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Purified immunoglobulin F(ab')$_2$ could protect mice and rhesus monkeys from lethal ricin intoxication

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Abstract

Objective: Ricin is a highly toxic ribosome–inactivation lectin derived from castor beans. So far, there has been no antidote to treat ricin–poisoned patients, and the development of a safe and effective antidote is urgently needed.

Methods: Firstly, the ricin was prepared and used to construct the ricin intoxication mouse and rhesus monkey model. Secondly, the pepsin–digested $F(ab')_2$ fragments of serum IgGs from horses inoculated with Freund's–adjuvanted purified ricin were prepared. Thirdly, the protective efficacy was evaluated in a lethal ricin intoxication mouse and rhesus monkey model.

Results: The purity quotient of the prepared ricin and $F(ab')_2$ fragments were more than 90% and 85%, respectively. The LD_{50} of ricin in mice and rhesus monkeys was 2.7 and 9 μg/kg, respectively. 6.25 and 1.85 mg/kg $F(ab')_2$ could treat the lethal ricin intoxication mice and rhesus monkeys models, respectively. Finally, the effect of this therapeutic antibody on peripheral blood immune cells was examined by analyzing peripheral blood immune cells through single cell sequencing technique, and its

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associated mechanism was elucidated, by restraining neutrophil activation, proliferation and differentiation.

Conclusion: Purified $F(ab')_2$ fragments using needle–free devices can completely protect mice and rhesus monkeys from a lethal dose of ricin intoxication.

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

Introduction

Ricin is an extremely potent toxin derived from the castor bean plant and a high toxic ribosome–inactivating glycoprotein produced in castor seeds (1). In most cases, ricin intoxication in humans and livestock occurred accidentally with ingestion of castor seeds and other improperly–detoxified castor–derived products (2). Patients suffering from ricin intoxication often exhibited vomiting, diarrhea and sometimes lapsed into a coma and even death (3). Clinical toxicological studies showed that ricin severely damaged liver and kidney and often caused the cardiovascular system and respiratory

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center injury(4–6). Ricin, as a potential bioterrorism agent, is a cytotoxin easily purified in large quantities (7). With the growing awareness and concerns of the "white powder incident" in recent years, it is indispensable to develop an effective countermeasure against ricin intoxication (8, 9).

Despite the grave challenge to public health, the approved prevention and treatment of ricin intoxication is currently unavailable. RiVax^{Tm} and RVEc^{Tm}, as RTA–based ricin subunit vaccines, are now investigated in the phase I clinical trials (10). Many monoclonal antibodies (such as huPB10) with potent ricin–neutralization activity and small molecule inhibitors/blockers with inhibiting the interaction of ricin with ribosomes had some therapeutic effects on ricin poisoned cells and/or animals models(12–15). However, until now, the treatment of patients suffering from ricin intoxication is supportive without the specific antidotes (11).

Therefore, it's still a long way to develop an effective ricin–specific antidote. Based on our equine immunoglobulin F(ab')$_2$ fragments preparation and evaluation technology(16, 17), the biological products related to ricin were developed, assayed and

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evaluated according to the Chinese Pharmacopoeia, which laid a foundation for the development of an effective antidote candidate against ricin intoxication.

Methods

1. Cells and animals

Vero cells (ATCC NO. CCL–81) were provided by the cell bank of the fifth medical center of PLA General Hospital. The 4–6 years old, healthy horses were provided by Inner Mongolia Huaxi Bio–technology Co., LTD (Inner Mongolia, China). Four weeks old female BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The rhesus monkeys were purchased from Beijing Institute of Xieerxin Biology Resource (Beijing, China). All the animals were approved for the Animal Experiment Committee of Laboratory Animal Center, Academy of Military Medical Sciences, China (Assurance Number: IACUC–DWZX–2017–048).

2. Preparation and identification of purified ricin

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Castor cake (500g) was de–husked and homogenized into a slurry with 5 mmol/L PBS. Centrifuged at 20000g for 30 min, the supernatant was taken and added with solid $(NH_4)_2SO_4$ to 60% saturation, stirred at 4℃ for 3 hours with magnetic stirrers, and centrifuged at 20000g for 30 min to precipitate the ricin. The precipitate was dissolved in PBS, dialyzed at 4℃ for 24 hours, and centrifuged at 20000g for 10 min. The supernatant was the crude ricin. Using the agarose derived 4–Aminophenyl β–D–Galactopyranoside as affinity chromatography mediums, the crude ricin was eluted with galactose. The lectin was separated from crude ricin by using ion–exchange resin. The purified ricin was obtained and stored at 4℃. The purified ricin was assayed by HPLC and SDS–PAGE.

