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ORIGINAL ARTICLE

Purified Immunoglobulin F(ab′)$_2$ Protects Mice and Rhesus Monkeys against Lethal Ricin Intoxication

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ABSTRACT

Objective: Ricin is a highly toxic ribosome-inactivating lectin derived from castor beans. To date, no antidote is available to treat ricin-poisoned patients, and the development of a safe and effective antidote is urgently needed.

Methods: First, ricin was prepared and used to construct a mouse model and a rhesus monkey model of ricin intoxication. Second, pepsin-digested F(ab′)$_2$ fragments of serum IgG from horses injected with Freund's-adjuvanted purified ricin were prepared. Third, the protective efficacy was evaluated in mouse and rhesus monkey models of lethal ricin intoxication.

Results: The purity quotient of the prepared ricin and F(ab′)$_2$ fragments exceeded 90% and 85% in the mouse and monkey models, respectively. The LD_{50} of ricin in mice and rhesus monkeys was 2.7 and 9 μg/kg, respectively. A quantity of 6.25 and 1.85 mg/kg F(ab′)$_2$ was sufficient to treat lethal ricin intoxication in the mice and rhesus monkeys, respectively. Finally, the effect of this therapeutic antibody on peripheral blood immune cells was examined by analysis of peripheral blood immune cells through single cell sequencing. The underlying mechanism was found to involve restraining neutrophil activation, proliferation, and differentiation.

Conclusion: Purified F(ab′)$_2$ fragments administered with needle-free devices fully protect mice and rhesus monkeys against lethal doses of ricin intoxication.

Keywords: Ricin, Equine immunoglobulin F(ab′)$_2$ fragments, Animal models, Needle-free, Single-cell sequencing

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INTRODUCTION

Ricin is an extremely potent toxin derived from the castor bean plant and a highly toxic ribosome-inactivating glycoprotein produced in castor seeds [1]. In most cases, ricin intoxication in humans and livestock occurs accidentally through ingestion of castor seeds and other improperly detoxified castor-derived products [2]. Patients with ricin intoxication often exhibit vomiting and diarrhea, and may lapse into a coma or even die [3]. Clinical toxicological studies have indicated that ricin severely damages the liver and kidneys, and often causes cardiovascular system and respiratory center injury [4–6]. Ricin, a potential bioterrorism agent, is a cytotoxin easily purified in large quantities [7]. With growing awareness and

concern regarding "white powder incidents" in recent years, effective countermeasures against ricin intoxication must be developed [8,9].

Despite the grave challenge to public health, approved prevention and treatment measures against ricin intoxication are currently unavailable. RiVax^{TM} and RVEc^{TM} are RTA-based ricin subunit vaccines currently being investigated in phase I clinical trials [10]. Many monoclonal antibodies (such as huPB10) with potent ricin-neutralization activity and small molecule inhibitors/blockers inhibiting the interaction of ricin with ribosomes have shown therapeutic effects in ricin poisoned cells and/or animal models [11-14]. However, to date, the treatment of patients with ricin intoxication is supportive, and specific antidotes are lacking [15].

Therefore, much work will be needed to develop an effective ricin-specific antidote. Using our equine immunoglobulin F(ab′)$_2$ fragment preparation and evaluation technology [16,17], we developed biological products associated with ricin, and assayed and evaluated them according to the Chinese Pharmacopoeia, thus laying a foundation for the development of an effective antidote candidate against ricin intoxication.

METHODS

Cells and animals

Vero cells (ATCC NO. CCL-81) were provided by the cell bank of the Fifth Medical Center of PLA General Hospital. Healthy horses 4–6 years old were provided by Inner Mongolia Huaxi Bio-technology Co., LTD (Inner Mongolia, China). Four week old female BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Rhesus monkeys were purchased from Beijing Institute of Xieerxin Biology Resource (Beijing, China). All animal experiments were approved by the Animal Experiment Committee of the Laboratory Animal Center, Academy of Military Medical Sciences, China (assurance number: IACUC-DWZX-2017-048).

Preparation and identification of purified ricin

Castor cake (500 g) was de-husked and homogenized into a slurry with 5 mmol/L PBS. After centrifugation at 20000 g for 30 min, the supernatant was collected, and solid $(NH_4)_2SO_4$ was added to 60% saturation, stirred at 4°C for 3 hours with magnetic stirrers, and centrifuged at 20000 g for 30 min to precipitate the ricin. The precipitate was dissolved in PBS, dialyzed at 4°C for 24 hours, and centrifuged at 20000 g for 10 min. The supernatant contained crude ricin. With agarose derived 4-aminophenyl β-D-galactopyranoside as affinity chromatography medium, the crude ricin was eluted with galactose. The lectin was separated from crude ricin with ion-exchange resin. The purified ricin was obtained and stored at 4°C. The purified ricin was assayed with HPLC and SDS-PAGE.

