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Pathogenic epitope-specific monoclonal antibody-based immunoassay for accurate diagnosis and monitoring of tetranectin in sepsis

Juncheng Wang [a], Meng Liu [a], Zecheng Cai [a], Rukhshan Zahid [a], Wenjie Zhang [a], Dan Ma [a], Die Li [a], Yan Liang [a], Lei Zha [b], Yun Zhou [c], Lina Wang [d], Gang Yang [c,1], Shuai Zheng [e,*], Yuekang Xu [a,*]

[a] Anhui Provincial Key Laboratory for Conservation and Exploitation of Biological Resources, College of Life Sciences, Anhui Normal University, Wuhu, Anhui 241000, China
[b] The First Affiliated Hospital of Wannan Medical College, Wuhu, Anhui 241001, China
[c] Respiratory Department, The Affiliated Hospital to East China Normal University, Wuhu Second People's Hospital, Wuhu, Anhui 241000, China
[d] Anhui Medical University Affiliated Conch Hospital, Wuhu Conch Hospital, Wuhu, Anhui 241001, China
[e] Department of Endocrinology and Metabolism, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

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ABSTRACT

Sepsis is a fatal consequence of compromised host immunity due to widespread infection. Its pathogenesis has recently been found to be associated with tetranectin (TN), a monocyte-produced plasma protein with a critical disease-associated epitope, P5-5. To develop a rapid and simple method for early monitoring of the disease in clinical settings, a purified monoclonal antibody (12F1 mAb) with high affinity for the human TN pathogenic epitope P5-5 was produced in this study. The linear range of the indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) based on the mAb to detect TN-P5-5 was 4.8–312 ng/mL, and the half-maximal inhibitory concentration (IC_{50}) was 26.99 ng/mL, with a limit of detection of 2.4 ng/mL. Furthermore, the average recovery of intra- and inter-assay were 103.253 ± 2.803 % and 107.778 ± 7.490 %, respectively. Importantly, the competitive ELISA method established using 12F1 revealed signals corresponding to disease severity in patients with sepsis. Furthermore, the specific *in vivo* recognition of a pathogenic epitope by mAbs can be extended to therapeutic applications. Collectively, the development of an epitope-specific mAb against disease-associated proteins could be utilized accurately and quantitatively for diagnosing and monitoring diseases in clinical blood samples.

1. Introduction

Sepsis is an infection-induced disease characterized by compromised host immunity and critical organ dysfunction [1]. Annually, more than 48 million cases of sepsis occur worldwide [2]. Owing to its rapid onset, clinical mortality is up to 80 %, with complications of septic shock [3]. Moreover, the pathogenic mechanisms underlying this fatal disease unclear, constituing a great barrier to early diagnosis and effective treatment [4–6]. Currently, clinical confirmation of the disease is purely based on late-stage symptoms, temperature changes, routine blood test results, or blood culture detection of pathogens [7,8]. Blood cultures typically require at least 72 h to yield results in favorable cases. However, in many instances, even two consecutive cultures may fail to determine the sources of infection, hindering the timely diagnosis and treatment of acute inflammatory diseases, such as sepsis [7,9].

Owing to expensive and time-consuming conventional diagnostic practices with poor sensitivity and selectivity, developing alternative diagnostic systems for sepsis is necessary. Currently, the biomarkers for

Abbreviations: BSA, bovine serum albumin; OVA, ovalbumin; ELISA, enzyme-linked immunosorbent assay; MBP, maltose-binding protein; ic-ELISA, indirect competitive ELISA; IgG, immunoglobulin G; ALDH4A1, aldehyde dehydrogenase 4 family member A1; GFP, green fluorescent protein; PBS, phosphate buffered saline; TMB, tetramethylbenzidine.

* Corresponding authors at: College of Life Sciences, Anhui Normal University, No. 1, Beijing East Road, Wuhu, Anhui 241000, China; Department of Endocrinology and Metabolism, The First Affiliated Hospital of Nanjing Medical University, No. 300 Guangzhou Road, Nanjing, Jiangsu 210029, China.
E-mail addresses: zhengshuai89@njmu.edu.cn (S. Zheng), yuekang.xu@hotmail.com (Y. Xu).
[1] Senior author.

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sepsis diagnosis mainly include C-reactive protein, procalcitonin, neutrophil surface receptor (CD64) expression, tumor necrosis factor-alpha, and interleukin-6 [7,10–15]. These biomarkers are also present in noninfectious cases, making the disease diagnosis nonspecific and inaccurate [16–18]. Interestingly, a recent study found that plasma and tissue proteins, called tetranectin (TN), present in the plasma of patients with sepsis or septic shock, were associated with their morbidity, and their concentrations were significantly decreased compared with those in healthy individuals [19,20]. Mechanistically, TN can bind to high-mobility group box 1 (HMGB1) secreted by activated leukocytes during the initial phases of sepsis to form a complex that induces pyroptosis of phagocytic immune cells, impairing the body's ability to eradicate microbial infections and leading to immune exhaustion [19,21–23]. A peptide library spanning the entire sequence of human TN was screened by dot blotting and it was found that the two polyclonal antibodies uniquely recognized a peptide containing the epitope sequence NDALYEYLRQ, termed P5-5 [19,24]. Additionally, they experimentally demonstrated that the P5-5 is required for the interaction and endocytosis of TN/HMGB1 by phagocytes, leading to reduced TN concentrations in the plasma of patients with sepsis [19]. Therefore, these findings suggest that TN is a good disease-associated biomarker of sepsis.

Only enzyme-linked immunosorbent assay (ELISA) kits based on polyclonal antibodies against the whole TN protein are available on the market to date. The low specificity of polyclonal antibodies and their potential cross-reactivity with non-disease-related epitopes in the total TN protein have severely limited their clinical application as diagnostic tools.

Compared to polyclonal antibodies, monoclonal antibodies (mAbs) have higher purity, more uniform physicochemical properties, and stronger antigen-binding specificity, resulting in better assay performance [25]. Immunoassays based on mAbs have clear advantages in target disease detection [26–28]. Furthermore, mAbs against a particular epitope over the entire protein may have better diagnostic accuracy and quantitation [29]. Given the disease-inducing nature of the P5-5 epitope for sepsis and the possible cross-reactivity of other peptide sequences in the whole TN protein, we hypothesized that a mAb specific for the P5-5 peptide could not only accurately detect the existence but also monitor the severity of the sepsis by quantifying the disease-inducing epitopes. Therefore, the present study was designed to develop a mAb with high affinity and specificity against P5-5 and to establish a quick and accurate system for detecting and quantitating the pathogenic TN epitope from patients with sepsis at different disease stages.

2. Materials and Methods

2.1. Materials

Bovine serum albumin (CAS: 9048–46-8) was purchased from Regal Biology Technology (Shanghai, China). Albumin from chicken egg white, ovalbumin (OVA) was bought from Sigma-Aldrich (CAS: A5378). The P5-5 (Human TN antigen peptide NDALYEYLRQ) were synthesized in Sangon (Shanghai, China). Goat anti-mouse IgG conjugated with horseradish peroxidase (IgG-HRP) (CAS: BF03001X) and Mouse Monoclonal Antibody Subtyping Kit (CAS:BF06002X) were purchased from Biodragon (Suzhou, China). TIANamp Genomic DNA Kit (CAT:DP304) was purchased from TIANGEN BIOTECH (Beijing, China). Mouse interleukin-1β (IL-1β) ELISA Kit (CAT: JLW18442) and IL-18 ELISA Kit (CAT: JLW20253) were purchased from Shanghai Jianglai Biologicals (Shanghai, China). The bicinchoninic acid (BCA) protein concentration assay kit (CAT: P0010S) was purchased from Beyotime (Shanghai, China). All other reagents were of analytical grade.