To measure the half lethal dose (LD_{50}) of ricin, the mice (8 mice per group) were injected intraperitoneally with 0.03, 0.04, 0.05, 0.06, 0.07 or 0.08 μg ricin, respectively, and the rhesus monkeys (4 rhesus monkeys per group) were injected intraperitoneally with 9, 18, 36 or 72 μg ricin, respectively. The mice and the rhesus monkeys were

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monitored for 7 days and 36 hours, respectively. The LD_{50} of ricin was measured by the Reed–Muench method.

3. Detoxification of the purified ricin

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

4. Preparation and identification of the F(ab')2 fragments of equine serum IgGs against ricin

The 4–6 years old, healthy horses (5 horses) without detectable pathogens were inoculated subcutaneously with 4.0, 4.0, 5.0 and 6.0 mg purified ricin adjuvanted with Freund's adjuvant into the sites near the inguinal and submandibular lymph nodes every 21 days, respectively. After the last immunization for 14 days, the horse sera

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were detected for the titer of ricin specific antibody by MTT method. When the titers of sera were more than 1:6400, the sera were collected and stored at 4℃.

The $F(ab')_2$ fragments of equine serum IgGs were prepared by the GMP plant of Shanghai Serum Bio-technology Co., LTD. The products were identified by HPLC and SDS-PAGE and stored at 4℃.

5. Identification of the neutralization effects of the F(ab')2 fragments against ricin in Vero cells with MTT method

The neutralization titers of the $F(ab')_2$ fragments against ricin were determined in Vero cells. The Vero cells were transferred to a 96-well plate with 2×10^4 cells in 100 μl per well and incubated at 37℃ for 24 hours. The $F(ab')_2$ fragments were diluted by two-fold serial dilution with DMEM, initially with 1:400. After the $F(ab')_2$ fragments were mixed with isopycnic suspension containing 100 TD_{50} of purified ricin and incubated at 37℃ for 1 hour, the $F(ab')_2$ fragments and ricin mixtures were added into the Vero cells. 'normal $F(ab')_2$ fragments control' (with 100 TD_{50} of ricin and $F(ab')_2$ fragments of equine serum IgGs without inoculation), and 'ricin control' (with 100 TD_{50} of ricin only)

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were set as controls. When CPE developed in 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 into the Vero cells with 200 µl per well for 8 hours. The OD value at 570 nm was determined with an ELISA reader. Compared with normal $F(ab')_2$ fragments control, the dilution of the cells that showed the same values of the normal cell was calculated as the neutralization antibody titer of the $F(ab')_2$ fragments against ricin.

6. Effective dosages of F(ab')2 fragments against ricin in mice and rhesus monkeys

To investigate the effective dosages of the $F(ab')_2$ fragments against ricin, the mice (8 mice per group) were weighed and injected intraperitoneally with 5 LD_{50} of purified ricin. After 4 hours, the mice were injected subcutaneously into the abdomen with 31.25, 62.5 or 125 µg of the $F(ab')_2$ fragments against ricin, respectively, using a needleless injection device (POK–V Dart, Boke BioTech, China) FIG(FIG.4A). The mice were monitored for mortality daily for 7 days. The mice injected with isopycnic normal equine $F(ab')_2$ fragments, meanwhile, were set as a control group.

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To further investigate the effective dosages of the $F(ab')_2$ fragments against ricin, the rhesus monkeys (4 rhesus monkeys per group) were injected 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, respectively, using a needleless injection device. The rhesus monkeys were monitored for physical condition and mortality daily for 7 days. The rhesus monkeys injected with isopycnic normal equine $F(ab')_2$ fragments, meanwhile, were set as a control group.

7. Histopathological analysis

After the moribund monkeys from the control group and the ones from injected with 12.5 mg F(ab')2 fragments on the 7th day were euthanized, the lung, liver and kidney tissues were collected, fixed with 4% formalin, paraffin–embedded, 5–μm sectioned, stained with hematoxylin and eosin (H&E) and observed in accordance with the standard procedure.

