MRSA infection, the bacterial load in the kidney, spleen and enterocelia was significantly decreased in TgV_H 3B4 mice ($P < 0.05$) compared with wild-type mice. Levels of IL-6, TNF-α, and IFN-γ were increased after MRSA infection. The levels of IL-6 and TNF-α in TgV_H 3B4 mice were decreased by 49.1% and 59.4% compared to wild-type mice. Additionally, the neutrophil ratio in the PLF of TgV_H 3B4 mice was decreased by 65.9%. The CH50 value was significantly higher in TgV_H 3B4 mice than in wild-type mice, indicating that 3B4 promoted the activation of the complement system in MRSA infected mice. The results reveal an important role of 3B4 in the anti-MRSA immune response, and the complement activation contributes to this effect.

1. Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an increasingly common pathogen in hospital and community settings [1]. It remains a major public health problem worldwide because of its strong resistance to a variety of antibiotics [1]. Penicillin-binding proteins (PBPs) of bacteria are important for penicillin-like antibiotics to exert their antimicrobial properties. The mechanism leading to methicillin resistance of MRSA is based on the expression of PBP2a, which is a mutated PBP [2]. The expression of PBP2a significantly reduces the binding affinity of β-lactam antibiotics [2]. Since excessive antibiotic use has become one of the top contributors to the development of resistance, new therapies are needed in this ongoing struggle, such as vaccines and antibodies.

Natural antibodies are a type of antibody that are produced without external antigenic exposure [3]. Natural antibodies are mostly of the immunoglobulin (Ig) M isotype and play crucial roles in the immune system [4,5]. IgM plays an important role in enhancing IgG responses and protecting against the development of autoreactive IgG and autoimmune diseases [3]. Natural IgM antibodies have also been reported to protect against microbial infections by recognizing a wide range of different microbial components [6,7]. In a previous study, we reported a monoclonal natural antibody, 3B4, which was isolated from the serum of a unimmunized mice [14]. We demonstrated that 3B4 recognized keratin and a surface antigen located at germ tubes of *Candida*

Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; Ig, immunoglobulin; FCM, flow cytometry; TgV_H 3B4, a μ chain transgenic mouse (TgV_H 3B4) using the V_H gene from 3B4; PLF, peritoneal lavage fluid; MBL, mannose-binding lectin; NMS, normal mouse serum; CFU, colony-forming units; MgEGTA, magnesium ethylene glycol tetra-acetic acid; TMB, 3,3′,5,5′-tetramethylbenzidine; HRP, horse reddish peroxidase; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; BSA, bovine serum albumin.

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albicans, and the isotype of 3B4 was IgM [14]. 3B4 inhibited germ tube formation of *C. albicans in vitro* and protected mice from *C. albicans*-induced death *in vivo* [14]. We also established a μ chain transgenic mouse (TgV_H 3B4) using the V_H gene from 3B4, and demonstrated that this mice possessed high level of anti-keratin/*C. albicans* IgM antibodies and were resistant to *C. albicans* infections [14].

The immune system is comprised of two branches: innate and acquired immunity. The innate immune system is the first line of host defense against pathogens, and acquired immunity is involved in the late phase of infection [8]. The complement system plays important roles in both innate and adaptive immune defense by defending against microbial infections, bridging innate and adaptive immunity, and disposing of immune complexes and the products of inflammatory injury [9]. The complement system can be activated by three different pathways: the classic pathway, the mannose-binding lectin (MBL) pathway, and the alternative pathway [10]. It has been reported that natural IgM can activate complement by the classical and lectin pathways under several conditions, such as pathogen infection, ischemia/reperfusion injury and hypoxic stress [11–13]. The effect of IgM in MRSA infection and complement activation needs to be investigated.

In the present study, we evaluated the protective effect of a monoclonal natural anti-keratin antibody IgM (3B4) during MRSA infection and its role in complement activation. The results show that 3B4 can bind to MRSA and activate the classic pathway and MBL pathway *in vitro*. In TgV_H 3B4 mice, the anti-MRSA effect and complement activation were greater than in wild-type mice. These results indicate that complement activation contributes to the anti-MRSA infection effect of the natural antibody 3B4.