2.2. Preparation of complete antigen and animal immunization

The three-dimensional (3D) structure of tetranectin was analyzed using the Swiss Model (https://swissmodel.expasy.org/) and PyMOL software. The P5-5 was conjugated to BSA or OVA using the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) method. First, the P5-5 solution (2 mg peptide dissolved in 500 μL of 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer, pH 4.7) and BSA or OVA solution (10 mg/mL dissolved in 0.1 M MES buffer, pH 4.7) were mixed. Subsequently, 100 μL EDC solution (10 mg EDC in 1 mL deionized water) was added to the above mixture and reacted at 25 ℃ for 2 h. The coupling molar ratio of hapten P5-5 to BSA (or OVA) was 10:1. After reacting for 2 h, the reaction solution was dialyzed with 0.01 M phosphate-buffered saline (PBS, pH 7.4) for 48 h. Finally, the product was stored at − 80 ℃ for further use.

The P5-5-BSA/OVA conjugates were analyzed using non-denaturing gel electrophoresis, and a BCA protein concentration assay kit was used to determine the concentration of the complete antigen. To obtain a high titer of antiserum, BALB/c mice (6–8 weeks old) were immunized by subcutaneous injections of P5-5-BSA conjugates (0.1 mL, 100 μg), which were emulsified with the same volume of Freund's complete adjuvant for the first injection. The follow-up immunization dose was adjusted to 50 μg (0.05 mL) and administered 8 times at two-week intervals. The antibody titer in the antiserum from immunized mice was determined using indirect ELISA. All animal experiments were performed in accordance with the guidelines of the Animal Experimentation Ethics Committee of Anhui Normal University(AHNU-ET2022015).

2.3. Cell fusion and identification of positive hybridoma

To screen for hybridoma cells that stably secreted mAbs against the P5-5 epitope, cell fusion was performed. After an intensified immunization schedule, fresh spleen cells were isolated from the immunized mouse, washed with RPMI-1640 incomplete medium, and fused with cultured Sp2/0 myeloma cells at a ratio of 4:1 in the presence of polyethylene glycol (50 % PEG-1450, 1 mL) [30]. After culturing in the selective hypoxanthine-aminopterin-thymidine (HAT) medium, the hybridoma supernatants were tested using indirect ELISA to screen for positive cells, which were then subcloned by limited dilution for monoclonal hybridomas. The subclass of antibodies secreted by the monoclonal hybridomas was identified using a mouse monoclonal antibody Ig subclass kit.

2.4. Preparation, purification, and characterization of monoclonal antibody against TN-P5-5

Female BALB/c mice aged 10 to 11 weeks were sensitized by intraperitoneal injection of 500 μL of ascites adjuvant. After sensitization, well-grown hybridoma cells from a 6-well cell culture plate were counted and injected into the abdomens of pre-sensitized BALB/c mice (approximately 10^6 cells). The ascites were collected and centrifuged, and the intermediate phase of the centrifuged ascites was used as the monoclonal antibody layer for purification. The monoclonal antibody was purified by Protein G Resin affinity chromatography to obtain high-purity mAbs. A BCA protein concentration assay kit was used to determine the antibody concentration, and an indirect ELISA was used to determine the affinity and specificity of the purified anti-P5-5 mAb. Different protein antigens, such as P5-5-OVA, BSA, OVA, aldehyde dehydrogenase 4 family member A1 (ALDH4A1), maltose-binding protein (MBP), P5-5-BSA, and skimmed milk (MILK), were diluted and coated in 96-well plates (20 μg/mL) to test the specificity of the 12F1 mAb. To further determine the affinity of 12F1 mAb, different antigen and antibody concentrations were serially diluted and analyzed using indirect ELISA. The optimal working concentration for mAb-mediated detection of TN-P5-5 was determined using an indirect ELISA checkerboard assay. Briefly, in ELISA plates, P5-5-OVA antigen was 2-fold serially diluted (20–1.25 μg/mL) and coated to react with the anti-P5-5 mAb at concentrations ranging from 199-12.4375 ng/mL. After the last washing step, an anti-mouse secondary antibody labeled with HRP was added,

and the reaction was stopped with a termination solution.

Recombinant glutathione S-transferase (GST)-fused full-length TN (GST-TN) was expressed by transient plasmid (pcDNA3.1-TN) transfection in HEK293T cells, and the secreted supernatant (recombinant human TN) was purified using a GST resin. Full-length TN was also tested against the 12F1 antibody using western blotting to determine the combining capacity.

2.5. Establishment and evaluation of ic-ELISA for P5-5 detection

To effectively determine plasma TN levels, an indirect competitive ELISA (ic-ELISA) was established. First, the optimal concentrations of the coating antigens P5-5-OVA and 12F1 antibodies were determined by checkerboard titration. Subsequently, P5-5-OVA dissolved in coating buffer (20 μg/mL, pH 9.6) was added to 96-well microplates at 100 μL/well and incubated at 4 ℃ overnight. After washing with PBS, the unbound sites on the plates were blocked with 300 μL/well of PBSM (5 % skim milk powder dissolved in PBS) for 2 h at 37 ℃. The plates were washed with PBS again. The synthesized peptide P5-5 solution (50 μL) was added at different concentrations (0, 0.002384186, 0.004768372, 0.009536743, 0.019073486, 0.038146973, 0.076293945, 0.152587891, 0.305175781, 0.610351563, 1.220703125, 2.44140625, 4.8828125, 9.765625, 19.53125, 39.0625, 78.125, 156.25, 312.5, 625, 1250, 2500, 5000, 10000, and 20000 ng/mL) and mixed with 50 μL of diluted 12F1 mAb solution in PBSM. The plates were incubated at 37 ℃ for 1 h and washed with PBST and PBS 3 times. Goat anti-mouse IgG-HRP (100 μL/well) at 1:5000 in PBSM was added, incubated for 1 h at 37 ℃, and washed. Freshly prepared tetramethylbenzidine (TMB) solution (100 μL) was added to each well and incubated.

for 15 min for color development. Following this, 100 μL of the stop solution (2 M H_2SO_4) was added, and data were collected at 450 nm using a plate reader.

The typical calibration curve and linear portion of the standard curve were illustrated using Origin 8.0, with the value of B/B0 over the P5-5 concentration.

2.6. Intra-assay and inter-assay of ic-ELISA for spiked P5-5 detection

The intra-assay and inter-assay precision of the ic-ELISA were tested using three replicates of different dilutions of the synthesized P5-5 peptide (50, 100, 200, and 300 ng/mL). The average recovery value and coefficient of variation (ratio of standard deviation to mean) were calculated. Each sample was tested in four replicates.