To measure the half lethal dose (LD_{50}) of ricin, eight mice per group were injected intraperitoneally with 0.03, 0.04, 0.05, 0.06, 0.07, or 0.08 μg ricin, and four rhesus monkeys per group were injected intraperitoneally with 9, 18, 36, or 72 μg ricin. The mice and rhesus monkeys were monitored for 7 days and 36 hours, respectively. The LD_{50} of ricin was measured with the Reed-Muench method.

Detoxification of purified ricin

Purified ricin was detoxified with formaldehyde at 1:4000 at ambient temperature for 48, 60, 72, 84, 96, 108, or 120 hours. The detoxified ricin was concentrated by dialysis and sterilized through a 0.22 μm filter. The detoxified ricin was measured with the Lowry method, and the survival rate of Balb/c mice injected with 0.1 mg detoxified ricin was observed.

Preparation and identification of the F(ab′)$_2$ fragments of equine serum IgG against ricin

Five healthy horses 4–6 years old without detectable pathogens were subcutaneously injected with 4.0, 4.0, 5.0, or 6.0 mg purified ricin with Freund's adjuvant into sites near the inguinal and submandibular lymph nodes every 21 days. At 14 days after the last immunization, the titer of ricin specific antibody in horse sera was detected with the MTT method. When the serum titers exceeded 1:6400, the sera were collected and stored at 4°C.

The F(ab′)$_2$ fragments of equine serum IgG were prepared by the GMP plant of Shanghai Serum Biotechnology Co., Ltd. The products were identified with HPLC and SDS-PAGE and stored at 4°C.

Identification of the neutralization effects of F(ab′)$_2$ fragments against ricin in Vero cells with the MTT method

The neutralization titers of the F(ab′)$_2$ fragments against ricin were determined in Vero cells. Vero cells were transferred to a 96-well plate with 2×10^4 cells in 100 μl per well, then incubated at 37°C for 24 hours. The F(ab′)$_2$ fragments were two-fold serially diluted with DMEM, initially at 1:400. After the F(ab′)$_2$ fragments were mixed with isopycnic suspension containing 100 TD_{50} of purified ricin and incubated at 37°C for 1 hour, the F(ab′)$_2$ fragments and ricin mixtures were added to the Vero cells. Normal F(ab′)$_2$ fragment control (100 TD_{50} of ricin and F(ab′)$_2$ fragments of equine serum IgG without injection) and ricin control (with 100 TD_{50} of ricin only) served as control groups. When CPE developed in the ricin control, the Vero cells were stained with 200 μl MTT (0.5 mg/ml) per well for 4 hours. After the supernatant was discarded, 10% SDS mixed with 0.01 M HCl was added to the Vero cells at 200 μl per well and incubated for 8 hours. The OD value at 570 nm was determined with an ELISA reader. The dilution of cells that showed the same values as the normal F(ab′)$_2$ fragment control was calculated as the neutralization antibody titer of the F(ab′)$_2$ fragments against ricin.

Effective dosas of F(ab′)$_2$ fragments against ricin in mice and rhesus monkeys

To investigate the effective dosas of the F(ab′)$_2$ fragments against ricin, we weighed eight mice per group and them injected intraperitoneally with 5 LD_{50} of purified ricin. After 4 hours, the mice were subcutaneously injected in the abdomen with 31.25, 62.5, or 125 μg of F(ab′)$_2$ fragments against ricin, with a needleless injection device (POK-V Dart, Boke BioTech, China) (Fig 4A). The mice were monitored for mortality daily for 7 days. The mice injected with isopycnic normal equine F(ab′)$_2$ fragments served as a control group.

To further investigate the effective doses of the F(ab′)$_2$ fragments against ricin, we injected four rhesus monkeys per group intraperitoneally with 2 LD_{50} of purified ricin [2]. After 4 hours, the rhesus monkeys were injected subcutaneously with 3.75, 7.5, or 12.5 mg of the F(ab′)$_2$ fragments against ricin with a needleless injection device. The rhesus monkeys' physical condition and mortality were monitored daily for 7 days. The rhesus monkeys injected with isopycnic normal equine F(ab′)$_2$ fragments served as a control group.

Histopathological analysis

After the moribund monkeys from the control group and those injected with 12.5 mg F(ab′)$_2$ fragments on the 7th day were euthanized, lung, liver and kidney tissues were collected, fixed with 4% formalin, paraffin-embedded, cut into 5-μm sections, stained with hematoxylin and eosin, and observed according to standard procedures.