8. Isolation of PBMC

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Two groups of mice (10 mice each group) were used, and 200μl 2LD50 ricin was injected intraperitoneally. After 4 hours, 0.5ml 125μg anti-ricin IgGs and PBS were injected subcutaneously into the abdomen as the treatment and control groups, respectively, using a needleless injection device. After 24h, peripheral blood was taken from the fundus vein, 1ml of whole blood was added to 3 ml of erythrocyte lysis solution, lyse on ice for 5 min, add 5 ml of PBS containing 10% fetal bovine serum, and centrifuge at 500g for 5 min; discard the supernatant, wash the precipitate with PBS containing 10% fetal bovine serum twice, centrifuge at 500g for 5 min; discard the supernatant, resuspend the precipitate with PBS, blow and mix to make a cell suspension, and 5 copies of the cells in the experimental group that met the standard were taken and mixed as treatment group samples, and 5 copies of the control group cells were taken and mixed as control group samples for single-cell sequencing.

9. Single-cell sequencing library construction

The sequencing and data analysis were done by Shanghai Ouyi Biomedical Technology Co. The raw data generated by high-throughput sequencing were analyzed using

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CellRanger, the official software of 10×genomics, followed by further quality control and processing of the data based on the preliminary quality control results of CellRanger using the Seurat software package. PCA (principal component) linear dimensionality reduction analysis was performed using gene expression, and PCA results were visualized in two dimensions by tSNE (non–linear dimensionality reduction). By SingleR package based on single cell reference expression quantitative public dataset, the cell expression profile to be identified is calculated correlation with the reference dataset, and the cell type with higher correlation in the reference dataset is assigned to the cell to be identified, which to some extent excludes the interference of human subjective factors. The marker genes are identified using the FindAllMarkers function in the Seurant package to find the genes that are up–regulated for each cell classification relative to the differentially expressed genes of other cell populations, and these genes are the potential marker genes for each cell classification. The identified marker genes were visualized by the Vlnplot and FeaturePlot functions. Significantly different genes were screened using the FindMarkers function in the

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Seurat package, and significantly different genes were screened according to the conditions of P–value less than 0.05 and difference multiplicity greater than 1.5–fold.

10. Statistical analysis

Statistical analyses were carried out using Prism software (GraphPad5.0).

Results

1. Preparation and identification of purified ricin

The purity quotients of the prepared ricin (64 kDa) were more than 90% (Fig. 1A) by SDS–PAGE and 92.2% (Fig. 1B) by HPLC., respectively. 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), respectively. Estimated by observing the Balb/c mice survival rate, the purified ricin could be completely detoxified within 96 hours by formaldehyde at 1:4000. No significant adverse effects were observed in the inoculated mice, and only a few showed a transient increase in body temperature.

2 Identification of the $F(ab')_2$ fragments of equine serum IgGs against ricin

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The purity quotients of the $F(ab')_2$ fragments (100 kDa) were more than 80% (Fig. 3A) by SDS–PAGE and 88.7% (Fig. 3B) by HPLC., respectively. The neutralization antibody titers of the $F(ab')_2$ fragments against ricin were 1:12800 with the MTT method.

3. Effective dosages of the $F(ab')_2$ fragments against ricin in mice and rhesus monkeys

To confirm the effective dosages of the $F(ab')_2$ fragments against ricin, the mice were injected with 5 LD_{50} ricin to construct a ricin intoxication mouse model at first. After 4 hours, the mice were injected with 31.25, 62.5 or 125 μg of the $F(ab')_2$ fragments, respectively. The survival rates of the mice were showed in Fig. 4A. All the mice of the control group were dead within 48 hours. All of the mice injected with 31.25 μg of the $F(ab')_2$ fragments were dead within 3 days. One of the mice injected with 62.5 μg of the $F(ab')_2$ fragments was dead on day 3, the others survived and recovered at day 7. All the mice injected with 125 μg of the $F(ab')_2$ fragments against ricin survived and recovered at day 7.

To further confirm the effective dosages of the $F(ab')_2$ fragments against ricin, the rhesus monkeys were injected with 2 LD_{50} of the purified ricin to construct a ricin