2.7. Detection of TN in patient plasma samples based on ic-ELISA

Blood samples of patients with sepsis and healthy controls were obtained from the First Affiliated Hospital of Wannan Medical College, Wuhu Conch Hospital, and Wuhu Second People's Hospital. Whole blood was placed in anticoagulation tubes, and centrifuged at 2000 g for 10 min. The upper layer of transparent plasma (100 μL per sample from each of the seven healthy individuals and seven patients with sepsis listed in Table 2) was collected, mixed with glycerin, and heat-treated at 100 ℃ for 10 min. Following this, 50 μL of the plasma analytes was mixed with 50 μL of diluted antibody solution in PBSM and added to enzyme-labeled 96-well plates to react for 1 h and washed with PBST and PBS. The goat anti-mouse HRP was added for incubation. The remaining steps were performed as described in Section 2.5.

2.8. Therapeutic effects of 12F1 mAb in vivo

To evaluate the blocking effect of 12F1 mAb *in vivo*, a septic mouse model was established using BALB/c mice (male, 5–6 weeks old, 20–25 g), which were intraperitoneally injected with the bacterial toxin lipopolysaccharide (LPS) at 7.5 mg/kg for each sepsis induction group. Six hours after LPS administration, the mice demonstrated typical septic symptoms such as chills, fever, rapid heart rate, and shortness of breath. They were treated intraperitoneally with either 12F1 mAbs at 2.5 mg/kg (6 h and 24 h after LPS administration) or PBS as a control. The experiments were terminated 24 h after the mAb/PBS treatment, and their survival rate and macrophage counts were assessed.

Macrophage genomic DNA was extracted using the TIANamp Genomic.

DNA Kit and RNA was extracted from macrophages using the TRIzol total.

RNA Extraction method. Complementary DNA (cDNA) was synthesized using Prime Script RT reagent and the Bio-Rad iCyCler real-time PCR7500 system with SYBR Green technology was used to quantify mRNA expression levels. Gene expression was analyzed quantitatively using the $2^{-\Delta\Delta Cq}$ method. The primer sequences were as follows: NLRP3 forward: 5′- CCGCGTGTTGTCAGGATCTC-3′, reverse: 5′-AAGGGCATTGCTTCGTAGATAGA-3′; Caspase-11 forward: 5′-GGTGGGAACTCTGGAGAAATGTGG-3′, reverse: 5′-ATCAATGGTGGGCATCTGGGAATG-3′; IL-1β forward: 5′- AAATGCCACCTTTTGACAGTGA-3′,

reverse: 5′-AAAGAAGGTGCTCATGTCCTCATCC-3′ [31]. The IL-1β and IL-18concentrations were measured in the serum of all mouse groups using ELISA.

kits (Shanghai Jianglai Biologicals, China), following the manufacturer's guidelines.

2.9. Statistical analysis

All experiments were performed at least 3 times. Data are presented as the mean+/- standard deviation (SD), with n representing the number of animals or experiments. Statistical differences were determined using an unpaired two-tailed Student's *t*-test for two groups, or a one-way analysis of variance (ANOVA) for more than two groups that followed a normal distribution and satisfied variance homogeneity. Prism 9.0 (GraphPad Software) was used for statistical analysis of all data, and a P value < 0.05 was considered statistically significant.

3. Results

3.1. Complete antigen preparation and animal immunization

As P5-5 is a hapten within the TN protein consisting of 10 amino acids, the carrier protein needs to be conjugated to effectively induce an immunogenic response in mice for antibody production. For this purpose, the carbodiimide crosslinker EDC was used. Two carrier proteins, BSA and OVA, were successfully conjugated to the human TN-P5-5 peptides, P5-5-BSA and P5-5-OVA, for immunization and detection, respectively (Fig. 1A). After eight subcutaneous immunizations (Table S1) with P5-5-BSA at two-week intervals, antiserum from the immunized animals was examined using indirect ELISA to detect the antigen P5-5-OVA. While the P5-5-BSA immunized serum did not react with OVA, it reacted with P5-5-OVA in a dose-dependent manner, suggesting the presence of antibodies against P5-5 (Fig. 1B). Notably, the antibody titer of the immunized serum exceeded 1:64000, far beyond the threshold titer of 1:8000 [27], indicating successful production of high-affinity polyclonal antibodies against TN-P5-5.

3.2. Cell fusion and selection of hybridomas

Using the polyclonal antiserum from P5-5-BSA immunized mouse, we prepared mAbs against P5-5 by hybridizing the splenocytes from the immunized mouse with hypoxanthine–guanine phosphoribosyltransferase (HGPRT)-deficient Sp2/0 myeloma cells *in vitro*. This was carried out in the presence of optimal amounts of feeding cells (10^4 cells/well in a 96-well culture plate), which not only stimulated the growth of fusion cells through cytokine secretion, but also facilitated the removal of apoptotic cell fragments via phagocytosis. Approximately

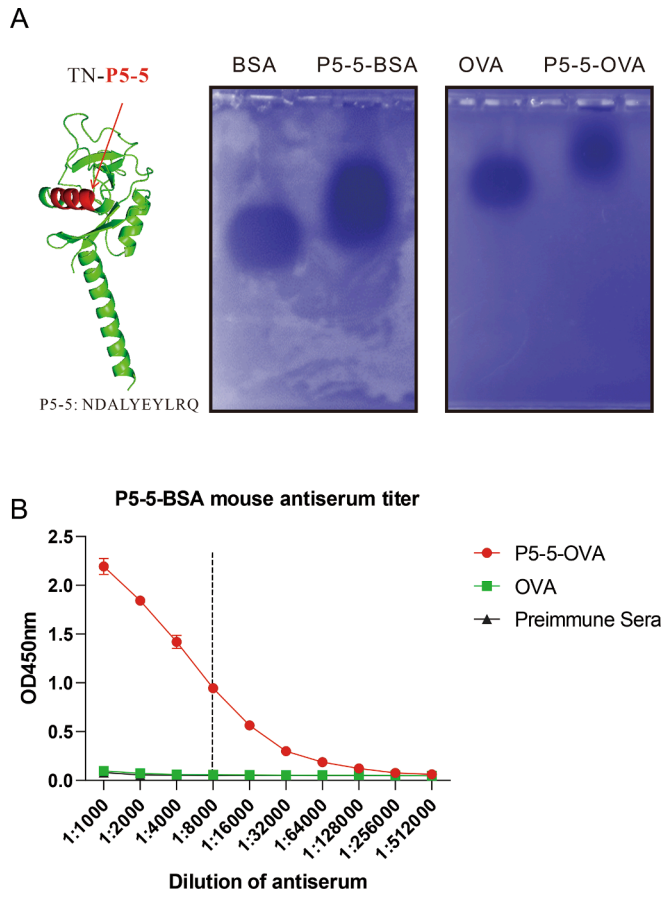

Fig. 1. Tetranectin (TN) is used as complete antigen to immunize mice to select high titer anti-P5-5 serum. (A) Preparation of complete antigen. The three-dimensional (3D) structures of TN were analyzed using Swiss Model and the PyMOL software. The highest level of optimization was used. (B) Determination of anti-P5-5 serum titer. After eight immunizations with P5-5-BSA, one immunized mouse displayed high titer by indirect ELISA compared to the control serum (preimmune). The cut-off line indicates the threshold dilution ratio for the most effective antiserum at 1:8000, suggesting that a higher anti-P5-5 antibody titer is obtained by multi-point subcutaneous injection The error bar represents the standard deviation (SD) of three replicates. Data are presented as mean ± SD.