Isolation of PBMCs

In two groups of mice (ten mice per group), 200 μl containing 2 LD_{50} ricin was injected intraperitoneally. After 4 hours, 0.5 ml containing 125 μg anti-ricin IgG or PBS was injected subcutaneously into the abdomen in the treatment or control groups, respectively, with a needleless injection device. After 24 h, peripheral blood was collected from the fundus vein, and 1 ml of whole blood was added to 3 ml of erythrocyte lysis solution and lysed on ice for 5 min. Subsequently, 5 ml of PBS containing 10% fetal bovine serum was added and centrifuged at 500 g for 5 min. The supernatant was discarded, and the precipitate was washed twice with PBS containing 10% fetal bovine serum, then centrifuged at 500 g for 5 min. After the supernatant was discarded, the precipitate was resuspended in PBS and mixed thoroughly to make a cell suspension. Five replicates of experimental group cells meeting the sequencing requirements were selected as the treatment group samples, and a mixture of five replicates of control group cells were selected as the control group samples for single cell sequencing.

Single-cell sequencing library construction

The sequencing and data analysis were performed by Shanghai Ouyi Biomedical Technology Co. The raw data generated by high-throughput sequencing were analyzed in CellRanger, the official software from 10x Genomics. Further quality control and processing of the data were performed according to the preliminary quality control results of CellRanger in the Seurat software package. Principal component analysis linear dimensionality reduction was performed according to gene expression. The principal component analysis results were visualized in two dimensions through non-linear dimensionality reduction (tSNE). With the SingleR package and a single cell reference expression quantitative public dataset, the correlation between the cell expression profile of interest and the reference dataset was calculated, and the cell type with the highest correlation in the reference dataset was assigned to the cell to be identified, thus decreasing interference due to subjective human factors. The marker genes were identified with the FindAllMarkers function in the Seurat package to identify genes up-regulated in each cell classification relative to the differentially expressed genes in other cell populations. These genes were potential marker genes for each cell classification. The identified marker genes were visualized with the Vlnplot and FeaturePlot functions. Significantly differentially expressed genes were screened with the FindMarkers function in the Seurat package, and significantly differentially expressed genes were screened according to a P-value less than 0.05 and a difference multiplicity greater than 1.5-fold.

Statistical analysis

Statistical analyses were performed in Prism software (GraphPad5.0).

RESULTS

Preparation and identification of purified ricin

The purity quotients of the prepared ricin (64 kDa) exceeded 90% (Fig 1A) in SDS-PAGE and 92.2% (Fig 1B) in HPLC. The LD_{50} of the purified ricin was 2.7 μg/kg in mice (Fig 2A) and 9 μg/kg in rhesus monkeys (Fig 2B). On the basis of the Balb/c mice survival rate, the purified ricin was completely detoxified within 96 hours by formaldehyde at 1:4000. No significant adverse effects were observed in the injected mice, and only several showed a transient increase in body temperature.

Identification of the F(ab′)$_2$ fragments of equine serum IgG against ricin

The purity quotients of the F(ab′)$_2$ fragments (100 kDa) exceeded 80% (Fig 3A) in SDS-PAGE and 88.7% (Fig 3B) in HPLC. The neutralization antibody titer of the F(ab′)$_2$ fragments against ricin was 1:12800, on the basis of the MTT method (Table 1).

Effective doses of F(ab′)$_2$ fragments against ricin in mice and rhesus monkeys

To confirm the effective doses of the F(ab′)$_2$ fragments against ricin, we injected the mice with 5 LD_{50} ricin to construct a ricin intoxication mouse model. After 4 hours, the mice were injected with 31.25, 62.5, or 125 μg