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intoxication rhesus monkey model at first. The rhesus monkeys were injected with 3.75, 7.5 or 12.5 mg of the $F(ab')_2$ fragments after 4 hours, respectively. The survival rates of the rhesus monkeys were showed in Fig. 4B. All the rhesus monkeys in the control group had the symptoms of anorexia and agitation and died within 2 days. The histopathological results (Fig. 5) showed that there was the exudation of neutrophils in the lung, a large amount of liver stromal cells necrosis and the exudation of the inflammatory cells in the kidney. After being injected with 3.75 mg of the $F(ab')_2$ fragments for 6 hours, all the rhesus monkeys had the symptoms of anorexia and agitation, two of them were dead at day 3 and day 4, and the others returned to normal after 3 days. After being injected with 7.5 mg of the $F(ab')_2$ fragments for 8 hours, all the rhesus monkeys had the symptoms of anorexia and agitation, but they all returned to normal after 48 hours. After being injected with 12.5 mg of the $F(ab')_2$ fragments for 10 hours, only one of the rhesus monkeys had the symptom of anorexia and returned to normal after 24 hours, the others seemed healthy all the time. The histological morphology (Fig. 5) of all the tissues from the rhesus monkeys injected with 12.5 mg of the $F(ab')_2$ fragments was normal.

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4. Anti–Ricin IgGs are involved in reversing lethal neutrophil toxicity in mice based on single cell sequencing

The number of cells was 10132 and 7892 in the antibody treatment group and model group samples after quality control, respectively. After clustering and differentially expressed gene analysis, we showed that Neutrophils, T cells, Natural killer (NK) cells, Monocytes, Epithelial cells, Basophils and B cells were involved in the RT virulence process (Figure 6ab). Subsequently, we performed a statistical analysis of the percentage of the seven cell types in PBMCs (Figure 6c), which showed a slightly downregulation of Neutrophils in the antibody–treated mice, leading to the hypothesis that anti–Ricin antibody is involved in the decline of maturation, differentiation and proliferation of neutrophils, rescuing the lethal outcomes of Ricin. Further by transcriptome characterization, the neutrophils were identified as Neutrophils–Camp, Neutrophils–Csf3r, Neutrophils–Cxcl3, Neutrophils–Gm26917, Neutrophils–Hbb–bt, Neutrophils–Ifitm1, Neutrophils–Ltf, Neutrophils–Orm1, Neutrophils–Pf4, Neutrophils–Stfa2. IL–1β, Csf3r and S100a9, which are characteristic transcriptional phenotypes of

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neutrophils, were significantly elevated among the Neutrophils clusters (Figure 6d). As shown in Figure 6e, the ratio of subtypes of neutrophils was significantly altered after antibody treatment. Neutrophils–Camp and Neutrophils–Hbb–bt were dominant in the control group; in contrast, Neutrophils–Csf3r and Neutrophils–Ifitm1 were dominant in the antibody–treated group. Furthermore, the neutrophil transcriptome pattern was significantly altered (Figure 6f): 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 against ricin, the equine $F(ab')_2$ fragments were prepared and evaluated in the lethal ricin intoxication animal models in this study. The purity quotients and bioactivities of the preparations meted the requirements of the Chinese Pharmacopoeia. It is certain that treatment with a full dose of purified $F(ab')_2$ fragments can protect animal models from a lethal dose of ricin intoxication. Thus far, the equine $F(ab')_2$ fragments against ricin are being assayed in the clinical trials.

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Although the crudely purified ricin and equine $F(ab')_2$ fragments were easily made, the ricin antigen for immunization and the $F(ab')_2$ fragments for treatment need high–purity in order to improve safety and reduce side effects of the $F(ab')_2$ preparations. Besides the reported methods in the literatures (18), the ricin was further purified by affinity and ion–exchange chromatography to achieve more than 90% purity. The equine $F(ab')_2$ fragments were purified by size exclusion chromatography in addition to the common methods in order to meet the requirements.

The bioactivities were extremely important. In order to improve the protective bioactivity ingredients of the $F(ab')_2$ preparations, the detoxified antigens, the immunization regimens and blood collection benchmark were optimized again and again. Take the methods in the literatures for reference(1, 19), the ricin detoxification is finally by use of formaldehyde at 1:4000 within 96 hours, which could completely inactivate the toxicity and retain the immunogenicity of the ricin. Furthermore, the adult and healthy horses were inoculated with the elevated adjuvanted purified ricin into the sites near the lymph nodes many times until the titers of sera were more than

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1:6400. Compared with the reported many types of antibodies(20, 21), the protective efficacy of the $F(ab')_2$ preparations were satisfactory, which could protect animal models from a lethal dose of ricin intoxication in a low protein dose.