one week after cell fusion, distinct hybridized cell colonies formed in the HAT selection medium. The wild-type splenic cells provided HGPRT, enabling the hybrid cells to survive, while HGPRT-deficient Sp2/0 myeloma cells died due to defects in the DNA synthesis rescue pathway. Primary splenic cells typically survive for only 5–7 days *in vitro*. After 10 days, the culture supernatants from each well containing the surviving hybridoma cells were tested by indirect ELISA using P5-5-OVA as the coating antigen. Hybridoma cells with a strong positive response were subcloned three to four times. Cell fusion and subclone hybridoma growth are depicted in Fig. S1. The culture supernatants from each single hybridoma colony was assessed for their reactivity with the P5-5-OVA antigen, and only those colonies whose supernatants could specifically recognize P5-5 conjugated on the OVA protein were further subcloned three to four times to ensure homogeneity of the antibody-producing cells. A single positive clone, 12F1, which consistently secreted mAbs against P5-5 with the highest antigen-binding activity, was obtained. Furthermore, antibody isotyping confirmed that the 12F1 mAb belonged to the IgG1 subtype (Fig. 2A), an optimal IgG type for antibody binding.

3.3. Large-scale in vivo production and in vitro characterization of anti-P5-5 mAb

The selected hybridoma cell, 12F1, was expanded *in vitro* and injected intraperitoneally into BALB/c mice for large-scale *in vivo* production. After 10 days, the mice were sacrificed, and the anti-P5-5 mAb, 12F1, was purified using protein G affinity chromatography. The purity and size of the antibodies were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), revealing clean bands corresponding to a heavy chain (approximately 50 kDa) and a light chain (approximately 25 kDa) (Fig. 2B). To select the optimal concentration for both the P5-5 antigen coating and 12F1 mAbs in the assay, an ELISA checkerboard assay was performed, with the results represented as a heat map based on OD values (Fig. 3). The brightest red in the heat map represents the highest OD value, whereas the darkest red represents the lowest OD value. The pair of 20 μg/mL coated antigens corresponding to 24.875 ng/mL antibodies were chosen, as their absorbance values were close to 1.0, the optimal working concentrations for antigen–antibody reactions [32]. Furthermore, the affinity and specificity of the chosen IgG1 mAb were assessed using indirect ELISA; the purified anti-P5-5 IgG1 exhibited specific binding to the TN-P5-5 antigen, with no cross-reactivity to its carrier proteins, BSA and OVA, or other proteins, such as green fluorescent protein (GFP), MILK, and ALDH4A1 (Fig. 4A). Additionally, western blotting results revealed that the 12F1 antibody reacted only with the GST-fused TN (approximately 46 kDa), but not with the GST protein (Fig. S2). The affinity constant (K_{aff}) was determined using indirect ELISA (Table S2) using both coated P5-5-OVA and 12F1 mAb at appropriate dilutions (Fig. 4B). The K_{aff} was calculated using the formula $K_{aff} = (n\text{-}1)/\{2(n\ [Abt]\text{-}[Ab])\}$. The affinity constant of 12F1 mAb was 1.38×10^9 L/mol, substantially higher than the threshold level of 10^7 L/mol (low-affinity antibody), suggesting high-affinity. Collectively, these results confirm the successful development of a high affinity, TN-P5-5 specific mAb.

3.4. Detection of TN-P5-5 by ic-ELISA in spiked and clinical plasma samples

Using the validated 12F1 mAb, we established an effective detection system. As the ic-ELISA method is widely utilized for detecting haptens, it was employed in the current study. The typical calibration curve was plotted, and the half inhibitory concentration (IC_{50}) established at 26.99 ng/mL (Fig. 5A), well above the μg/mL range that was reported for some newly generated mAb [33]. The linear equation $y = 1.325–0.5317x$, with a correlation coefficient (R^2) of approximately 0.9685 is depicted in Fig. 5B. The linear detection range of the typical calibration curve was 4.8–312 ng/mL, with a lowest limit of detection (LOD) of 2.4 ng/mL. Therefore, these data indicate that the 12F1 mAb exhibits high sensitivity, and the ic-ELISA method developed in this study can be utilized to quantify the TN-P5-5 epitope in samples using a linear equation. To validate the repeatability and accuracy of detection with ic-ELISA, recovery experiments were conducted by spiking samples with various concentrations of the P5-5 peptide. As presented in Table 1, the mean recoveries of detection for the intra-assay (precision of the same measurement) and inter-assay (precision of different measurements) were 103.253 ± 2.803 % and 107.778 ± 7.490 %, respectively. The intra- and inter-assay coefficients of variation (CV) were 2.577 % and 6.845 %, respectively, indicating that the ic-ELISA had high repeatability and accuracy and could be used for quantitatively detecting P5-5.

After establishing the ic-ELISA as an effective detection method with satisfactory sensitivity and recovery in spiked samples, we attempted to determine its efficacy for blood detection in clinical cases with confirmed sepsis. Blood samples from patients with sepsis were collected from the First Affiliated Hospital of Wannan Medical College, Wuhu Conch Hospital, and Wuhu Second People's Hospital. Blood samples from healthy individuals were used as controls. Notably, the average