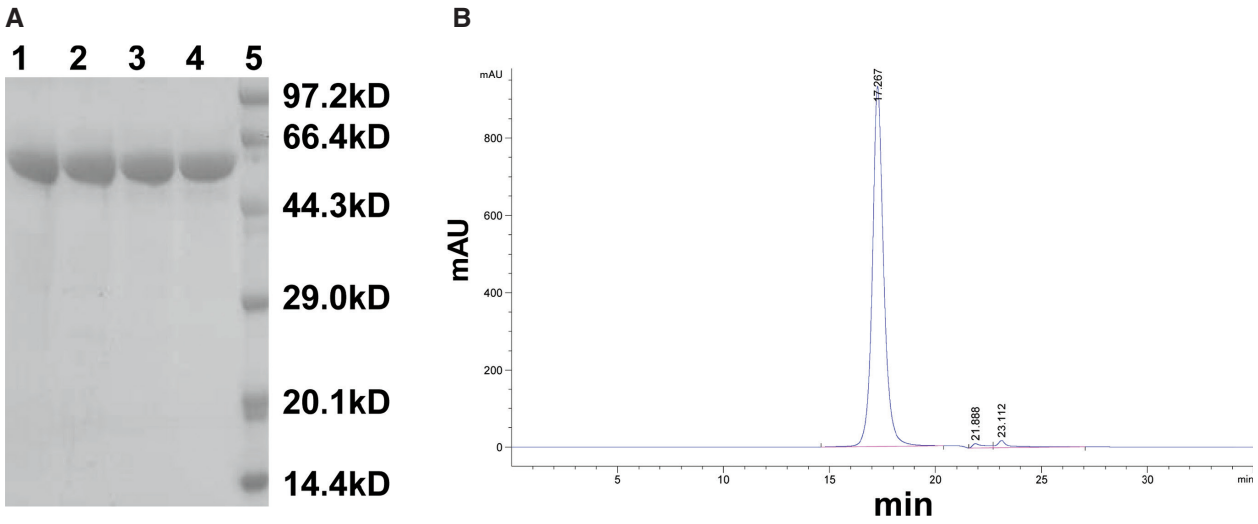

FIGURE 1 | Characterization of purified ricin. (A) SDS-PAGE of purified ricin. Lane 1, ricin standards. Lane 2–4, purified ricin. Lane 5, protein molecular weight standards with MW indicated for each band. (B) Analysis of purified ricin by HPLC.

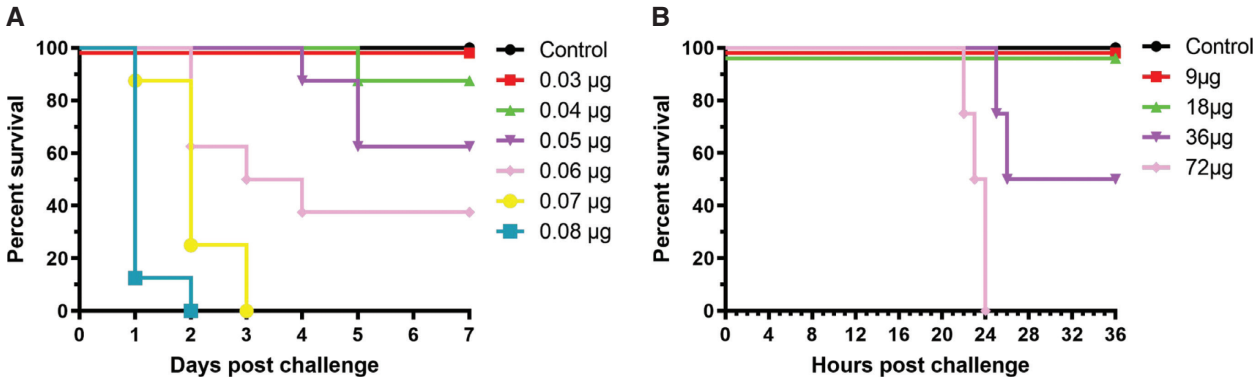

FIGURE 2 | The half lethal dose (LD_{50}) of ricin. (A) In the mouse model, mice (n=8/group) were injected intraperitoneally with six concentrations of purified ricin. The mice were monitored daily for survival for 7 days. (B) In the rhesus monkey model, rhesus monkeys (n=4/group) were injected intraperitoneally with four concentrations of purified ricin. The rhesus monkeys were monitored for survival for 36 hours. The LD_{50} of ricin was measured with the Reed-Muench method.