2. Materials and methods

2.1. Monoclonal antibody and MRSA

The monoclonal natural anti-keratin antibody IgM was produced as previously described [14]. The clone 3B4, which preferentially recognizes keratin, was used in the present study. The MRSA strain used was obtained from ATCC (Rockville, Maryland, USA).

2.2. In vitro MRSA-3B4 binding assay

For the immunofluorescence assay, 100 μL of MRSA solution (1×10^7/mL) was incubated with 1 μL of 3B4 (1 mg/ml) for 1 h. After washing twice with PBS containing 5% FBS, FITC-labeled anti-IgM (Sigma, St. Louis, MO, USA) was added and incubated in the dark for 30 min. After washing three times, the bacteria were detected by fluorescence microscopy (Olympus, Tokyo, Japan). A purified IgM from murine myeloma (TEPC, 1 mg/ml, Sigma) was used as the isotype control.

Flow cytometry (FCM) was also performed to detect the binding of MRSA and 3B4. Briefly, 1 μL of 3B4 (1 mg/ml) was added to 100 μL of the MRSA solution (1×10^7/mL) and incubated for 1 h. The system was washed twice and the supernatant was removed. FITC-labeled anti-IgM (Sigma) was added to the system and incubated for 1 h. After washing twice, the samples were analyzed by a FACSCalibur instrument (BD Biosciences, San Jose, CA).

2.3. In vitro MBL binding assay

Purified 3B4 and TEPC were coated at different concentrations. The wells were washed three times and blocked for 1 h at room temperature with 0.1% bovine serum albumin (BSA, Invitrogen, Carlsbad, CA, USA). After washing three times, normal mouse serum (NMS, Invitrogen) was added to the incubation system. Following incubation overnight at 4 °C, the wells were washed three times. Biotinylated anti-MBL-A or anti-MBL-C (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the wells and incubated for 1 h. HRP-labeled streptavidin (Santa Cruz Biotechnology) was added and incubated for 30 min. Then, the substrate solution was added and incubated for 10 min. The reaction was stopped by adding 0.5 M H_2SO_4 to each well. The plates were read at 450 nm using a BioRad (Hercules, CA, USA) ELISA reader.

2.4. Analysis of complement activation

To elucidate which complement pathways are activated by 3B4, several complement inhibitors were evaluated. The magnesium ethylene glycol tetra-acetic acid (MgEGTA, 5 mmol/l, Sigma) was used to block complement activation. An antibody against C1q (IgG1, Abcam, Cambridge, UK) was used to block the activation of classic pathway. An antibody 20B4 (IgG1, Abcam) was used as isotype control of anti-C1q antibody. D-mannose (200 mmol/l, Sigma), a ligand for MBL which can inhibit the binding of MBL to its ligands, was used for the blockade of MBL pathway. Since the alternative pathway is inactive at serum concentrations below 5%, 2% serum was used to block the alternative pathway. Complement activation was measured by the detection of C3 deposition. The plates were coated with TEPC (1 mg/ml) or 3B4 (1 mg/ml), blocked with BSA, and incubated with MRSA (for classic pathway activation). Subsequently, plates were incubated with 2% or 5% normal mouse serum (NMS, pre-incubated with anti-C1q/D-mannose), with/without the presence of MgEGTA, for 1 h at 37 °C. The plates were washed, then incubated with rat anti-mouse C3 (Abcam) followed by horse reddish peroxidase (HRP)-conjugated rabbit anti-rat IgG (Abcam). Bound antibody was detected using the chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB).

2.5. Animal and treatment

The μ chain transgenic mice (TgV_H 3B4) using the V_H gene from 3B4 used in this study was kindly provided by Prof. Yufeng Liu (Fourth Military Medical University, Xi'an, China) [14]. The mice in the infection group were administered 0.5 ml of MRSA (10^8/ml) by intraperitoneal injection. The mice in the control group were injected with the same volume of normal saline. The animals were kept in cages with free access to food and water until they were used. All procedures in this study were approved by the Animal Care Committee of the Fourth Military Medical University.