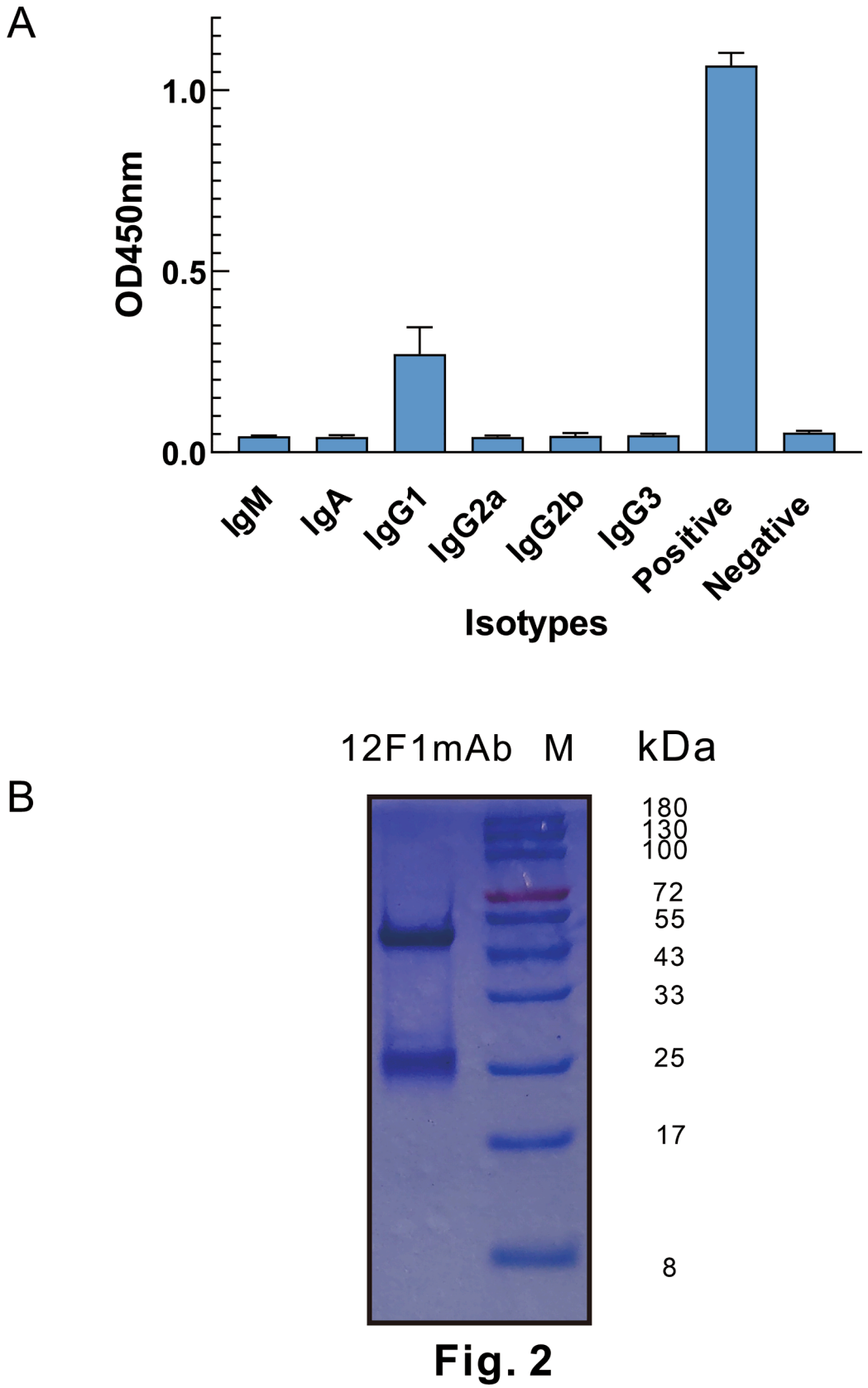

Fig. 2

Fig. 2. The Isotye of mouse monoclonal antibody (mAb) produced by hybridoma 12F1 is identified to be IgG1, and purified as two distinct protein bands. (A) Mouse monoclonal antibody subtyping kit was used to determine the subtype of mAb secreted by hybridoma. After P5-5-OVA was coated into the ELISA plate, ~~and~~ mAb-containing supernatants were reacted with six different antibody subtyping reagents (IgG1, IgG2b, IgG2a, IgG3, IgA, and IgM) with absorbance measured at 450 nm. (B) SDS-PAGE of purified mAb 12F1. Two distinct protein bands at 50 kDa and 25 kDa, indicate successful ascites purification of the mAb. SDS-PAGE, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

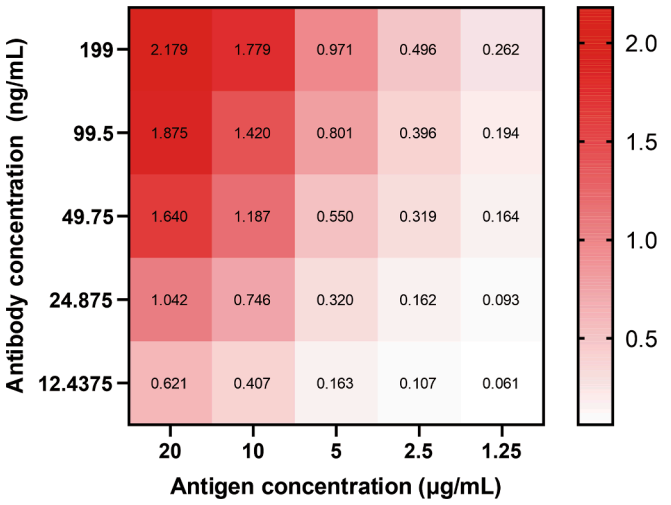

Fig. 3. The optimal antigen–antibody concentrations of mAb 12F1 is determined by indirect ELISA checkerboard assay. Five concentrations of P5-5-OVA antigen (20, 10, 5, 2.5and 1.25 μg/mL) and five concentrations of detection antibody (199, 99.5, 49.75,24.875 and 12.4375 ng/mL) were tested in different combinations. Cells shaded in bright red indicate maximum OD values, while cells shaded in the lightest red stand for minimum OD values. Values represent the average OD values of three assays (n = 3). ELISA, enzyme-linked immunosorbent assay; OD, optical density, OVA, ovalbumin.

results indicated that the TN-P5-5 epitope concentration in the plasma of seven patients with sepsis were 464.369 ± 19.018, 544.773 ± 14.809, 348.294 ± 40.581, 593.222 ± 21.253, 278.929 ± 12.320, 190.802 ± 7.452, and 447.386 ± 7.050 ng/mL, which were all lower than that of healthy controls (Table 2). After taking the molecular weight ratio of P5-5 over TN protein into the calculation, the concentration of TN in the healthy individual tested as normal control was worked out to be 10.237 ± 0.072–11.234 ± 0.709 μg/mL, consistent with the reported values of 10–12 μg/ml in healthy subjects [19], further validating the accuracy of our assay. Interestingly, patients with lower plasma TN-P5-5 concentrations had more severe symptoms and higher Acute Physiology and Chronic Health Evaluation version II (APACHE-II) scores than patients with higher TN-P5-5 concentrations and lower APACHE-II scores (Fig. 6), demonstrating that the quantity of the disease-inducing P5-5 epitope measured by the 12F1 mAb can indeed reflect disease severity. Furthermore, there was a good linear relationship between APACHE-II scores and TN-P5-5 levels in patients with sepsis (Fig. 6). The control value (the average of healthy individuals) was 682.186 ± 22.862 ng/mL and can be normalized. Values lower than the control values indicated that the patient may have suffered from sepsis (Fig. S3). Thus, these data indicate that the ic-ELISA method based on the 12F1 mAb developed in the current study, can be employed for the accurate quantitative detection of septic cases in clinical settings.

3.5. 12F1 mAb specifically blocks the disease-inducing epitope and reduces the demise of macrophages in a mouse model of sepsis

To examine the pathogenic nature of P5-5-TN *in vivo* and the disease-blocking property of the 12F1 mAb developed, an experimental septic mouse model was established using lipopolysaccharide (LPS), a bacterial endotoxin frequently employed as a simple and sterile method of sepsis induction and shares many similarities with the initial stages of sepsis in humans. The 12F1 mAb was injected intraperitoneally 6 and 24 h after LPS treatment. As shown in Fig. 6, compared with the control group (injected with PBS), in which mice died at 46, 67, and 96 h after LPS-induced sepsis, the mice in the antibody-injected group were still alive at 192 h after which the experiments were terminated, demonstrating the optimal therapeutic and disease-blocking properties of the