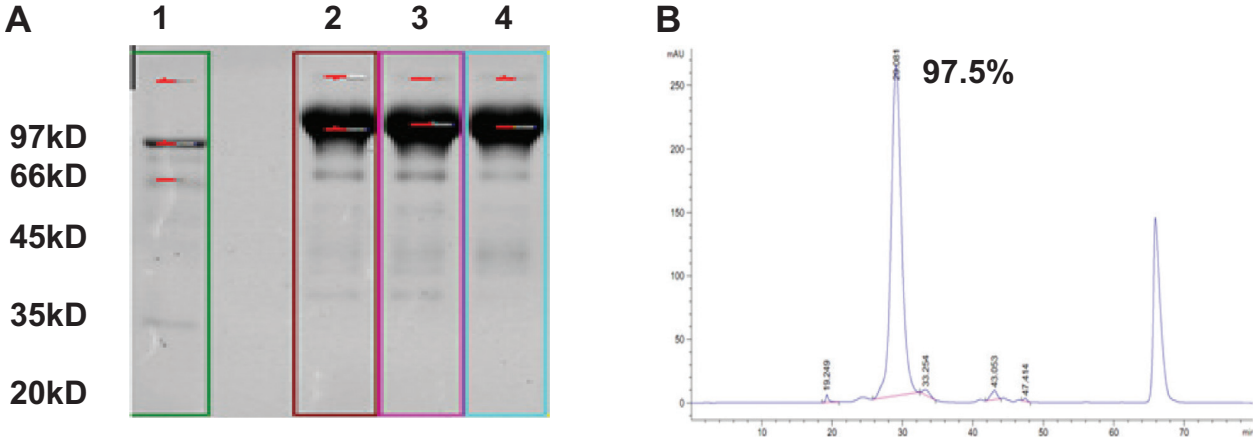

FIGURE 3 | Characterization of $F(ab')_2$ fragments of equine serum IgG against ricin. (A) SDS-PAGE of $F(ab')_2$ fragments. Lanes 1, protein molecular weight standards with MW indicated for each band. Lane 2–4, purified $F(ab')_2$ fragments. (B) Analysis of purified $F(ab')_2$ fragments by HPLC.

TABLE 1 | Neutralization effects of horse anti smallpox virus purified F(ab′)$_2$ on Tiantan strain vaccinia virus by MTT.

Antibody dilution	OD value (570 nm)	
	Purified equine F(ab′)$_2$[a]	Normal horse immunoglobulin control[a]
1/800	1.684±0.568	0.726±0.220
1/1600	1.781±0.327	0.798±0.210
1/3200	1.521±0.507	0.640±0.295
1/6400	1.029±0.600	0.565±0.212
1/12800	0.960±0.493	0.653±0.282
1/25600	0.793±0.190	0.587±0.517
Normal cell control[b]	1.763±0.467	
Virus infected cell control[b]	0.663±0.374	

[a]The immunoglobulin F(ab′)$_2$ of equine anti smallpox virus and normal horse immunoglobulin control (with virus and normal horse immunoglobulin) were both diluted twice with DMEM. The initial titer was 1:800, with six dilution in total. The vaccinia virus Tiantan strain at 10^5 PFU was diluted with serum-free DMEM, and 200 ml/well was successively added after supernatant was removed from wells. Each dilution was added to four wells.
[b]Normal cell control (without virus and without F(ab′)$_2$) and virus control (with virus and without F(ab′)$_2$) were used. When the virus was in Virus infected cell control, 200 ml MTT (0.25 mg/ml) was used to stain cells for 4 h and was then aspirated. Termination solution (10% SDS + 0.01 M HCl; 200 ml) was added and incubated for 8 h. The OD value at 570 nm was determined with an ELISA reader.

of F(ab′)$_2$ fragments. The survival rates of the mice are shown in Fig 4A. All the mice in the control group died within 48 hours. All mice injected with 31.25 μg of F(ab′)$_2$ fragments died within 3 days. One mouse injected with 62.5 μg of F(ab′)$_2$ fragments died on day 3, and the others survived and recovered by day 7. All mice injected with 125 μg of F(ab′)$_2$ fragments against ricin survived and recovered by day 7.

To further confirm the effective doses of the F(ab′)$_2$ fragments against ricin, we injected rhesus monkeys with 2 LD_{50} of purified ricin to construct a ricin intoxication rhesus monkey model. The rhesus monkeys were injected with 3.75, 7.5, or 12.5 mg of F(ab′)$_2$ fragments after 4 hours. The survival rates of the rhesus monkeys are shown in Fig 4B. All rhesus monkeys in the control group had symptoms of anorexia and agitation, and died within 2 days. The histopathological results (Fig 5) indicated the exudation of neutrophils in the lungs, large amounts of liver stromal cell necrosis, and exudation of inflammatory cells in the kidneys. Six hours after injection with 3.75 mg of F(ab′)$_2$ fragments, all rhesus monkeys had symptoms of anorexia and agitation; two died at day 3 and day 4, whereas the others returned to normal after 3 days. Eight hours after injection with 7.5 mg of F(ab′)$_2$ fragments, all rhesus monkeys had symptoms of anorexia and agitation, but they all returned to normal after 48 hours. After injection with 12.5 mg of F(ab′)$_2$ fragments for 10 hours, only one rhesus monkey had anorexia symptoms, and the monkey returned to normal condition after 24 hours. The other monkeys appeared healthy at all time points. The histological morphology (Fig 5) of all tissues from the rhesus monkeys injected with 12.5 mg of F(ab′)$_2$ fragments was normal.