2.6. Detection of inflammatory cytokines

After 48 h of infection, the mice were subjected to lavage of the peritoneal cavity. The peritoneal lavage fluid (PLF) was centrifuged for 5 min at 200 g. The supernatant was collected for the detection of interleukin (IL)-6, IL-10, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. Levels of these cytokines were detected using ELISA kits (Sigma) according to the manufacturer's instructions.

2.7. Detection of neutrophils ratio

The PLF was washed with PBS containing 5% FBS. The cells in the PLF (5×10^5) were stained with FITC-labeled anti-Gr-1 (Biolegend, San Diego, CA, USA)) for 30 min in dark. After washing twice, the cells were analyzed by FCM.

2.8. Detection of bacterial load

Kidneys and spleens from infected and uninfected mice were surgically removed and homogenized in PBS. Serial dilutions of

tissue homogenates and PLF were plated onto LB agar plates and cultured at 37 °C for 16 h. The colony-forming units (CFU) were determined on the following day.

2.9. Collection of blood and the CH50 assay

At each time point, i.e. 0, 0.5, 1, 2, 3, and 5 h after MRSA infection, three mice from each group were bled. Blood samples were collected, clotted and centrifuged for 15 min at 1000 *g*. The obtained serum was stored at −20 °C for the CH50 assay. Total serum hemolytic activity (CH50) was determined using an ELISA kit (Sangon Biotech, Shanghai, China).

2.10. Statistics analysis

All data are expressed as means ± SD. Statistical analysis was performed using unpaired t-test and one-way ANOVA with the GraphPad Prism 6 software (GraphPad Prism Software, Inc.). $P < 0.05$ was considered significant.

3. Results

3.1. Natural antibody IgM 3B4 binds to MRSA and MBL in vitro

Preliminary work found that the natural antibody IgM 3B4 binds to MRSA in a dose-dependent manner (data not shown). In the present study, the binding of 3B4 to MRSA was confirmed using immunofluorescence assays and FCM. As shown in Fig. 1A, 3B4 bound directly to MRSA, but the control antibody TEPC did not. The FCM results showed that the rate of FITC-positive cells in the 3B4 group was 92.1 ± 15.5%, while the control group was 9.3 ± 6.1% (Fig. 1B and C), also indicating that 3B4 bound to MRSA.

Previous studies have shown that monoclonal IgM binds to MBL and activates the MBL pathway *in vitro* [13]. To evaluate the binding