Due to the advent and advancement of single–cell experimental methods, thousands of cells were able to be sequenced in a single experiment thanks to microfluidics technology and combinatorial indexing methods. Ricin can cause a lethal inflammatory response, which is partially attributed to the massive activation and proliferation of neutrophils. Our results show that this anti–ricin antibody has potent protectiveness to inhibit the neutrophils development, proliferation and differentiation and to attenuates the systemic inflammatory–immune response. The reason for this is due, on the one hand, to its potent neutralization and, on the other hand, to the absence of the FC segment. The FC segment of the antibody is known to have 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 the undesired Fc activities of an antibody is the rational

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design of human IgG1 antibodies missing ADCC or other Fc functions. IL–1, initially discovered as the major endogenous pyrogen, induces prostaglandin synthesis, neutrophil influx, and activation. Receptor for granulocyte colony–stimulating factor (CSF3), essential for granulocytic maturation, plays a crucial role in the proliferation, differentiation, and survival of cells along the neutrophilic lineage. In addition, it may function in some adhesion or recognition events at the cell surface. S100A9 is a calcium– and zinc–binding protein that plays a prominent role in the regulation of inflammatory processes and the immune response. It can induce neutrophil chemotaxis, adhesion, increase the bactericidal activity of neutrophils by promoting phagocytosis via activation of SYK, PI3K/AKT, and ERK1/2, and induce degranulation of neutrophils by a MAPK–dependent mechanism. From the preliminary analysis results of single cell sequencing, it is easy to see that the antibody can reverse the trend of neutrophil infantilization caused by Ricin by regulating gene transcription. This is difficult to accomplish with common assays. In order to further explore the therapeutic mechanism of the antibody and optimize the technological process of anti–ricin antibodies, we will predict the effect of IL–1b, csf3r and S100a9 on neutrophil

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differentiation by proposed time series analysis in the subsequent analysis, and further validate their change characteristics in temporal and spatial dimensions by flow cytometry.

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

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Competing Interests

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The authors declare that the 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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Figure 1. Characterization of purified ricin. (A) SDS-PAGE of purified ricin. Lane 1, the ricin standards. Lane 2-4, the purified ricin. Lane 5, protein molecular weight ladder with indicated MW of each band. (B) The purified ricin was determined by HPLC.

Figure 2. The half lethal dose (LD_{50}) of ricin. (A) In the mouse model, the mice (n=8/group) were injected intraperitoneally with 6 concentrations of purified ricin. The mice were monitored daily for survival for 7 days. (B) In the rhesus monkey model, the rhesus monkeys (n=4/group) were injected intraperitoneally with 4 concentrations of purified ricin. The rhesus monkeys were monitored for survival for 36 hours. The LD_{50} of ricin was measured by the Reed-Muench method.

Figure 3. Characterization of the $F(ab')_2$ fragments of equine serum IgGs against ricin. (A) SDS-PAGE of the $F(ab')_2$ fragments. Lanes 1, protein molecular weight ladder with indicated MW of each band. Lane 2-4, the purified $F(ab')_2$ fragments. (B) The purified $F(ab')_2$ fragments were determined by HPLC.

Figure 4. Effective dose of $F(ab')_2$ fragments against ricin in mice and rhesus monkeys. (A) After being injected with 0.2 ml of 5 LD_{50} ricin for 4 hours, the groups of mice (n=8/group) were injected subcutaneously by needle free devices with 0.2 ml of 31.25 μg, 62.5 μg and 125 μg of $F(ab')_2$ fragments, respectively. The mice injected subcutaneously by needle free devices with PBS were set as a control group. The mice were monitored for mortality daily for 7 days. (B) After being injected with 72 μg ricin for 4 hours, the groups of rhesus monkeys (n=4/group) were injected subcutaneously by needle free devices with 3.75 mg, 7.5 mg and 12.5 mg $F(ab')_2$ fragments, respectively. The rhesus monkeys injected subcutaneously by needle free devices with PBS were set as a control group. The rhesus monkeys were monitored for physical condition and mortality daily for 7 days.

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Figure 5. Histopathological examinations of the lung, liver and kidney tissues from the rhesus monkeys injected with PBS or 12.5 mg F(ab')2 fragments from Fig.4. After the moribund monkeys from the control group and the ones from injected subcutaneously by needle free devices with 12.5 mg F(ab')2 fragments on the 7th day were euthanized, the lung, liver and kidney tissues were collected, handled and observed by pathological section.

Figure 6. Effects of ricin antibodies on peripheral blood revealed by single cell sequencing. (A) tSNE plot of cellular populations; (B) t-SNE plot showing cells sample in mouse with ricin toxin and healthy controls; (C) comparison of the relative proportion of each subpopulation between the experimental group and the control group; (D) Bubble plot of the identified gene 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;