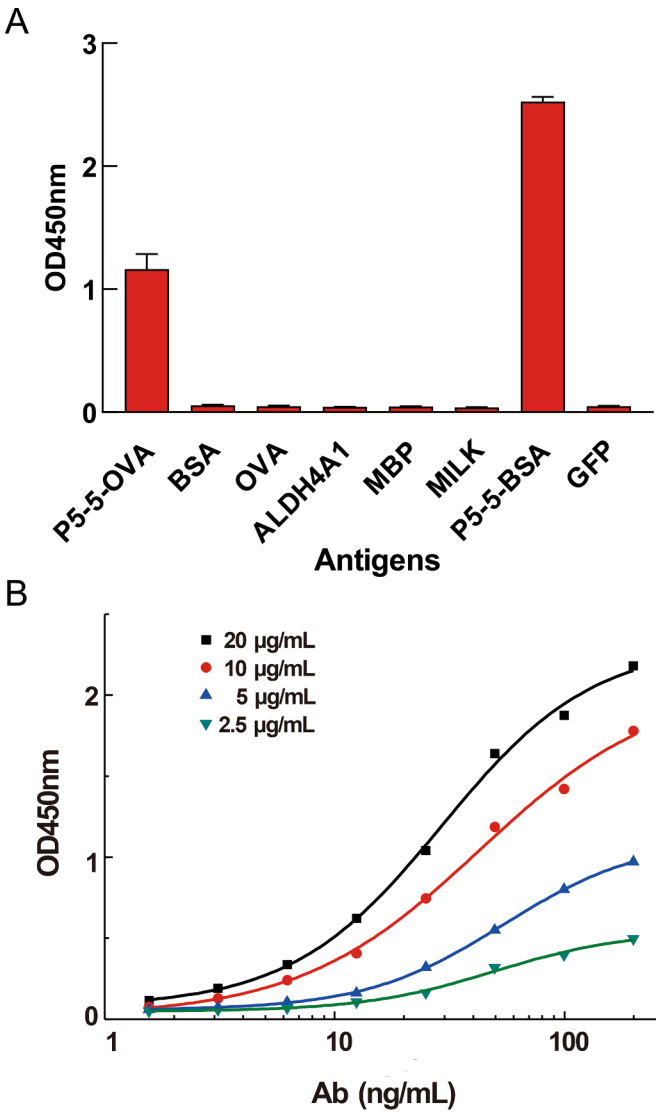

Fig. 4. 12F1 mAb demonstrates high specificity and affinity. (A) Specificity analysis of mAb 12F1. Indirect ELISA shows that 12F1 mAb specifically reacts with P5-5 epitope-containing samples, with no cross-reactivity to any other antigens such as BSA, OVA, ALDH4A1, MBP, MILK, and GFP. (B) Affinity determination of mAb 12F1 by indirect ELISA using P5-5-OVA coated at different concentrations and 12F1mAb at appropriate dilutions. BSA, bovine serum albumin; OVA, ovalbumin; ALDH4A1, aldehyde dehydrogenase 4 family member A1; MBP, maltose-binding protein; MILK, skimmed milk; GFP, green fluorescent protein.

mAb developed in the current study.

To further verify that the disease-retardation effect of the mAb was caused by reduced pyroptosis of macrophages that uptake the TN-HMGB1 complex, as previously reported [19], peritoneal macrophages from experimental mice were examined using flow cytometry. We found that after the mice developed experimental sepsis, their peritoneal macrophages greatly reduced in number, presumably dying by pyroptosis. The 12F1 mAb treatment, however, prevented this rapid reduction in macrophages (Fig. 8, $P < 0.0001$), which is consistent with their improved survival rate (Fig. 7, long rank $P < 0.001$). Since pyroptosis is accompanied by stochastic DNA degradation, inflammasome activation, and the release of the inflammatory cytokines IL-1β and IL-18 [34], we further explored these parameters in the mAb treatment regimen for any signs of pyroptosis. In the LPS-induced sepsis group, macrophages displayed all parameters of pyroptosis in terms of stochastic DNA degradation (Fig. S4), activation of caspases and the NRPL3 inflammasome

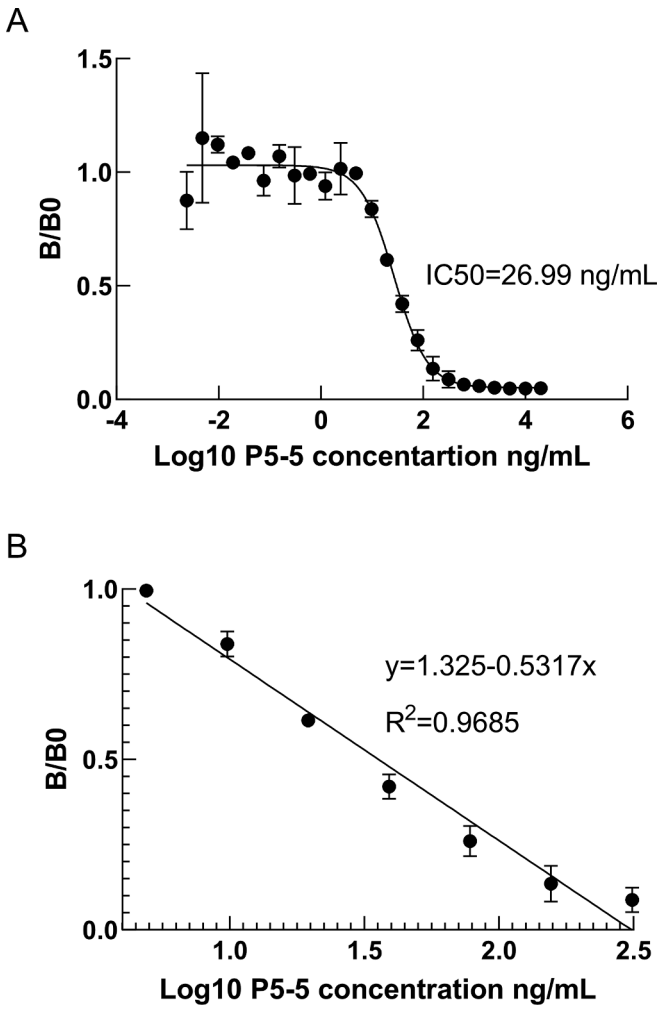

Fig. 5. Standard curves of 12F1 mAb is eatablished for TN-P5-5 detection. (A) Calibration curve plotting B/B0 against P5-5 concentration. The data obtained in the presence or absence of various inhibitor concentrations are referred to as B and B0, respectively. (B) Linear portion of the standard curve. The equation is y = 1.325–0.5317x, with a correlation coefficient (R^2) of 0.9685.

(Fig. S5), and release of IL-1β and IL-18 (Fig. S6). Notably, these signs of LPS-elicited pyroptosis were suppressed by treatment in the 12F1 mAb group (Figs. S4–6). These findings underscore the therapeutic potential of 12F1mAb to specifically inhibit the P5-5 epitope from inducing macrophage pyroptosis during sepsis.

4. Discussion and conclusion

In this study, we successfully produced an IgG1 mAb (12F1) using hybridoma technology, that exhibited a high specificity and affinity for the pathogenic epitope P5-5 in the sepsis-associated protein TN, with no cross-reactivity observed with other related proteins present in human plasma. Furthermore, an ic-ELISA method was established with an LOD of 2.4 ng/mL for the TN-P5-5 epitope, superior to the LOD of other reported mAbs [33,35], and outperforming commercial ELISA kits with ranges from μg to pg/mL. Furthermore, the total assay time was only two hours because the P5-5 antigen was precoated onto the ELISA plate and blocked in advance. Additionally, our established ic-ELISA method can be used to examine both fresh whole blood samples or treated serum or plasma with a wide target detection range (4.8–312 ng/mL), which is much lower than the levels of TN-P5-5 in healthy individual's plasma (641–769 ng/mL). Notably, quantitation of the P5-5 epitope by the 12F1 mAb in patient sera corresponded with disease severity, and direct HRP conjugation allowed the competitive ELISA method to detect P5-5 in patient plasma within one hour (data not shown). To our knowledge, this is the first report of an immunoassay developed specifically for sepsis detection based on the restrictive P5-5 epitope in TN.