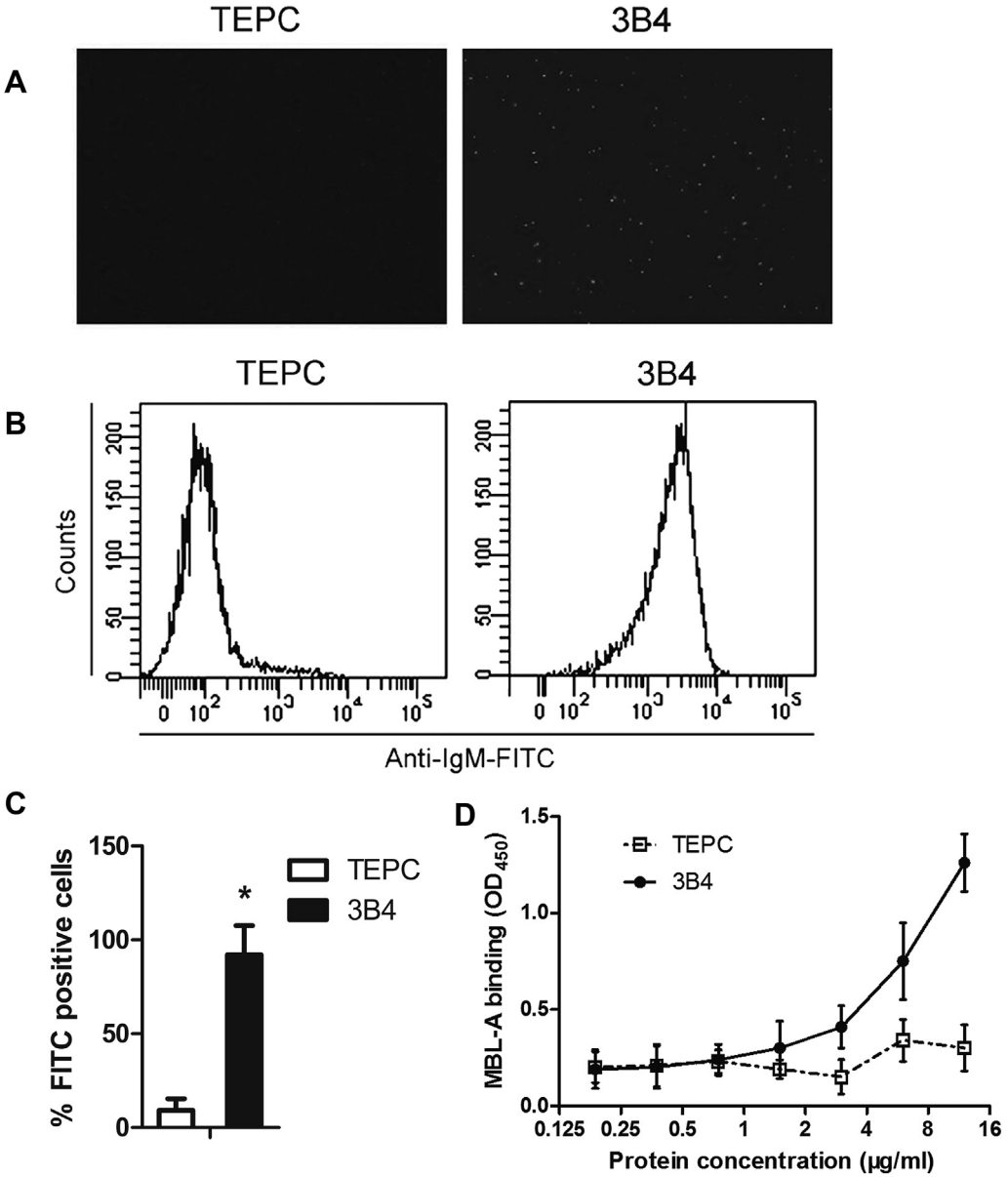

Fig. 1. Natural antibody IgM 3B4 binds to MRSA and MBL *in vitro*. The binding of 3B4 to MRSA was studied using an immunofluorescence assay (A) and FCM (B). TEPC was used as the isotype control. The quantification data of the FCM assay was shown in (C). (D) MBL binding was detected by ELISA using OD values measured at 450 nm. Wells were coated with purified 3B4 or TEPC at different concentrations, followed by the addition of NMS. Data are presented as mean ± SD of three independent experiments. $^{*}P < 0.05$ vs. TEPC group.

of purified 3B4 to MBL, plates were coated with purified 3B4 or TEPC at different concentrations, followed by the addition of NMS. The results showed dose-dependent binding of 3B4 to MBL-A as well as MBL-C (data not shown), but not in the control plates coated with TEPC (Fig. 1D).

3.2. Natural antibody IgM 3B4 activates the complement system via the classical and lectin pathways in vitro

Since 3B4 could bind to both MRSA and MBL *in vitro*, whether the interaction of 3B4 with MRSA/MBL induces complement activation was determined. Wells were coated with 3B4 or TEPC and then incubated with MRSA. Then, 2% NMS pre-incubated with mannose was added into the wells. As depicted in Fig. 2, there was no significantly difference in the levels of C3 deposition between the none pre-coated group and TEPC coated group. In the 3B4 group, C3 deposition was significantly elevated ($P < 0.05$), indicating the complement system was activated by 3B4 (Fig. 2). To block the MBL pathway, mannose was added. But C3 deposition level was still significantly higher than that in the none pre-coated group (Fig. 2, $P < 0.05$). As the alternative pathway was inactive at serum concentrations of 2%, it revealed that the binding of 3B4 to MRSA activated the classic pathway.

To assess activation of the MBL pathway, wells were pre-incubated with anti-C1q antibody in 2% NMS to blocked the classic pathway. The antibody 20B4 was used as isotype control. The result demonstrated that C3 deposition was still significantly elevated (Fig. 2, $P < 0.05$), suggest 3B4 binding also induced the activation of the MBL pathway. When wells were treated with MgEGTA or both mannose and anti-C1q antibody, C3 deposition was reduced to the level of none pre-coated group (Fig. 2).