Anti-ricin IgG reverses lethal neutrophil toxicity in mice, according to single cell sequencing

The numbers of cells were 10132 and 7892 in the antibody treatment group and model group samples after quality control. After clustering and differentially expressed gene analysis, neutrophils, T cells, natural killer cells, monocytes, epithelial cells, basophils, and B cells were involved in the RT virulence process (Fig 6A,B). Subsequently, we performed a statistical analysis of the percentages of the seven cell types in PBMCs

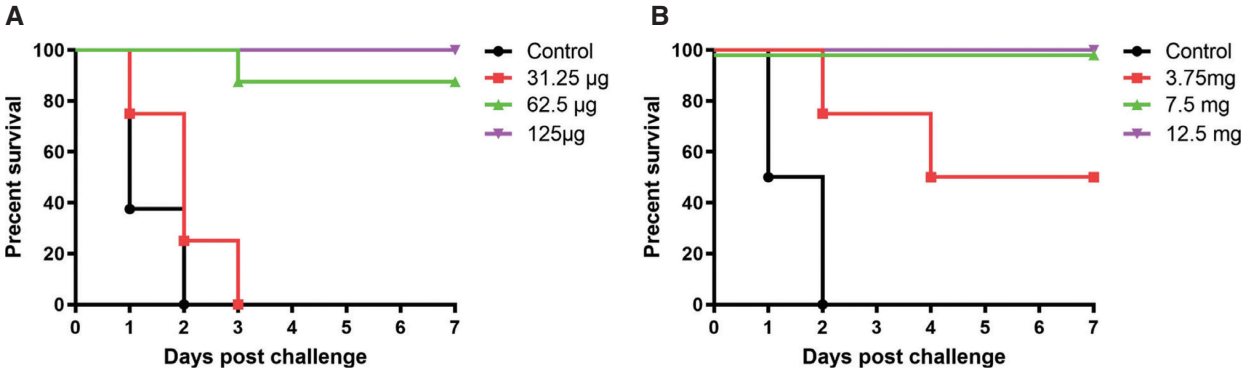

FIGURE 4 | Effective doses of F(ab′)$_2$ fragments against ricin in mice and rhesus monkeys. (A) At 4 hours after injection with 0.2 ml of 5 LD_{50} ricin, the groups of mice (n=8/group) were injected with 0.2 ml of 31.25 μg, 62.5 μg, or 125 μg of F(ab′)$_2$ fragments subcutaneously with needle free devices. The mice injected with PBS subcutaneously with needle free devices served as a control group. The mice were monitored for mortality daily for 7 days. (B) At 4 hours after injection with 72 μg ricin, the groups of rhesus monkeys (n=4/group) were injected with 3.75 mg, 7.5 mg, or 12.5 mg F(ab′)$_2$ fragments subcutaneously with needle free devices. The rhesus monkeys injected with PBS subcutaneously with needle free devices served as a control group. The rhesus monkeys were monitored for physical condition and mortality daily for 7 days.

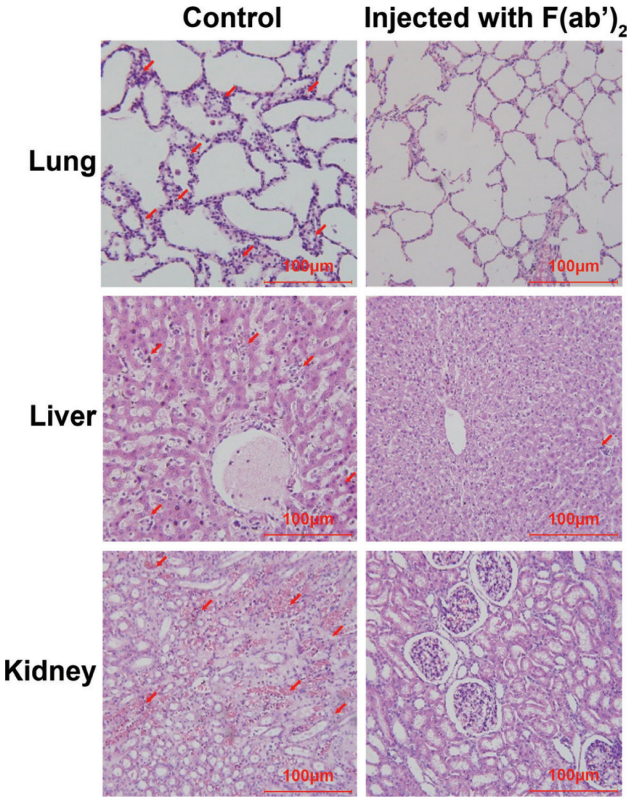

FIGURE 5 | Histopathological examination of lung, liver and kidney tissues from the rhesus monkeys injected with PBS or 12.5 mg $F(ab')_2$ fragments from Fig. 4. After moribund monkeys from the control group and those from injected with 12.5 mg $F(ab')_2$ fragments subcutaneously with needle free devices on the 7th day were euthanized, the lung, liver and kidney tissues were collected and processed, and sections were subjected to pathological analysis.