Compared to our ic-ELISA, the capture and detection antibodies from commercially available human TN ELISA Kits (Abcam ab213832) were all sheep polyclonal antibodies, whose specificity was not as good as that of the 12F1 mAb developed in the current study, as they were raised against total TN protein, which could potentially cross-react with common epitope(s) in non-diseased individuals. Furthermore, these polyclonal antibodies detect any epitope in the TN protein that may or may not be pathogenic; therefore, they cannot detect the intensity of the disease as effectively as the 12F1 mAb.

Sandwich ELISA uses two types of antibodies (capture and detection) to react with different epitopes on the target antigen. Therefore, it can detect the presence of a large antigen with multiple epitopes that may or may not be pathogenic. Conversely, ic-ELISA is a competitive ELISA designed to detect and quantify small-molecule antigens in immunoassays where a sample antigen competes with a coated antigen for antibody binding. Therefore, the ic-ELISA developed in this study is more suitable for detecting haptens with one active epitope in complex samples (plasma or serum), such as the pathogenic TN-P5-5 hapten, providing high specificity and sensitivity for disease diagnosis and monitoring.

The mAb developed in the current study differed from that used in another study, where the immunogen was a full-length TN protein [19]. First, since P5-5 represents the major epitope within the 20 kDa TN protein, the immunogenicity of enriched P5-5 epitopes should theoretically be stronger than that of the whole TN protein, where minor epitopes might interfere [19]. This is supported by the data in Fig. 1B, which shows that the anti-P5-5 antibody titer reached as high as 1:64000, well above the threshold level of 1:8000, indicating a high-affinity mAb towards the targeted antigen [27]. Second, P5-5 in the

Table 1
Recovery and coefficient of variation detection in spiked samples with TN-P5-5 (n = 4).

spiked conventration		Intra-assay Measured	Recovery	CV		Inter-assay Measured	Recovery	CV
(ng/mL)	n	(ng/mL)	(%)	(%)	n	(ng/mL)	(%)	(%)
50	4	51.664 ± 1.654	103.328 ± 3.308	3.202	4	55.893 ± 4.206	111.786 ± 8.412	7.526
100	4	116.260 ± 5.816	116.260 ± 5.816	5.003	4	128.689 ± 8.994	128.689 ± 8.994	6.989
200	4	207.900 ± 2.987	103.950 ± 1.494	1.437	4	213.772 ± 16.458	106.886 ± 8.229	7.699
300	4	268.422 ± 1.784	89.474 ± 0.595	0.665	4	251.244 ± 12.975	83.748 ± 4.325	5.164
Average			103.253 ± 2.803	2.577			107.778 ± 7.490	6.845

Recovery= (detection concentration / spiked concentration) *100%.
CV= (Standard Deviation/mean) *100%.
Intra-assay variation was determined by four replicates of each spiked level on the first day.
Inter-assay variation was determined in independent days (4 times).
Data was given as the mean value.

Table 2
The plasma samples detection results of TN-P5-5 (n = 4).

Blood samples	Gender	Age	Ethnicity	APACHE-II Score	P5-5 epitope concentration (ng/mL)	TN concentration (μg/mL)
Healthy individual 1	Female	40	Han	0	720.127 ± 45.458	11.234 ± 0.709
Healthy individual 2	Male	57	Han	3	701.362 ± 23.202	10.941 ± 0.362
Healthy individual 3	Female	63	Han	3	691.625 ± 37.696	10.789 ± 0.588
Healthy individual 4	Male	79	Han	6	656.232 ± 4.622	10.237 ± 0.072
Healthy individual 5	Male	65	Han	5	671.041 ± 6.562	10.468 ± 0.102
Healthy individual 6	Female	75	Han	6	665.427 ± 15.576	10.381 ± 0.243
Healthy individual 7	Female	72	Han	5	669.488 ± 26.268	10.444 ± 0.410
Patient 1 with sepsis	Female	73	Han	23	464.369 ± 19.018	7.244 ± 0.297
Patient 2 with sepsis	Male	58	Han	18	544.773 ± 14.809	8.498 ± 0.231
Patient 3 with sepsis	Female	84	Han	27	348.294 ± 40.581	5.433 ± 0.633
Patient 4 with sepsis	Male	80	Han	12	593.222 ± 21.253	9.254 ± 0.332
Patient 5 with sepsis	Male	97	Han	32	278.929 ± 12.320	4.351 ± 0.192
Patient 6 with sepsis	Female	65	Han	39	190.802 ± 7.452	2.977 ± 0.116
Patient 7 with sepsis	Female	78	Han	24	447.386 ± 7.050	6.979 ± 0.110

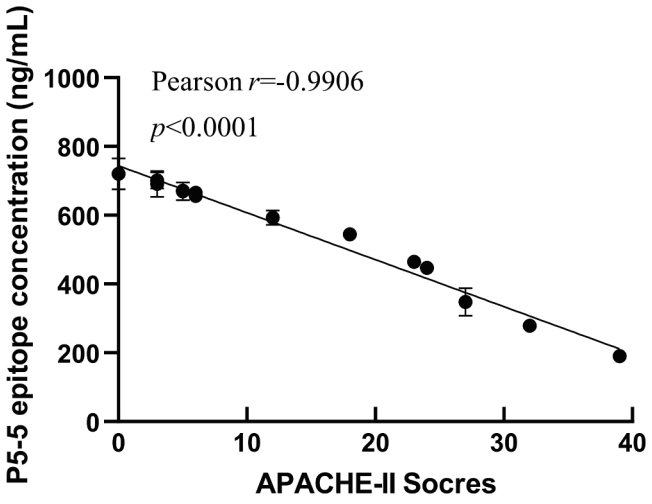

Fig. 6. APACHE score in relation to TN-P5-5 levels in patients with sepsis is plotted. The higher the APACHE-II score of patient with sepsis with severer condition corresponds to the lower the patient's plasma P5-5 levels (n = 14 individual subjects). The error bar indicates the standard deviation (SD) of four repetitions. Pearson r = -0.9906, Data are mean ± SD. **** $P < 0.0001$. APACHE, acute physiology and chronic health evaluation; TN, tetranectin.

TN is a linear epitope, and our 12F1 mAb against linear epitope is more suitable to for use as a clinical diagnostic tool than antibodies targeting conformational epitopes, as clinical samples are often denatured by storage conditions and various other factors that disrupt conformational epitopes. Consistently, the ic-ELISA method based on a linear epitope-specific monoclonal antibody has demonstrated higher accuracy [36], and anti-linear peptide epitope antibodies have been widely used in clinical applications with good specificity and affinity [37–45]. Importantly, the P5-5 sequence chosen for mAb development is a critical pathogenic epitope of the TN protein. Although total TN protein plays a role in wound healing, tissue remodeling, and serves as a diagnostic aid in multiple sclerosis, the mAb-based ic-ELISA developed in this study specifically targets the pathogenic P5-5 fragment of TN. This is believed to trigger sepsis by binding to HMGB1 for macrophage pyroptosis induction [19]. As the P5-5 fragment is a causal factor for the disease and is specific to sepsis onset, the more P5-5 binds to HMGB1, the less P5-5 peptide is available for detection.