For the study on the alternative pathway activation, 5% NMS was used. In the 3B4 coated group, there was no significant difference in C3 deposition between the 5% NMS and 2% NMS (Fig. 2). Both mannose and anti-C1q antibody treatment reduced the C3 deposition to the none pre-coated group level, and no significant difference was observed as compared to the MgEGTA group (Fig. 2). These results indicated that 3B4 binding activated the classical and MBL pathways, but not the alternative pathway.

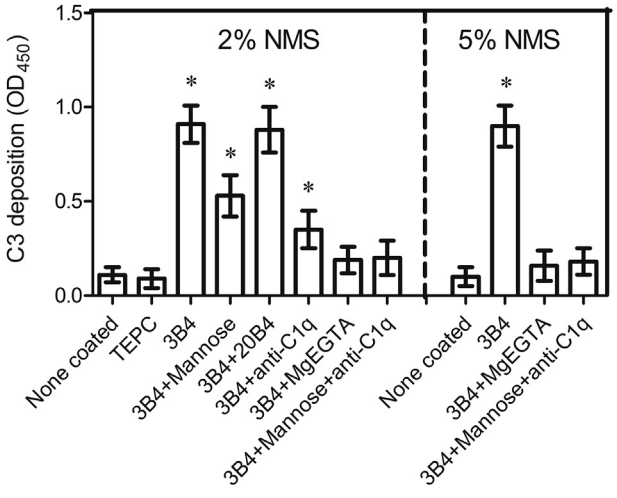

Fig. 2. Complement activation by 3B4 *in vitro*. Wells were coated with 3B4 or TEPC. The wells were then incubated with MRSA (only in the study on the classic pathway activation), followed by the addition of 2% NMS (pre-incubated with mannose, anti-C1q or the isotype control 20B4) or 5% NMS (in the presence of MgEGTA). C3 deposition was detected at 450 nm. Data are presented as mean ± SD of three independent experiments. *$P < 0.05$ vs. none pre-coated group. #$P < 0.05$ compared 3B4 group between 2% and 5% NMS & $P < 0.05$ the 3B4 + MgEGTA group vs. the 3B4 + Mannose + anti-C1q group.

3.3. Natural antibody IgM 3B4 protects mice from MRSA infection

To detect the protective effect of 3B4 on MRSA infection *in vivo*, TgV_H 3B4 and wild-type mice were used. After 48 h of MRSA infection, the bacterial load was detected in the kidney, spleen and enterocelia of mice. As shown in Fig. 3A, the bacterial load was significantly decreased in TgV_H 3B4 mice ($P < 0.05$) compared with wild-type mice. Inflammatory cytokine levels in the PLF of mice were detected by ELISA. The results showed that levels of IL-6, TNF-α, and IFN-γ were increased after MRSA infection, but the levels of IL-6 and TNF-α were decreased by 49.1% and 59.4%, respectively, in TgV_H 3B4 mice compared to wild-type mice (Fig. 3B). Additionally, the neutrophil ratio in the PLF of TgV_H 3B4 mice was decreased by 65.9% (Fig. 3C and D). The results suggested that 3B4 protects mice from MRSA infection.

3.4. Natural antibody IgM 3B4 promotes the activation of the complement system in MRSA infected mice

At different time points, three mice were bled and the serum activity of the complement system was determined. As shown in Fig. 4, at the early stage of MRSA infection, the CH50 value in TgV_H 3B4 mice was significantly higher than in wild-type mice. The results indicated that complement depletion in TgV_H 3B4 mice was greater than in wild-type mice, suggesting that 3B4 promoted the activation of the complement system in MRSA infected mice.

4. Discussion

The present study evaluated the function of a monoclonal IgM natural antibody (3B4) in MRSA infection. We found that the interaction between 3B4 and MRSA/MBL led to activation of the classic pathway and MBL pathway *in vitro*. 3B4 protected mice from MRSA infection and promoted the activation of the complement system at an early stage of MRSA infection. We propose that complement activation contributes to the anti-MRSA effect of 3B4.