(Fig 6C). The results indicated a slight downregulation of neutrophils in the antibody-treated mice, thus leading to a hypothesis in which anti-ricin antibody might be involved in the decline in maturation, differentiation, and proliferation of neutrophils, thereby avoiding the lethal outcomes of ricin. Furthermore, through transcriptome characterization, we identified neutrophils as neutrophil-Camp, neutrophil-Csf3r, neutrophil-Cxcl3, neutrophil-Gm26917, neutrophil-Hbb-bt, neutrophil-Ifitm1, neutrophil-Ltf, neutrophil-Orm1, neutrophil-Pf4, and neutrophil-Stfa2. IL-1β, Csf3r, and S100a9, which are characteristic transcriptional phenotypes of neutrophils, were significantly elevated among the neutrophil clusters (Fig 6D). As shown in Fig 6E, the ratio of subtypes of neutrophils was significantly altered after antibody treatment. Neutrophil-Camp and neutrophil-Hbb-bt were dominant in the control group; in contrast, neutrophil-Csf3r and neutrophil-Ifitm1 were dominant in the antibody-treated group. Furthermore, the neutrophil transcriptome pattern was significantly altered (Fig 6F), such that IL-1β, csf3r, and S100a9 shifted from low to high expression in major subsets of neutrophils after antibody treatment.

DISCUSSION

To develop a safe and effective antidote to ricin, we prepared equine $F(ab')_2$ fragments and evaluated them in lethal ricin intoxication animal models. The purity quotients and bioactivities of the preparations met the requirements of the Chinese Pharmacopoeia. Treatment with a full dose of purified $F(ab')_2$ fragments protected the animals against a lethal dose of ricin intoxication. The equine $F(ab')_2$ fragments against ricin are currently being assayed in clinical trials.

Although the crudely purified ricin and equine $F(ab')_2$ fragments were easily produced, the ricin antigen for immunization and $F(ab')_2$ fragments for treatment required high purity to improve safety and avoid adverse effects of $F(ab')_2$ preparations. Extending the reported methods in prior studies [18], we further purified the ricin by affinity and ion-exchange chromatography to achieve greater than 90% purity. The equine $F(ab')_2$ fragments were purified by size exclusion chromatography in addition to commonly applied methods to meeting the sequencing requirements.

Bioactivity is extremely important. To improve the protective bioactive ingredients of $F(ab')_2$ preparations, we optimized the detoxified antigens, the immunization regimens, and the blood collection procedure repeatedly. Using the methods in prior studies as a reference [1,19], we determined that ricin detoxification was achieved with formaldehyde at 1:4000 within 96 hours, thereby completely inactivating the toxicity and retaining immunogenicity against ricin. Furthermore, healthy adult horses were injected in sites near the lymph nodes many times until the serum titers exceeded 1:6400. The protective efficacy of $F(ab')_2$ preparations was satisfactory with respect to that of the many types of antibodies previously reported [20,21]. Thus, the preparations protected animal models against a lethal dose of ricin intoxication at low protein doses.

With the advent and advancement of single-cell experimental methods, thousands of cells can be sequenced in a single experiment by using microfluidics technology and combinatorial indexing methods. Ricin can cause a lethal inflammatory response, which is partially attributable to the massive activation and proliferation of neutrophils. Our results indicated that this anti-ricin antibody, owing to its potent neutralization effects and the absence of the FC segment, exerts strong protection through inhibition of neutrophil development, proliferation, and differentiation, and through attenuation of the systemic inflammatory immune response. The FC segment of the antibody has various biological functions in the activation of the immune response, such as ADCC, complement activation, and induction of apoptosis. A particularly promising method of creating therapeutic antibodies without undesirable Fc activity is the rational design of human IgG1 antibodies lacking ADCC or other Fc functions. IL-1, initially discovered as the major