Compared to immunization with total TN protein, which may generate antibodies that cross-react with common proteins in the patient's serum and lack specificity for disease-related epitopes, immunization with the exclusive disease-related epitope of human P5-5 produces mAbs that can more accurately detect the presence of disease-relevant components, greatly reducing the misdiagnosis rate in clinics. Moreover, the amount of the disease-inducing peptides detected by the 12F1 mAb can be used to distinguish different developmental stages of the disease for tailored treatment plans, or to monitor the therapeutic

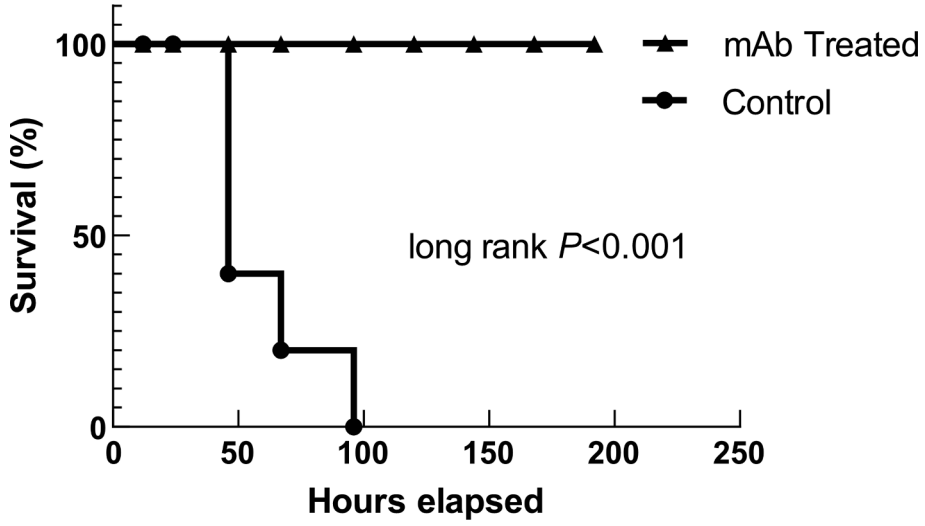

Fig. 7. Treatment with 12F1 mAb improves survival rate in septic mice. Following the lipopolysaccharide (LPS) challenge (subjected to lethal endotoxemia at 7.5 mg/kg), BALB/C mice were intraperitoneally administered mAbs (2.5 mg/kg) at 6 h and 24 h after LPS administration, and monitored for up to 9 days. (n = 5 mice/group). *** $P < 0.001$ (Long rank test).

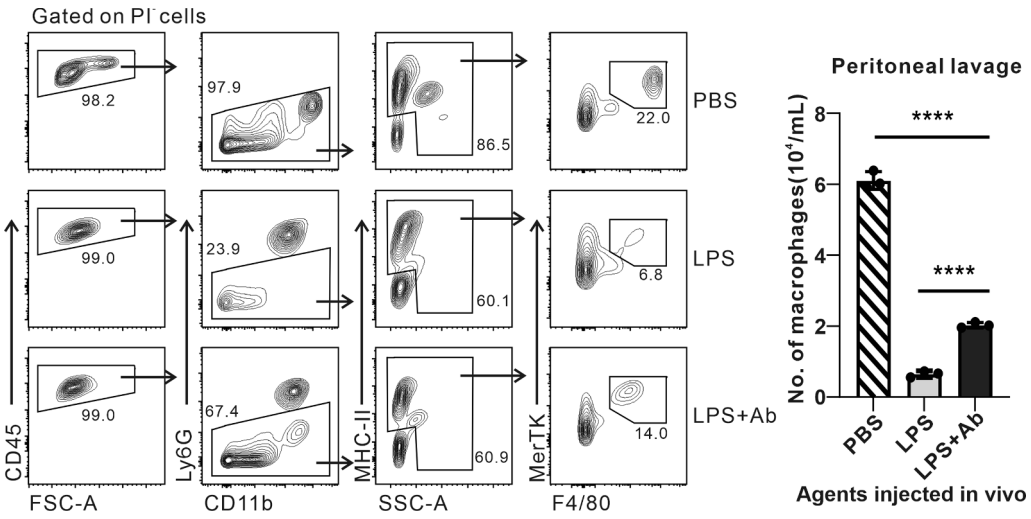

Fig. 8. 12F1 mAb treatment prevents rapid reduction in macrophage numbers from septic mice. BALB/c mice (male, 20–25 g) were intraperitoneally administered lipopolysaccharide (LPS) at 7.5 mg/kg for each group. Six and twenty-four hours after the LPS administration, mice received intraperitoneal mAbs (2.5 mg/kg) or PBS as control. Peritoneal macrophages were analyzed using flow cytometry 24 h after the mAb/PBS treatment, (n = 3 mice/group). Data are mean ± standard deviation (SD). **** $P < 0.0001$. PBS represents healthy control, LPS represents sepsis control, and LPS + Ab represents the treatment group. PBS, phosphate buffered saline, mAb, monoclonal antibodies.

effect after hospital admission. Additionally, the specific recognition and blocking properties of the mAb against the pathogenic epitope *in vivo* suggest potential for therapeutic applications. This is supported by the improved survival rates and increased macrophage numbers observed in septic mice treated with the 12F1mAb. Overall, the ic-ELISA method based on this mAb has demonstrated sensitivity in both the clinical diagnosis of sepsis and disease severity. This method holds potential as a valuable reference for detecting and studying other diseases.

4.1. Bioethics statement

Blood samples were obtained from healthy controls and patients with sepsis at the First Affiliated Hospital of Wannan Medical College, Wuhu Conch Hospital, and Wuhu Second People's Hospital (Wuhu, China). This study was reviewed and approved by the Human Ethics Committee of the Anhui Normal University (AHNU-ET2023029). All authors declare that this work involving the use of human subjects was conducted in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. This manuscript is in line with the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals. Representative 14 human subjects (six males and eight females, aged 40–97, all ethnic Han) were tested; please see Table 2 for details. Informed consent was obtained from the subjects during blood sample collection, and their privacy rights were observed.

BALB/c mice were purchased from Nanjing Bioengineering Ltd (Nanjing, China) and housed in a specific pathogen free environment in the Animal Facilities of Anhui Normal University with a temperature of 25 ± 2 ℃, humidity at 55 ± 5 %, under 12 h light–dark cycles, and free access to food and water. Five female BALB/c mice were used for immunization and monoclonal antibody production. Thirty male BALB/c mice were used as the sepsis model and for antibody treatment. Female and male BALB/c mice were 6 weeks old at the start of the experiment and were randomly divided into groups. The study protocols were reviewed and approved by the Animal Experimentation Ethics Committee of the Anhui Normal University (AHNU-ET2022015).

CRediT authorship contribution statement

Juncheng Wang: Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Meng Liu:** Validation, Methodology, Data curation. **Zecheng Cai:** Methodology, Data curation, Conceptualization. **Rukhshan Zahid:** Validation, Formal analysis. **Wenjie Zhang:** Methodology, Data curation. **Dan Ma:** Visualization, Validation. **Die Li:** Validation, Data curation. **Yan Liang:** Visualization, Validation. **Lei Zha:** Validation, Resources. **Yun Zhou:** Validation, Resources. **Lina Wang:** Validation, Resources. **Gang Yang:** Validation, Resources, Project administration. **Shuai Zheng:** Project administration, Funding acquisition, Validation. **Yuekang Xu:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2024.113473.

Data availability

Data will be made available on request.

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