MRSA is a versatile, well-equipped pathogen with the potential to evolve and adapt to its host [1]. It is a therapeutic challenge to treat MRSA. It has been reported that natural IgM can bind to a wide range of antigens and plays an important role in early defense against bacteria, viruses, and parasites [11]. In the present study, we found a monoclonal natural antibody IgM (3B4) that can bind directly to MRSA *in vitro*. However, the binding site on MRSA is unclear. Due to its polymeric structure, IgM possesses high affinity and a strong ability to activate complement [15]. We propose that the complex of 3B4 and MRSA can activate the classic pathway. The results show that 3B4 and MRSA successfully activated the classic pathway *in vitro*, which is in agreement with our hypothesis.

Although the MBL pathway is usually activated by pathogens with particular carbohydrate structures, it has been reported that some antibodies also activate the MBL pathway. For instance, human IgA has been reported to activate the complement system via the mannan-binding lectin pathway, which may contribute to the development of IgA nephropathy [16]. Moreover, it has been shown that IgM binds to MBL and activates the lectin complement pathway both *in vitro* and *in vivo* [13,17]. The binding of 3B4 and mouse MBL was investigated in this study. The results indicate that 3B4 bound to both MBL-1 and MBL-C *in vitro*, although it is unknown which part of 3B4 is the binding site for MBL. We next found that 3B4 could induce the MBL pathway activation, as demonstrated by activation of C3. MBL belongs to the collectin family and contains a carbohydrate recognition domain, which is able to bind to a number of saccharides to induce complement activation [18,19]. Whether the interaction between 3B4 and MBL is linked to carbohydrate binding needs to be investigated further.