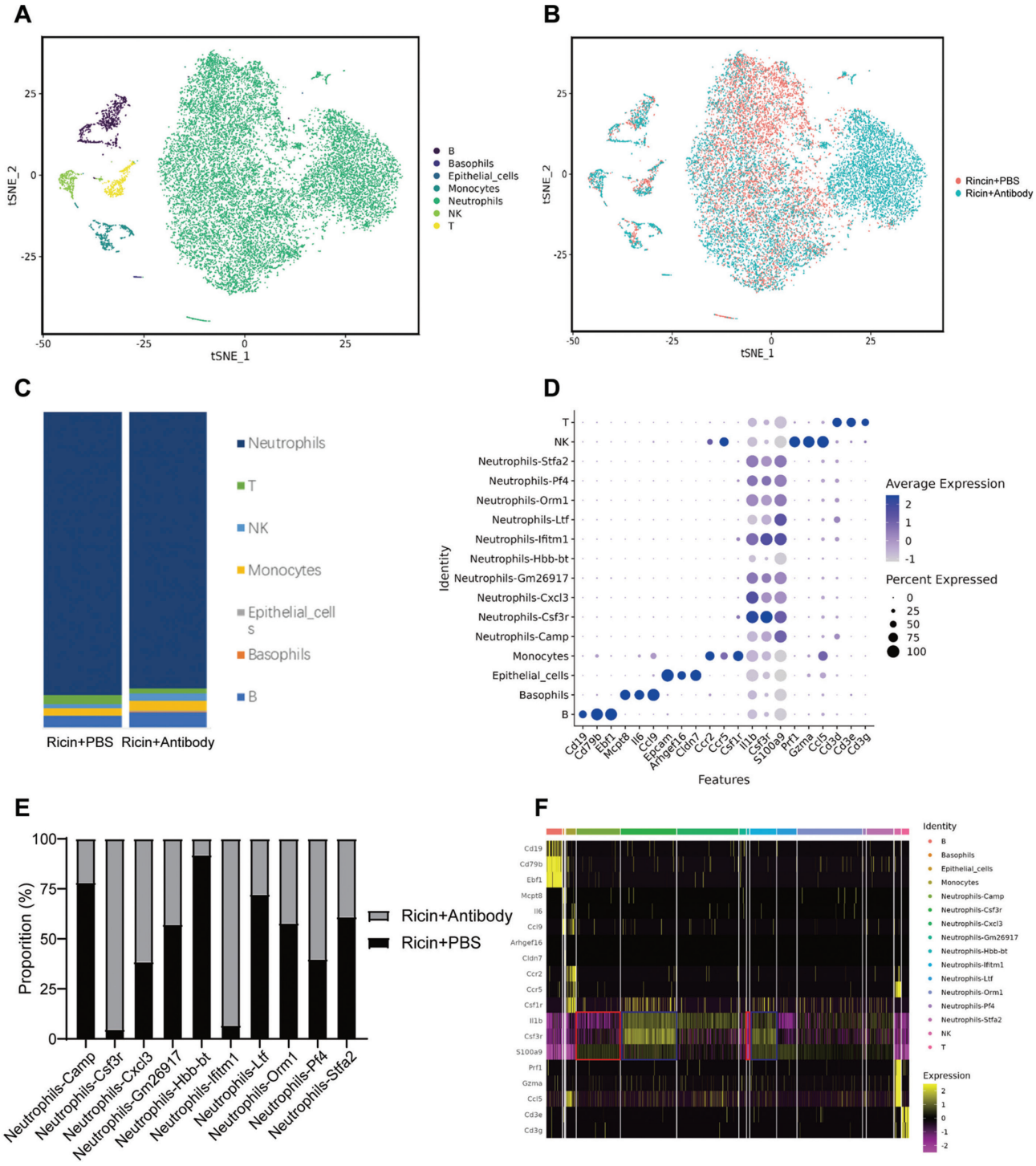

FIGURE 6 | Effects of anti-ricin antibodies on peripheral blood, revealed by single cell sequencing. (A) tSNE plot of cellular populations; (B) t-SNE plot showing cell samples from mice with ricin toxin exposure and healthy controls; (C) comparison of the relative proportions of each subpopulation between the experimental group and the control group; (D) bubble plot of the identified genes for each subpopulation; (E) comparison of the relative proportion of each cluster between the experimental group and the control group; (F) heat map showing the top three genes for each subpopulation.

endogenous pyrogen, induces prostaglandin synthesis, neutrophil influx, and activation. Receptor for granulocyte colony-stimulating factor (CSF3), which is essential for granulocytic maturation, plays a crucial role in the proliferation, differentiation, and survival of neutrophil lineage cells. In addition, it may function in adhesion or recognition events at the cell surface. S100A9 is a calcium- and zinc-binding protein with prominent roles in the regulation of inflammatory processes and the immune response. It induces neutrophil chemotaxis and adhesion; increases the bactericidal activity of neutrophils by promoting phagocytosis via activation of SYK, PI3K/AKT,

and ERK1/2; and induces degranulation of neutrophils through a MAPK-dependent mechanism. According to the preliminary analysis results of single cell sequencing, the antibody reversed the immature neutrophils caused by ricin by regulating gene transcription. This result is difficult to accomplish with commonly used assays. To further explore the therapeutic mechanism of the antibody and optimize the technological process of producing anti-ricin antibodies, we plan to predict the effect of IL-1b, csf3r, and S100a9 on neutrophil differentiation in time series analyses, and further validate their spatiotemporal changes with flow cytometry.

In summary, antibody bioproducts against ricin were successfully prepared and may be used to treat patients and animals poisoned by ricin in the near future.

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CONFLICTS OF INTEREST

The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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