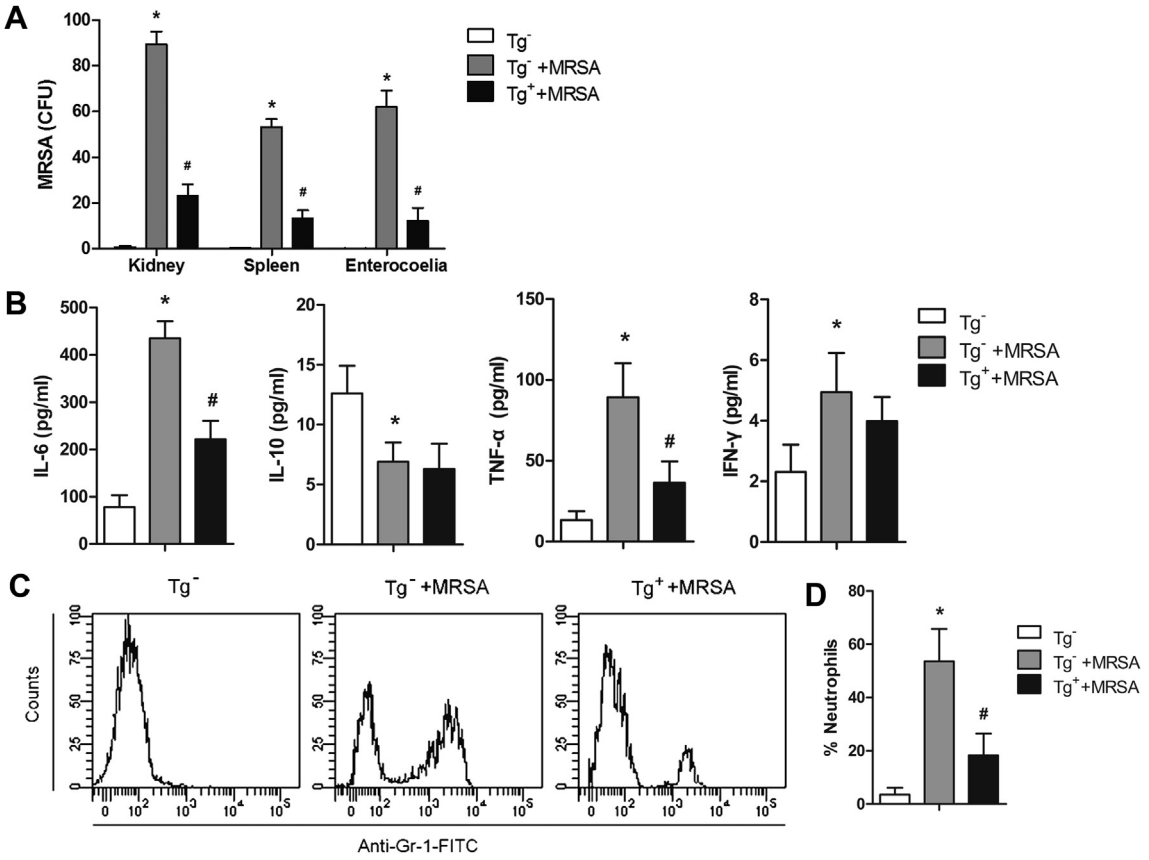

Fig. 3. Natural antibody IgM 3B4 protects mice from MRSA infection. (A) Quantification of the bacterial burden in the kidney, spleen and enterocelia of mice. Kidneys and spleens from mice were removed, weighed, and homogenized in PBS. Serial dilutions were plated onto LB agar plates. The colony-forming units (CFU) were counted the following day. (B) Inflammatory cytokine levels in the PLF of mice. The levels of IL-6, IL-10, TNF-α, and IFN-γ in PLF were detected using ELISA kits. (C) Neutrophil ratio in the PLF of mice in a representative experiment. The cells in the PLF were stained with FITC-labeled anti-Gr-1 and detected by FCM. (D) The quantitative description of the neutrophil ratio. Data are presented as mean ± SD of three animals. $^{*}P < 0.05$ vs. Tg^{-} group (wild-type mice without infection); $^{\#}P < 0.05$ vs. Tg^{-} + MRSA group (wild-type mice with infection).

Since 3B4 was demonstrated to bind to MRSA and activate the classic pathway and MBL pathway *in vitro*, the effect of 3B4 in MRSA infection was then evaluated *in vivo*. Transgenic mice with the VH gene from 3B4 (TgV_H 3B4) were used. The results show that the anti-MRSA effect in TgV_H 3B4 mice was stronger than in wild-type mice. Natural antibodies play an important role in the defense against pathogenic microorganisms during the early stage of infection [20]. As reported previously [14], the total serum IgM level in TgV_H 3B4 mice was higher than in wild-type mice; the IgM in TgV_H 3B4 mice was mostly keratin-reactive IgM (3B4 recognizes keratin). As the keratin-reactive IgM in TgV_H 3B4 mice possesses the V_H fragment of 3B4, we assumed that keratin-reactive IgM might also recognize MRSA and play an important role in protecting against MRSA infection *in vivo*. Additionally, the role of IgG which, may express the V_H fragment of 3B4 in TgV_H 3B4 mice, cannot be ignored. In addition, the CH50 value was significantly higher in TgV_H 3B4 mice than in wild-type mice, indicating that complement depletion in TgV_H 3B4 mice was greater than in wild-type mice. Based on these results, we inferred that keratin-reactive IgM promotes the activation of the complement system in MRSA infected mice. However, the pathway involved in this activation needs to be studied in the future.

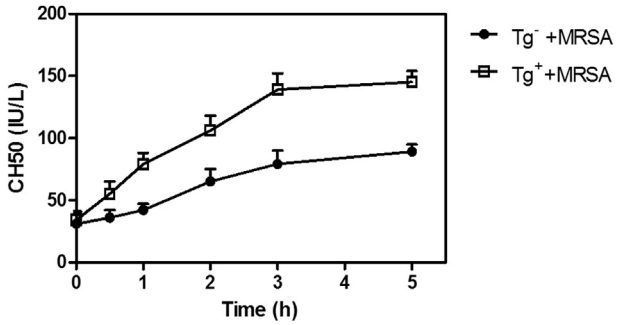

Fig. 4. Activity of the complement system in mouse serum. At different time points, three mice were bled and the serum activity of the complement system was determined. Data are presented as mean ± SD of three animals. $^{*}P < 0.05$ vs. Tg^{-} + MRSA group (wild-type mice with infection).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Transparency document

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